

Chemotherapy-Induced Gastrointestinal Dysfunction and Enteric Neuropathy

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This thesis is submitted in total fulfilment of the requirements for the degree of
Doctor of Philosophy

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FRONTISPIECE

Changes in myenteric neurons and colonic motility following 5-fluorouracil treatment. Published as a journal cover.

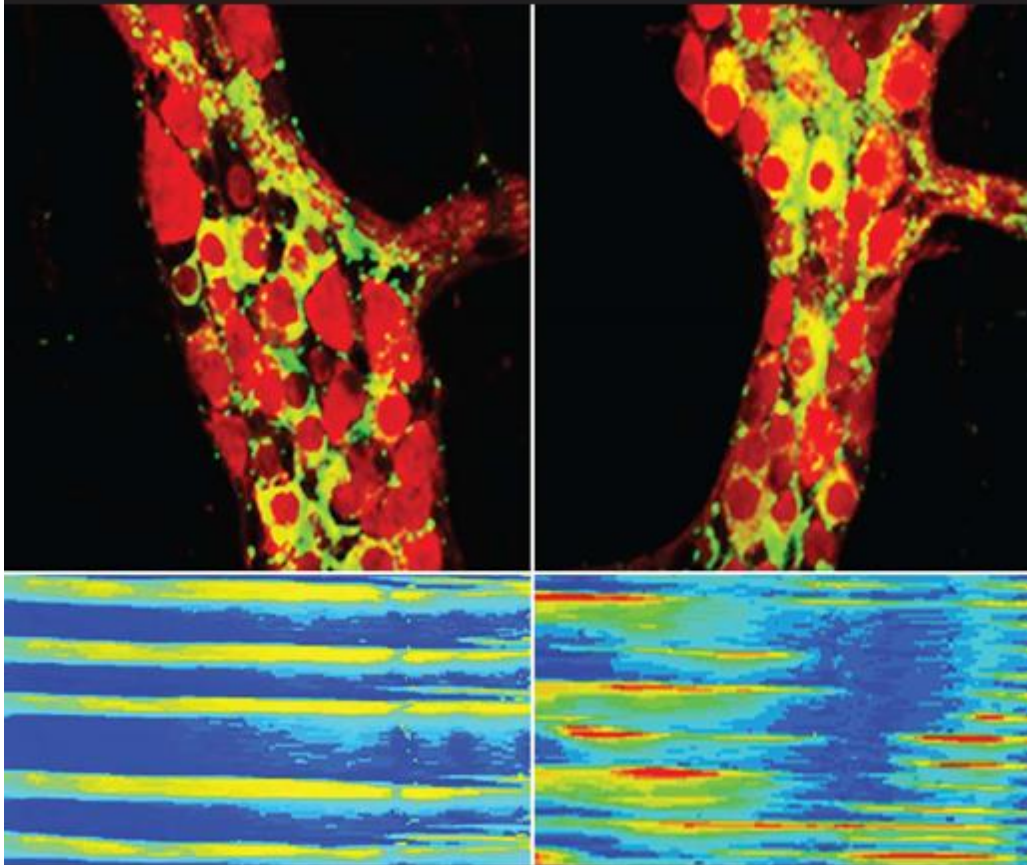
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ABSTRACT

Colorectal cancer (CRC) is a leading cause of morbidity and mortality affecting more than 1.4 million people annually worldwide. Due to the aggressive and asymptomatic nature approximately 60% of CRC sufferers are diagnosed at or beyond stage III resulting in prognostic outlook relying heavily on the successful application of chemotherapeutic treatment.

Chemotherapeutic agents oxaliplatin, 5-fluorouracil and irinotecan represent the backbone of CRC treatment, significantly enhancing tumour regression and patient survival. However successful application of these cytotoxic chemotherapies is hindered by undesirable neurological and gastrointestinal (GI) side-effects. Chronic GI side-effects often result in dose limitations and, in severe circumstances, cessation of anti-cancer treatment, presenting a constant challenge in efficient and tolerable treatment of CRC. It is believed that chemotherapy-induced GI side-effects are a direct result of intestinal mucositis; however adjacent systems such as the enteric nervous system have been overlooked.

This thesis aims to uncover the effects of *in vivo* administration of anti-cancer chemotherapeutics oxaliplatin, 5-fluorouracil and irinotecan on the enteric nervous system and GI function, and examine the neuroprotective efficacy of a cytoprotective agent BGP-15.

This study demonstrated that treatment with oxaliplatin, 5-fluorouracil and irinotecan resulted in significant neuronal loss in the myenteric plexus of the colon. This loss was correlated with dysfunction in both whole gut transit and colonic motility, resulting in altered faecal water content and symptoms of constipation and diarrhoea. This study has revealed that the mechanisms underlying myenteric neuropathy varied across chemotherapeutic treatments, whilst oxaliplatin-induced neuronal loss was associated with oxidative stress of the myenteric plexus, 5-fluorouracil and irinotecan-induced neuronal loss was correlated with inflammation of the colonic

mucosa. Co-treatment with BGP-15 alleviated oxidative stress and myenteric neuropathy associated with oxaliplatin treatment, resulting in normalisation of colonic motility and reduced symptoms of GI dysfunction.

Together, this study has established that enteric neuropathy following oxaliplatin, 5-fluorouracil and irinotecan administration plays a significant role in the development of GI dysfunction. Co-administration of BGP-15 with oxaliplatin, alleviated both enteric neuropathy and GI dysfunction, therefore, can be considered as a potential therapy to reduce GI side-effects associated with oxaliplatin treatment.

DECLARATION

I, Rachel McQuade, declare that the PhD thesis entitled "Chemotherapy-Induced Gastrointestinal Dysfunction and Enteric Neuropathy" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.



Rachel McQuade

January 2017

DECLARATION OF CONTRIBUTION TO WORK

Under my direction, the following people have made the stated contributions to this work:

Chapter 1:

Assistance in preparing the manuscripts that contributed to this chapter was provided by V Stojanovska, R Abalo, JC Bornstein and K Nurgali.

Chapter 2:

Intracellular electrophysiology was performed by SE Carbone and R Gwynne. Colonic force contraction was performed by A Rahman. Technical assistance with western blots was provided by V Stojanovska and C Goodman. Technical assistance with analysis was provided by A Robinson. Assistance in preparing the manuscript for this chapter was provided by JC Bornstein and K Nurgali.

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Reviews

1. **RM McQuade**, V Stojanovska, JC Bornstein, K Nurgali (2016) Chemotherapy-Induced Constipation and Diarrhoea: Pathophysiology, Current and Emerging Treatments. *Frontiers in Pharmacology; special edition "Adverse Effects of Cancer Chemotherapy: Anything New to Improve Tolerance and Reduce Sequelae?"*, Vol 7, p414-428.
2. **RM McQuade**, V Stojanovska, J C Bornstein, K Nurgali (2016) Colorectal Cancer Chemotherapy: The Evolution of Treatment and New Approaches. *Current Medicinal Chemistry; Special Edition "DNA damage as a strategy for anticancer therapy"*, accepted December 26th 2016.
3. **RM McQuade**, J C Bornstein, K Nurgali (2014) Anti-Colorectal Cancer Chemotherapy-Induced Diarrhoea: Current Treatments and Side-Effects. *International Journal of Clinical Medicine*, Vol 5, p393-406.

In Submission

1. **RM McQuade**, V Stojanovska, JC Bornstein, AC Petersen, E Rybalka, K Nurgali (2017) Oxaliplatin-induced enteric neuronal loss and gastrointestinal dysfunction is prevented by co-treatment with BGP-15. Submitted to *British Journal of Pharmacology*.
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Co-Authored Publications

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2. V Stojanovska, **RM McQuade**, E Rybalka, K Nurgali (2016) Neurotoxicity Associated with Platinum-Based Anti-Cancer Agents: What are the Implications of Copper Transporters? *Current Medicinal Chemistry; Special Edition "DNA damage as a strategy for anticancer therapy"*, accepted December 26th 2016.
3. E Donald, **RM McQuade**, AM Robinson, JC Bornstein, K Nurgali (2017) Resveratrol, but not SRT1720, alleviates oxaliplatin-induced damage to the myenteric neurons and colonic dysmotility. Submitted to *Neurogastroenterology and Motility*.
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2. V Stojanovska, **RM McQuade**, M Stewart, CA Timpani, JC Sorensen, J Orbell, E Rybalka, K Nurgali (2016) Platinum accumulation and changes in mitochondrial function of the longitudinal muscle & myenteric plexus following oxaliplatin administration. *Neurogastroenterology & Motility*, Vol 28, p55.
3. **RM McQuade**, J Sorensen, CA Timpani, K Nurgali, E Rybalka (2015) Idebenone therapy protects against oxaliplatin-induced gastrointestinal dysfunction. *Proceedings of the Australian Physiological Society*, Vol 46, p90.
4. **RM McQuade**, V Stojanovska, A Rahman, SE Carbone, JC Bornstein, K Nurgali (2015) Treatment with anti-cancer chemotherapeutic agent oxaliplatin causes oxidative stress-induced enteric neuropathy. *Proceedings of the 6th Annual Victorian Student Research Symposium*, PO22, p90.
5. V Stojanovska, M Stewart, **RM McQuade**, CA Timpani, JC Sorensen, J Orbell, E Rybalka, K Nurgali (2015) Platinum accumulation and changes in mitochondrial function of the longitudinal muscle & myenteric plexus following oxaliplatin administration. *Proceedings of the Australian Physiological Society*, Vol 46, p91.
6. **RM McQuade**, V Stojanovska, SE Carbone, A Rahman, V Jovanovska, JC Bornstein, K Nurgali (2013) Oxaliplatin-induced enteric neuropathy. *Neurogastroenterology and Motility*, Vol 25, p13-45.

Media Releases

1. Chemotherapy destroys more than just the cancer, 2014. The Australian Society for Medical Research

<http://www.asmr.org.au/MRW%20Media/2014Vic%20Meeting.pdf>

2. Chemo side-effects, 2014. The Daily Mail.

<http://www.dailymail.co.uk/wires/aap/article-2643486/Checkup-Friday-May-30.html>

PRESENTATIONS

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2. **RM McQuade**, V Stojanovska, AC Petersen, R Abalo, JC Bornstein, E Rybalka, K Nurgali (2016) Effects of cytoprotectant BGP-15 on Chemotherapy-Induced Gastrointestinal Dysfunction and Enteric Neuropathy, *Lifestyle Associated Diseases (LAD) Seminar*, Victoria University, Melbourne, December, 2016.
3. **RM McQuade**, Stojanovska V, Donald E, Bornstein JC, Nurgali K (2015) anti-cancer chemotherapeutic agent's 5-fluorouracil, oxaliplatin and irinotecan cause severe enteric neuropathy resulting in gastrointestinal dysmotility, *Australian Society of Medical Research 54th National Scientific Conference (ASMR-NCS)*, Adelaide, November, 2015.
4. V Stojanovska, Stewart M, **RM McQuade**, Timpani C, Orbell J, Rybalka E, Nurgali K (2015) Platinum-based chemotherapy: drug accumulation in neurons controlling gastrointestinal functions. *Australian Society of Medical Research (ASMR) Symposium Australian Society of Medical Research 54th National Scientific Conference (ASMR-NCS)*, Adelaide, November, 2015.
5. V Stojanovska, Stewart M, **RM McQuade**, Timpani C, Orbell J, Rybalka E, Nurgali K (2015) Oxaliplatin-induces neurotoxicity: platinum accumulation in the medulla oblongata and enteric neurons, *Australian Society for Medical Research*, Melbourne, May, 2015.
6. **RM McQuade**, V Stojanovska, JC Bornstein, K Nurgali (2014) Damage to Submucosal Neurons as a Result of Treatment with Anti-Cancer Treatment. Victoria University Student Conference, Melbourne, October, 2014.
7. **RM McQuade** & K Nurgali (2014), Damage to Submucosal Neurons from treatment with chemotherapeutic agents oxaliplatin, irinotecan and 5-fluorouracil. *Australian Society of Medical Research (ASMR)*, Adelaide, May, 2014.
8. V Stojanovska, **RM McQuade**, K Nurgali (2014) Neurotoxicity to the myenteric plexus associated with the anti-cancer drug 5-fluorouracil.

Australian Society for Medical Research Symposium, Melbourne, May 2014.

9. **RM McQuade**, V Stojanovska, JC Bornstein, K Nurgali (2013) Damage to Submucosal Neurons as a Result of Treatment with Anti-Cancer Agent Irinotecan. *Victoria University Student Conference*, Melbourne, November, 2013.

10. E Tuckett, **RM McQuade**, V Stojanovska, SE Carbone, S Brookes, A Rahman, K Nurgali (2013) Anti-cancer chemotherapy: effects on intrinsic and extrinsic innervation of the gastrointestinal tract. *International Society for Autonomic Neuroscience - European Federation of Autonomic Societies (ISAN-EFAS)*, Giessen, July 2013.

Posters Presentations

1. **RM McQuade**, V Stojanovska, JC Sorensen, JC Bornstein, AC Petersen, E Rybalka, K Nurgali (2016) BGP-15 co-treatment protects against oxaliplatin-induced neuronal loss and alleviates gastrointestinal dysfunction, *Federation of Neurogastroenterology and Motility Meeting*, San Francisco, August, 2016.

2. V Stojanovska, **RM McQuade**, M Stewart, CA Timpani, JC Sorensen, J Orbell, E Rybalka, K Nurgali (2016) Platinum accumulation and changes in mitochondrial function of the longitudinal muscle & myenteric plexus following oxaliplatin administration, *Neurogastroenterology & Motility*, San Francisco, August, 2016.

3. **RM McQuade**, JC Sorensen, CA Timpani, K Nurgali, E Rybalka (2015) Idebenone therapy protects against oxaliplatin-induced gastrointestinal dysfunction, *Australian Physiological Society*, Hobart, December, 2015.

4. V Stojanovska, M Stewart, **RM McQuade**, CA Timpani, JC Sorensen, J Orbell, E Rybalka, K Nurgali (2015) Platinum accumulation and changes in mitochondrial function of the longitudinal muscle & myenteric

plexus following oxaliplatin administration, *Australian Physiological Society*, Hobart, December, 2015.

5. **RM McQuade**, SE Carbone, A Robinson, A Rahman, JC Bornstein, K Nurgali (2015) Treatment with Anti-cancer chemotherapeutic oxaliplatin causes oxidative stress-induced enteric neuropathy. *Australian Society of Medical Research (ASMR) Symposium*, Melbourne, May, 2015.
6. **RM McQuade**, V Stojanovska, SE Carbone, AA Rahmen, K Nurgali (2015) Enteric Neuropathy Induced by anti-cancer chemotherapy drug oxaliplatin. *Australasian Neurogastroenterology and Motility Association*, Adelaide, February, 2015.
7. V Stojanovska, **RM McQuade**, K Nurgali (2015) Enteric Neuropathy Induced by anti-cancer chemotherapy drug oxaliplatin. *Australasian Neurogastroenterology and Motility Association*, Adelaide, February, 2015.
8. **RM McQuade** & K Nurgali (2014), Damage to Submucosal Neurons as a Result of Treatment with Anti-Cancer Agent Irinotecan, *Australian Neuroscience Conference (ANS)*, Adelaide, 2014.
9. SE Carbone, **RM McQuade**, V Stojanovska, K Nurgali (2014), Treatment with Anti-Cancer Drug Oxaliplatin Affects Neuromuscular Transmission in the Murine Distal Colon, *Australian Neuroscience Conference (ANS)*, Adelaide, January, 2014.
10. V Stojanovska, **RM McQuade**, K Nurgali (2014) Neuronal loss in the myenteric plexus associated with the anti-cancer agent irinotecan, *Australasian Neuroscience Society (ANS)*, Adelaide, January, 2014.
11. **RM McQuade**, V Jovanovska, JC Bornstein and K Nurgali (2013) Effects of oxaliplatin treatment on the submucosal neurons, *Australian Neuroscience Conference (ANS)*, Melbourne, February 2013.
12. Stojanovska V, **RM McQuade**, S Sakal, K Nurgali (2013) Oxaliplatin-induced enteric neuronal damage and changes in neuro-immune interactions. *Australasian Neuroscience Society (ANS)*, Melbourne, February, 2013.

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LIST OF ABBREVIATIONS

| | |
|--------------------------|--|
| 5-FU | 5-fluorouracil |
| 5-HT | 5-hydroxytryptamine |
| APC | adenomatous polyposis coli |
| ATP | adenosine triphosphate |
| BER | base excision repair |
| BRAF | B-Raf proto-oncogene serine/threonine kinase |
| CAPE | capecitabine |
| CB₁ | cannabinoid receptor 1 |
| CB₂ | cannabinoid receptor 2 |
| CH₂THF | folate 5, 10-methylenetetrahydrofolate |
| ChAT | choline acetyltransferase |
| CHT1 | high-affinity choline transporter 1 |
| CIC | chemotherapy-induced diarrhoea |
| CID | chemotherapy-induced constipation |
| CISP | cisplatin |
| CMMC | colonic migrating motor complex |
| COX | cyclooxygenase |
| CRC | colorectal cancer |
| CTL1 | choline transporter-like 1 |
| DACH | 1,2-diaminocyclohexane |
| DMSO | dimethyl sulfoxide |
| dNTP | deoxynucleotide |
| DRG | dorsal root ganglia |
| dTMP | deoxythymidine monophosphate |
| DTO | deodorized tincture of opium |
| dUMP | deoxyuridine monophosphate |
| dUTP | deoxyuridine triphosphate |

| | |
|-----------------------------------|--|
| EC | enterochromaffin |
| EJPs | excitatory junction potentials |
| ENS | enteric nervous system |
| EPSPs | excitatory postsynaptic potentials |
| FC | fragmented contraction |
| FdUDP | fluorodeoxyuridine diphosphate |
| FdUMP | fluorodeoxyuridine monophosphate |
| FdUTP | fluorodeoxyuridine triphosphate |
| fIJP | fast inhibitory junction potentials |
| fIJP | fast inhibitory junction potentials |
| FOLFIRI | 5-fluorouracil, irinotecan and leucovorin |
| FOLFOX | 5-fluorouracil, oxaliplatin and leucovorin |
| FUDP | fluorouridine diphosphate |
| FUDR | fluorodeoxyuridine |
| FUMP | fluorouridine monophosphate |
| FUTP | fluorouridine triphosphate |
| GFAP | glial fibrillary acidic protein |
| GI | gastrointestinal |
| GMCs | giant migrating contractions |
| H&E | haematoxylin and eosin |
| H₂O₂ | hydrogen peroxide |
| HOCl | hypochlorite |
| HSP | heat shock proteins |
| IBS-C | irritable bowel syndrome-constipation |
| IBS-D | irritable bowel syndrome-diarrhoea |
| ICC | interstitial cells of cajal |
| IFN-γ | Interferon γ |
| IL-1β | interleukin 1 β |
| iNOS | inducible nitric oxide synthase |

| | |
|-----------------------------------|---|
| IR | immunoreactive |
| IRI | irinotecan |
| ISNs | intrinsic sensory neurons |
| KRAS | Kirsten rat sarcoma viral oncogene homolog |
| LMMP | longitudinal muscle-myenteric plexus |
| L-NNA | N ω -Nitro-L-arginine |
| LV | leucovorin |
| MAPK | mitogen-activated protein kinases |
| mETC | mitochondrial electron transport chain |
| MMC | migrating motor complex |
| mPTP | mitochondrial permeability transition pore |
| mtDNA | mitochondrial DNA |
| NER | nucleotide excision repair |
| NF-κB | nuclear factor- κ B |
| nNOS | neuronal nitric oxide synthase |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| O₂ | oxygen |
| O₂^{-•} | superoxide |
| OCT1 | organic cation transporter 1 |
| OCT2 | organic cation transporter 2 |
| OCT3 | organic cation transporter 3 |
| OCTs | organic cation transporters |
| OH[•] | hydroxyl |
| ONOO⁻ | peroxynitrite |
| OXL | oxaliplatin |
| PACAP | pituitary adenylyl cyclase activating peptide |
| PAR | poly(ADP-ribose) |
| PARP | poly ADP ribose polymerase |

| | |
|-------------------------------|---|
| PBS | Phosphate buffered saline |
| PGE2 | prostaglandin E2 |
| PGP9.5 | Protein Gene Product 9.5 |
| RIRR | ROS-induced ROS release |
| ROS | reactive oxygen species |
| ROS | reactive oxygen species |
| SC | short contraction |
| sIJP | slow inhibitory junction potentials |
| SNP | sodium nitroprusside |
| SOD | superoxide dismutase |
| Tcf | T-cell factor |
| TNFα | tumour necrosis factor α |
| Top I | topoisomerase I |
| TS | thymidylate synthase |
| UGT1A1 | uridine diphosphate glucuronosyltransferase 1A1 |
| V600E | valine-to-glutamate change at the residue 600 |
| VACht | vesicular acetylcholine transporter |
| VIP | vasoactive intestinal peptide |

CHAPTER ONE: LITERATURE REVIEW

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RM McQuade, J C Bornstein, K Nurgali (2014) Anti-Colorectal Cancer Chemotherapy-Induced Diarrhoea: Current Treatments and Side-Effects. *International Journal of Clinical Medicine*, Vol 5, p393-406.

RM McQuade, V Stojanovska, E Rybalka, JC Bornstein, K Nurgali (2016) Chemotherapy-Induced Constipation and Diarrhoea: Pathophysiology and Treatments. *Frontiers in Pharmacology; special edition "Adverse Effects of Cancer Chemotherapy: Anything New to Improve Tolerance and Reduce Sequelae?"*, Vol 7, p414-428.

RM McQuade, V Stojanovska, E Rybalka, JC Bornstein, K Nurgali (2016) Colorectal Cancer Chemotherapy: The Evolution of Treatment and New Approaches. *Current Medicinal Chemistry; Special Edition "DNA damage as a strategy for anticancer therapy"*, accepted 26th of December 2016.

1.1 Colorectal Cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide (Ferlay et al., 2015). With a global incidence of approximately 1.4 million and mortality rates approaching 700,000 people per year, it is one of the highest contributors to cancer related death (World Health Organization, 2011; Ferlay et al., 2015). Globally the incidence of CRC is highly variable, with rates fluctuating up to 10-fold between countries (Wilmink, 1997; Parkin et al., 2010), ranging from more than 40 per 100,000 people in the United States, Australia, New Zealand, and Western Europe to less than 5 per 100,000 in Africa and some parts of Asia (Marmot et al., 2007). Lower rates of CRC in developing countries may be due to lack of screening and diagnostics of population over 50 years of age, something implemented in many western countries (Haggard and Boushey, 2009).

Several risk factors are associated with the incidence of CRC. Non modifiable risk factors include those that an individual cannot control like age and hereditary factors (Haggard and Boushey, 2009). Additionally, a substantial number of environmental and lifestyle factors, considered as modifiable risks factors, may play an important role in the development of CRC (Haggard and Boushey, 2009; Khan et al., 2010). CRC is widely considered to be an environmental disease and is associated with a diverse range of ill-defined cultural, social, and lifestyle factors including abdominal obesity (Kim et al., 2007; Kim et al., 2009), alcohol consumption (Ferrari et al., 2007; Mizoue et al., 2008), western diet and smoking (Buc et al., 2006; Paskett et al., 2007; Botteri et al., 2008). Thus, many CRC cases may theoretically be preventable (Boyle and Langman, 2000; Johnson and Lund, 2007).

The histopathogenesis of CRC revolves heavily around development of mucosal colonic polyps. The most common histological types of colonic polyps are hyperplastic and adenomatous (Mitchell et al., 2004). Approximately 75% of colorectal tumours do not appear to be due to inherited genetic mutations and are referred to as non-familial or sporadic

tumours, however a small proportion of colorectal tumours have been correlated with inherited mutations in specific oncogenes such as adenomatous polyposis coli (APC) and defective DNA mismatch repair genes MLH1, MSH2, PMS2 and MSH6 (Berg and Søreide, 2011).. Approximately 30-50% of colorectal tumours are known to have a mutated V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) gene. A further 10% of CRC patients are characterized by a mutation in the B-Raf proto-oncogene serine/threonine kinase (BRAF) gene resulting in a valine-to-glutamate change at the residue 600 (V600E) (Barras, 2015). Presence or absence of KRAS and BRAF mutations has been found to greatly impact the efficacy of certain anti-cancer agents; furthermore, disruption of several signalling pathways is associated with disease progression. Transforming growth factor- β (TGF β) has been found to promote tumour growth via Smad-independent mechanisms (Cheruku et al., 2015), and is involved in crosstalk with several other pathways including the mitogen-activated protein kinases (MAPK) and Wnt signalling pathway. Deregulation of Wnt and T-cell factor (Tcf) signalling and accumulation of β -catenin contributes to accretion of undifferentiated cells in colonic crypts which when combined with subsequent mutations, involving genes such as KRAS and BRAF, may eventually lead to carcinoma (Armaghany et al., 2012).

The clinical manifestations of CRC are highly dependent on the location and characteristics of the tumour regardless of preceding condition. Approximately 65% of colon cancers are distal to the splenic flexure and thus may be detectable via sigmoidoscopy, conversely 35% of primary tumours are located proximal to the sigmoid and are essentially imperceptible (McCallion et al., 2001; Church, 2005). Exacerbating this diagnostic dilemma, the discernible symptoms of CRC are non-specific and not well defined, with a recent review of literature indicating that rectal bleeding and weight loss are the only definitive symptoms associated with CRC (Adelstein et al., 2011). Whilst the 5 year survival rate for patients at the early stage of CRC (I and II) currently sits at 63%, that rate drops to below 10% in patients with metastatic form of the disease (stage III) (Goldberg, 2005; Jemal et al., 2008; Ries et al., 2008; Hagggar and Boushey,

2009). Currently, patients with chemotherapy-refractory colorectal liver metastases have a median survival of only 4–6 months with optimal supportive care (Amado et al., 2008). Approximately 60% of CRC sufferers are diagnosed at or beyond stage III resulting in a prognostic outlook relying heavily on the successful application of chemotherapeutic treatment (Siegel et al., 2014).

Approximately two thirds of CRC patients undergo surgical resectioning with or without adjuvant chemotherapy (Scheer and Auer, 2009). Unfortunately, 30-50% of patients develop recurrent disease, with greater than 90% of recurrences in the first 5 years following surgery (Böhm et al., 1993). Advances in preoperative chemotherapeutic therapies over the last several decades have led to development of several promising regimes resulting in improvement of 5 year survival rates for locally resected stage III CRC (Haller, 2000;Souglakos et al., 2006). Progress in the area of CRC chemotherapeutics over the last 70 years has, however, been limited with initial treatments such as 5-fluorouracil (5-FU) still representing the foundation of chemotherapeutic regimens. While modest, the evolution of CRC management has seen a transition in treatment structure and administration from singular cytotoxic agents to targeted and multi-agent regimes. Incorporation of platinum agents, plant alkaloids and antibiotics to adjuvant and neoadjuvant chemotherapy combination regimens resulted in an improvement in both 5 and 10 year survival rates for CRC sufferers (Al-Hajeili et al., 2016), highlighting the importance of continual advancement of CRC treatments (De Gramont et al., 2000;Douillard et al., 2000;Saltz et al., 2000;Cassidy et al., 2004;Grothey et al., 2004;Haller et al., 2011).

1.2 The Evolution of Colorectal Cancer Chemotherapeutics

Since the introduction of 5-FU ($C_4H_3FN_2O_2$) into clinical treatment of CRC in 1957, progress in the management of advanced CRC has been modest (Bertino, 1997). Trends in treatment for stage III and IV metastatic CRC over the last several decades may be categorised into several eras based on induction of new chemotherapeutics and novel combinations.

1.2.1 Fluoropyrimidines

1.2.1.1 5-Fluorouracil and Leucovorin

For many years following fluoropyrimidine discovery, 5-FU remained the backbone of therapy for CRC and until recently was considered the standard first-line treatment for metastatic CRC (Goldberg, 2005). Fluoropyrimidines were developed following the observation that rat hepatomas utilised pyrimidine uracil more rapidly than normal tissues (Rutman et al., 1954), signifying that uracil metabolism may be a potential target for antimetabolite chemotherapy. 5-FU enters the cell using the same facilitated transport mechanism as uracil (Wohlhueter et al., 1980) and is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). These active metabolites disrupt RNA synthesis triggering mis-incorporation of fluoro nucleotides into RNA and DNA alongside inhibition of the enzyme thymidylate synthase (Longley et al., 2003). Thymidylate synthase-catalysed reactions provide the sole source of thymidylate, a key molecule necessary for DNA replication and repair (Longley et al., 2003) (**Figure 1.1**).

5-FU has been used for more than 50 years in the treatment of CRC. Many early randomized studies of 5-FU in the adjuvant setting failed to show significant improvement in patient survival (Higgins et al., 1984;Buyse et al., 1988;Panettiere et al., 1988), with the overall response rate as a single agent in advanced CRC reported as approximately 10–15% (O'Connell, 1989). However, over the past 30 years important modulation strategies have been developed to reduce cytotoxicity, increase anti-cancer activity and overcome clinical resistance associated with 5-FU. Side-effects of 5-FU include stomatitis, esophagopharyngitis, diarrhoea, nausea, vomiting, dermatologic changes, alopecia, hematopoietic depression, fever, neurotoxicity, cardiotoxicity and death (Curreri et al., 1958;Diasio and Harris, 1989;Kennedy, 1999). Leukopenia is recognised as a major dose-limiting toxicity of 5-FU monotherapy with rates as high as 93% reported in

some studies (O'Connell, 1989; Petrelli et al., 1989). However, dose reduction from 15 mg/kg/day to 12 mg/kg/day has resulted in tolerable toxicity (O'Connell, 1989), allowing 5-FU to remain a key agent for the treatment of both advanced and early-stage CRC (Longley et al., 2003).

Leucovorin (LV) ($C_{20}H_{23}N_7O_7$) is a folinic acid derivative that enhances the cytotoxic effects of 5-FU by inhibiting the production of thymidylate synthase (Longley et al., 2003). Thymidylate synthase catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), using reduced folate 5, 10-methylenetetrahydrofolate (CH_2THF) as the methyl donor (Diasio and Harris, 1989) (**Figure 1.1**). This reaction provides the sole *de novo* source of thymidylate, which is necessary for DNA replication and repair (Longley et al., 2003).

High intracellular levels of the reduced folate CH_2THF are necessary for optimal binding of the 5-FU metabolite FdUMP to thymidylate synthase. Leucovorin increases intracellular concentrations of CH_2THF and has been shown to increase both *in vitro* (Matherly et al., 1990) and *in vivo* (Nadal et al., 1987) toxicity of 5-FU in many cancer cell lines. Synergistic administration of 5-FU/LV has been shown to improve response rates in CRC by up to 37% when compared to a single agent 5-FU bolus as well as increasing median and overall survival (Petrelli et al., 1987; Erlichman et al., 1988). Several studies have found the dose-limiting toxicity of both high and low dose 5-FU/LV regimens to be gastrointestinal (Petrelli et al., 1987; Petrelli et al., 1989) including severe ulcerative stomatitis occurring at rates as high as 30% of patients (Jebb et al., 1994). Although it has been reported that severe diarrhoea is not more frequent in patients treated with 5-FU/LV compared to patients treated with 5-FU alone (O'Connell, 1989), severe diarrhoea specifically has been described as a major treatment-related toxicity affecting approximately 40% of patients receiving 5-FU/LV, of these patients 52% not only required a dose reduction of 5-FU, but also hospitalization for intravenous hydration (Petrelli et al., 1987). Moreover,

treatment related diarrhoea has been correlated with the demise of 5% of the patient cohort (Petrelli et al., 1989).

Although modest, the improvements resulting from the combination of 5-FU with LV offered the possibility of enhancing the anti-cancer efficacy of 5-FU. Increased understanding of the mechanisms of action of 5-FU in the years to come led to development of strategies to successfully increase its anti-cancer activity (Longley et al., 2003), with 5-FU now used in clinical combinations with several new generation anti-cancer chemotherapeutics.

Figure 1.1 5-Flourouracil, Capecitabine and Leucovorin.

Capecitabine is an oral prodrug that is converted to its only active metabolite, 5-Fluorouracil (5-FU) by thymidine phosphorylase. The chief mechanism of 5-FU activation is conversion of 5-FU to fluorouridine monophosphate (FUMP). FUMP is phosphorylated to fluorouridine diphosphate (FUDP), which can either be further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or be converted to fluorodeoxyuridine diphosphate (FdUDP). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites fluorodeoxyuridine triphosphate (FdUTP) and fluorodeoxyuridine monophosphate (FdUMP), respectively. An alternative activation pathway involves the thymidine phosphorylase catalysed conversion of 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated to FdUMP. Thymidylate synthase (TS) catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5,10-methylene tetrahydrofolate (CH₂THF) as the methyl donor. The active metabolite FdUMP binds to TS and CH₂THF, blocking access of dUMP to the nucleotide-binding site and inhibiting dTMP synthesis. This results in imbalances to deoxynucleotide (dNTP) levels and accumulation of deoxyuridine triphosphate (dUTP). Accumulating dUTP triggers misincorporation of uracil into the DNA via DNA polymerase. Although the DNA repair mechanism can excise the misincorporated DNA, the existing dUMP is just re-phosphorylated to dUTP and re-incorporated back into the DNA. This process is known as futile cycling. DNA strand breakage and damage occur as repair mechanisms ultimately fail.

1.2.1.2 Capecitabine

Capecitabine (CAPE) ($C_{15}H_{22}FN_3O_6$) is an oral prodrug that is enzymatically converted to 5-FU by thymidine phosphorylase. Derived from a predecessor prodrug of 5-FU, doxifluridine, CAPE is metabolized to FU through 3 activation steps: i) CAPE is absorbed through the intestine and converted to 5'-deoxy-S-fluorocytidine (5'-DFCR) by carboxylesterase and then ii) to 5'-deoxy-S-fluorouridine (5'-DFUR) by cytidine deaminase, in the liver; iii) thymidine phosphorylase then converts 5'-DFUR to the active drug, FU (**Figure 1.1**). This occurs in both neoplastic and healthy tissues. However, thymidine phosphorylase is present at higher levels in tumour cells than in healthy tissues, allowing for selective activation of the drug and less systemic toxicity (Budman et al., 1998; Miwa et al., 1998; Walko and Lindley, 2005).

Initially approved in 1998 for use in patients with metastatic breast cancer whose tumours were resistant to standard chemotherapy with paclitaxel (Taxol) and an anthracycline-containing regimen, CAPE was first trialled for the use in metastatic CRC in the early 2000's. Results demonstrated preferential conversion of CAPE to 5-FU in colorectal tumours after oral administration to patients (Schüller et al., 2000). Several clinical trials have demonstrated that single agent CAPE administration has a significantly superior response rate compared to 5-FU/LV, even in patient subgroups with poor prognostic indicators. CAPE, compared with bolus 5-FU/LV treatment also demonstrated improved safety producing a significantly lower incidence of diarrhoea, stomatitis, nausea, alopecia and myelosuppression (Hoff et al., 2001; Twelves et al., 2001; Van Cutsem et al., 2004). However, severe hand-foot syndrome and hyperbilirubinemia were significantly more frequent in CAPE-treated patients compared to those treated with 5-FU/LV (Hoff et al., 2001). Regardless of this, CAPE patients required substantially fewer hospital visits following drug administration than 5-FU/LV patients and spent fewer days in hospital for the management of treatment-related adverse events than did patients treated with 5-FU/LV (Hoff et al., 2001; Twelves et al., 2001).

1.2.2 Platinum-Based Chemotherapeutics for CRC

The serendipitous rediscovery of biologically active platinum complexes in the early 1960's, (Rosenberg et al., 1965) triggered a resurrection in inorganic chemistry and re-evaluation of thousands of platinum analogues as potential chemotherapeutic agents (Kelland, 2007). As a result, platinum-based agents, cisplatin (CISP) and oxaliplatin (OXL), are now used to treat a broad range of malignant diseases.

1.2.2.1 Cisplatin

Following *in vivo* administration in murine sarcoma in the late 1960's CISP ($\text{Cl}_2\text{H}_6\text{N}_2\text{Pt}$) was the first member in the class of platinum-containing anti-cancer drugs to enter phase I clinical trials in 1971 (Kelland, 2007). The main biochemical mechanism of action of CISP involves the formation of mono-adducts as well as intra- and interstrand crosslinks at the nucleophilic site of the DNA, resulting in interference with normal transcription and/or DNA replication mechanisms (Jamieson and Lippard, 1999;Fuentes et al., 2003;Noll et al., 2006). These adducts cause distortions in DNA, including unwinding and bending (**Figure 1.2**), and are recognized by several cellular proteins; some of which are involved in DNA-repair pathways (Chaney et al., 2004). These disruptions trigger cytotoxic processes generally leading to apoptotic cell death (Eastman, 1989), although the pathways from platinum–DNA binding to apoptosis remain incompletely elucidated (Kelland, 2007). There is continued debate as to which of the various platinum-DNA adducts is more biologically significant in response to CISP treatment.

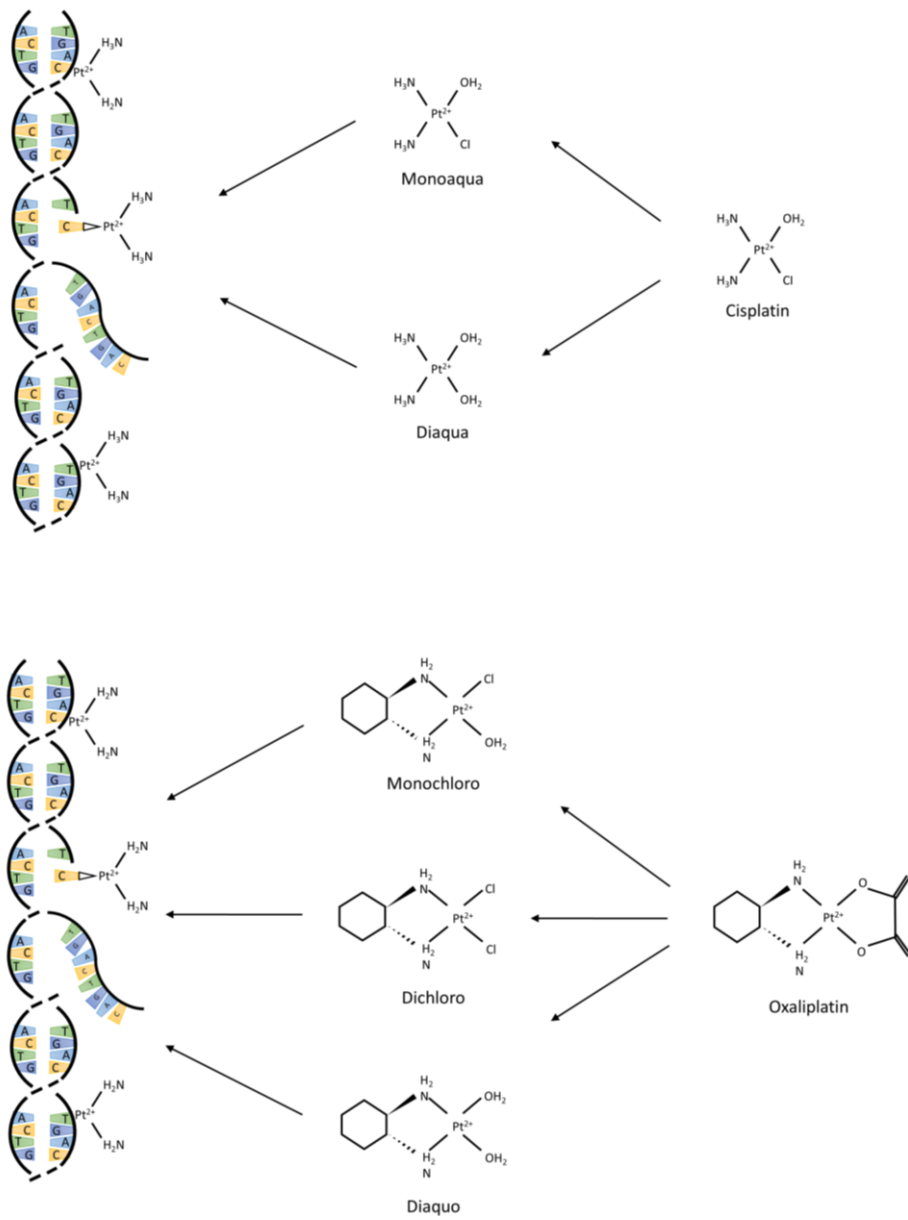
Early trials evaluating the efficacy of CISP in combination with 5-FU showed no significant improvement in median survival time when compared to 5-FU alone (Loehrer et al., 1988;Kemeny et al., 1990;Hansen et al., 1996), and although effective against some tumours, the administration of CISP was hindered by high rates of drug resistance and severe side-effects of nephrotoxicity, neurotoxicity and ototoxicity that limited its therapeutic

potential (Wong and Giandomenico, 1999;Fuentes et al., 2003). Although still regularly used for the treatment of lung cancer, bladder cancer and various gastrointestinal cancers, CISP alone and in combination with 5-FU has not shown any significant cytotoxic effect in CRC (Hansen et al., 1996). However, the discovery of platinum-DNA adducts through CISP stimulated the search for other anti-tumor active platinum complexes with improved pharmacological properties, resulting in the discovery of OXL.

1.2.2.2 Oxaliplatin

OXL ($C_8H_{14}N_2O_4Pt$), is a third generation platinum-based agent currently used as the first line of treatment for metastatic CRC (De Gramont et al., 2000;Petrioli et al., 2008). It exerts its cytotoxic effects via formation of platinum-DNA adducts that trigger immobilization of the mitotic cell cycle and stimulates apoptosis of dividing cells (Graham et al., 2000;Goodisman et al., 2006) (**Figure 1.2**). At clinically recommended doses, it is reported to be less toxic for the auditory, hematologic and renal systems than predecessor drugs like CISP and carboplatin (Raymond et al., 1998). However, OXL appears to have a unique pattern of side-effects unrelated to those observed with other platinum-derived chemotherapeutics. During the course of OXL clinical trials, commonly reported side-effects included hematologic toxicity, gastrointestinal toxicity and peripheral sensory neuropathy (Cassidy and Misset, 2002). Although these side-effects are prominent in OXL-treated patients, treatment with OXL has significantly improved efficacy for many tumours resistant to the first and second generation platinum-based agents (Machover et al., 1996;Louvet et al., 2002). Several key studies undertaken in the early 2000's evaluated the effectiveness of OXL, as both a first and a second-line treatment in combination with 5-FU/LV for CRC uncovering great therapeutic potential (Levi et al., 1993;Machover et al., 1996;Giacchetti et al., 2000;André et al., 2004). Incorporation of OXL into treatment regimens led to the innovation of a novel therapeutic pathway in which patients may undergo a secondary potentially curative surgery.

Figure 1.2 Cisplatin and Oxaliplatin. In aqueous condition cisplatin is converted into active metabolites monoaqua and diaqua. These metabolites bind with DNA to form 1,2-intrastrand cross-links with purine bases resulting in changes in the conformation of DNA that affect DNA replication. Oxaliplatin is a third generation coordination complex of platinum II with a 1,2-diaminocyclohexane (DACH) core. Oxaliplatin is activated through conversion to monochloro, dichloro and diaquo via non-enzymatic hydrolysis with the displacement of the oxalate group. Cytotoxicity of oxaliplatin is attributed to the formation of inter- and intra-strand cross links in DNA which prevent DNA replication and transcription.



1.2.3 Topoisomerase Inhibitors

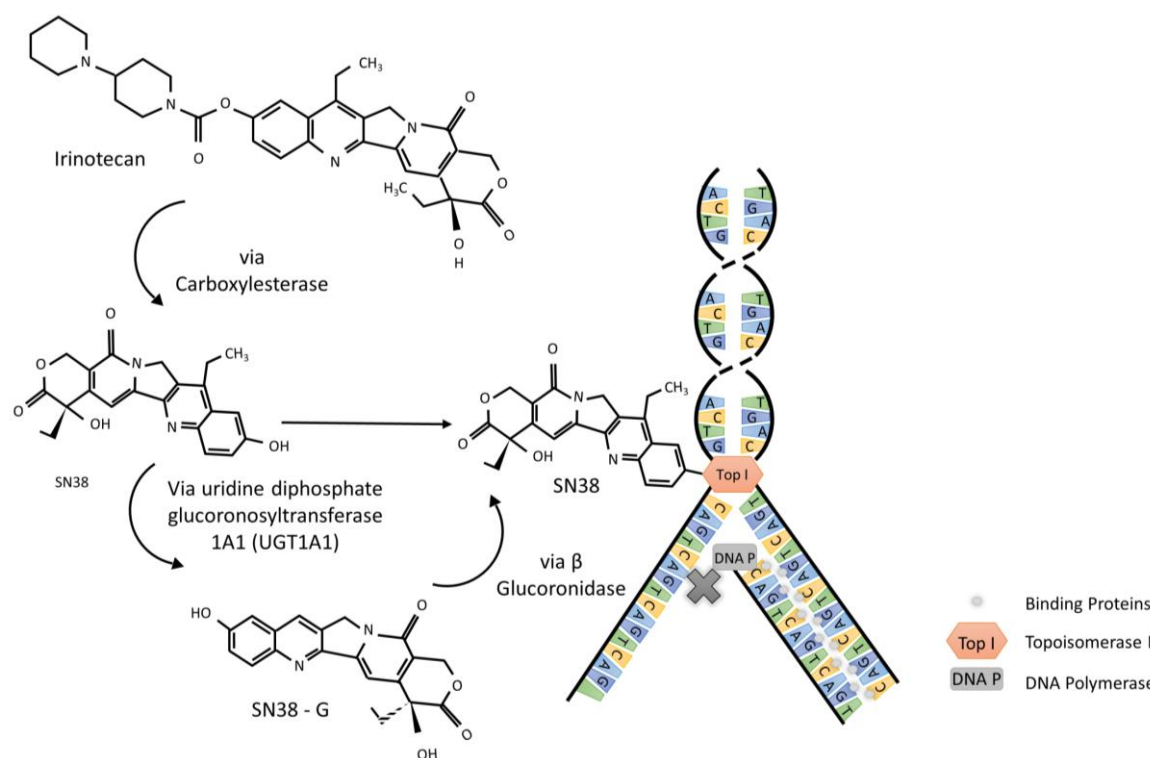
1.2.3.1 Irinotecan

Irinotecan ($C_{33}H_{38}N_4O_6$) (IRI) is a semi-synthetic analog of a naturally occurring quinoline alkaloid, camptothecin, which exerts its cytotoxicity through inhibition of topoisomerase I (Top I) (Xu and Villalona-Calero, 2002). Top I is essential for DNA transcription and acts to cut, relax and reanneal DNA strands. IRI's active metabolite SN-38 binds to Top I and its DNA complex resulting in the formation of a stable ternary structure that prevents DNA re-ligation and promotes DNA damage and apoptosis (**Figure 1.3**). Cellular toxicity from IRI administration is primarily a result of conversion from single-strand breaks into double-strand breaks during the S-phase of the cell cycle when the replication fork collides with the cleavage complexes formed by DNA and SN-38 (Grivicich et al., 2001; Pommier et al., 2003). Phase II trials investigating the overall benefit of IRI in the late 1990's highlighted the positive impact of IRI as a second line therapy in 5-FU refractory patients with advanced CRC (Grothey et al., 2004). These studies uncovered that patients, whose disease did not respond to first line 5-FU therapy, showed increased survival times when administered sequential IRI compared to patients receiving supportive care (Cunningham et al., 1998) and 5-FU/LV (Rougier et al., 1998).

Common side-effects associated with irinotecan administration include cholinergic syndrome, nausea, vomiting, constipation, dyspnoea and neutropenia (Cunningham et al., 1998; Rougier et al., 1998); however, the major dose limiting side-effect is diarrhoea. Acute diarrhoea experienced within the first 24 hours following irinotecan administration, occurs in approximately 60-80% of patients (Gibson and Keefe, 2006). This type of diarrhoea is thought to be primarily secretory with attenuation of symptoms by administration of atropine suggesting a cholinergic nature (Gibson et al., 2003; Gibson and Stringer, 2009). Delayed onset diarrhoea, experienced 24-48 hours following chemotherapeutic treatment occurs in approximately 80% of patients (Saliba et al., 1998). IRI-induced diarrhoea has been linked

to the enzymatic activity of β -glucuronidase in the intestinal microflora. Detoxified SN-38 (SN-38 glucuronide) is hydrolysed by β -glucuronidase in the lumen to reform an active metabolite SN-38, potentially contributing to delayed onset diarrhoea (Takasuna et al., 1998). Treatment with the broad spectrum antibiotic neomycin has been found to reduce fecal β -glucuronidase activity to undetectable levels and decreases fecal concentrations of the active SN-38. Although neomycin had no significant effect on haematological toxicity, it was found to ameliorate diarrhoea in 86% patients, indicating that bacterial β -glucuronidase plays a crucial role in IRI-induced diarrhoea (Kehrer et al., 2001).

Figure 1.3 Irinotecan. Irinotecan is converted into its active metabolite SN38 through hydrolysis by enzyme carboxylesterase. SN38 forms a complex with DNA and topoisomerase I (Top I). Top I is responsible for cleaving and unwinding the DNA for replication and transcription. Formation of SN38 and Top I complexes inhibits the activity of Top I causing obstruction of the re-ligation of DNA and blocking the movement of DNA polymerase resulting in inhibition of DNA replication and transcription. SN38 is deactivated through glucuronidation by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) to form SN38 glucuronide (SN38-G), but can be reactivated in the presence of β -glucuronidase to SN38.



1.2.4 Combination and Sequential Therapies

Moving forward almost a decade, the most effective current anti-CRC agents remain 5-FU, cisplatin, oxaliplatin, irinotecan, and capecitabine. Although proven to be individually effective, multi-agent regimens including leucovorin (LV) given in combination or sequentially are now the standard line of chemotherapeutic treatment and are the most promising approaches to curative and non-curative CRC management (De Gramont et al., 2000; Douillard et al., 2000; Saltz et al., 2000; Louvet et al., 2002; De Gramont et al., 2007; Seymour et al., 2007; Folprecht et al., 2008). A detailed analysis of outcomes and survival rates of the most pivotal studies for single agent and combination therapies for CRC has been published in a recent review (Gustavsson et al., 2015).

1.2.4.1 FOLFOX, FOLFIRI and FOLFOXIRI

Originally intended as salvage therapies for patients failing to respond to single agent 5-FU treatment, combination therapies containing oxaliplatin and irinotecan are now clinically established first-line, second-line and sequential treatment options for advanced CRC (De Gramont et al., 2000; Douillard et al., 2000; Saltz et al., 2000; Grothey et al., 2004; Tournigand et al., 2004; Goldberg, 2005). Several key trials conducted in the late 1990's established improved efficacy and overall survival of both FOLFOX (FOL = folinic acid (Leucovorin) + F = 5-fluorouracil (5-FU) + OX = oxaliplatin (Elotaxin)) and FOLFIRI (FOL = folinic acid (Leucovorin) + F = 5-fluorouracil (5-FU) + IRI = irinotecan (CPT-11) combinations over 5-FU/LV alone in metastatic CRC (Conti et al., 1996; Saltz et al., 2000).

Combination of OXL with 5-FU/LV (FOLFOX), in particular, has shown great therapeutic potential, improving progression-free survival by approximately 20% and overall survival rates by approximately 6% of stage II and III CRC patients (André et al., 2004; De Gramont et al., 2007; Kuebler et al., 2007b). Addition of OXL to 5-FU/LV significantly enhances antitumor efficacy, improving median progression free survival from 6.1 to 8.7 months in 5-

FU/LV treated and FOLFOX treated patients respectively (Giachetti et al., 1997). In a further study, FOLFOX treatment was found to increase response rates by almost 30% compared to 5-FU/LV (De Gramont et al., 2000), additionally 20-30% of patients from various trials were referred for surgery, the median survival of this group was 3-4 years (Lévi et al., 1992; Bertheault-Cvitkovic et al., 1996; de Gramont et al., 1997). It was found, however, that in comparison to 5-FU/LV, FOLFOX had higher frequencies of grade 3/4 neutropenia (5.3% v 41.7% of patients), grade 3/4 diarrhoea (5.3% v 11.9%), and grade 3 neurosensory toxicity (0% v 18.2%) (De Gramont et al., 2000).

Several phase III trials have demonstrated a significant survival advantage for IRI combined with 5-FU/LV (FOLFIRI), compared to 5-FU/LV alone in patients with metastatic CRC (Douillard et al., 2000; Saltz et al., 2000). A multicentre trial evaluating FOLFIRI as a first line therapy for metastatic CRC found that patients receiving FOLFIRI combination had significantly higher response rates when compared to 5-FU/LV (49% vs 31% respectively) and average time to progression was significantly longer in the FOLFIRI group (6.7 vs 4.4 months) (Douillard et al., 2000). These studies established IRI-based chemotherapy as the preferred comparator regimen and FOLFIRI was approved as a first-line therapy for advanced CRC by the United States Food and Drug Administration in 2000, replacing 5-FU/LV as the standard of care (Grothey et al., 2004; Souglakos et al., 2006).

Given increased efficacy demonstrated by FOLFOX and FOLFIRI when compared with 5-FU/LV alone in randomized studies (Giachetti et al., 1997; De Gramont et al., 2000; Douillard et al., 2000; Saltz et al., 2000) the notion of a triple combination therapy FOLFOXIRI (FOL = folinic acid (Leucovorin) + F = 5-fluorouracil (5-FU) + OX = oxaliplatin (Eloxatin) + IRI = irinotecan (CPT-11) emerged. Initial phase I-III studies found FOLFOXIRI treatment was well tolerated; with a response rate of 72%, and a median progression free survival of 10.8 and overall survival of 28.4 months. Furthermore, it was found that resection of residual metastatic disease could be performed in 26% of initially unresectable patients, with the 4 year

survival of these patients recorded as 37% (Falcone et al., 2002;Masi et al., 2004;Masi et al., 2006). A phase III trial comparing infusional FOLFOXIRI to FOLFIRI demonstrated superior response rates as well as significantly improved progression free survival and overall survival in FOLFOXIRI treated patients (Tournigand et al., 2004).

1.2.4.2 XELOX and XELIRI

Capecitabine's equivalent efficacy and clinically significant safety advantage compared to 5-FU treatment underpinned the rationale for capecitabine inclusive combination and sequential treatments (Hoff et al., 2001;Van Cutsem et al., 2001;Koopman et al., 2007). Early studies confirmed that the combination of capecitabine with oxaliplatin (XELOX) was feasible and established with the recommended dose regimen that safety was predictable and similar to the FOLFOX regimen (Diaz-Rubio et al., 2002). Phase II studies combining capecitabine with either irinotecan (XELIRI) (XEL = capecitabine (Xeloda) + IRI = irinotecan (CPT-11)) or oxaliplatin (XELOX) (XEL = capecitabine (Xeloda) + OX = oxaliplatin (Elotaxin)) have shown efficacy and toxicity comparable with combination schedules involving 5-FU (Borner et al., 2002;Rea et al., 2005). XELOX has shown to be a highly effective first-line treatment for metastatic CRC with response rates, time to progression and overall survival similar to those observed with FOLFOX combinations. Studies comparing XELOX with 5-FU/LV as adjuvant therapy for stage III CRC demonstrated that XELOX therapy improved disease free survival 70.9% when compared to 66.5% with FU/LV and had an increased overall survival rate of 77.6% compared to 5-FU/LV (74.2%) (Haller et al., 2011). XELOX treatment was associated with lower rates of neutropenia and stomatitis compared to patients treated with 5-FU/LV. However, it was associated with higher rates of hand-foot syndrome and thrombocytopenia than those seen in 5-FU/LV-treated patients (Haller et al., 2011).

Although XELIRI showed promise in early trials as an active first line treatment for metastatic CRC (Patt et al., 2007), further studies showed little benefit of XELIRI over FOLFIRI (Skof et al., 2009; Pectasides et al., 2012). No significant difference between XELIRI and FOLFIRI in grade 3 or 4 adverse events, including diarrhoea, neutropenia, ischemic stroke and acute coronary syndrome, was found (Skof et al., 2009). It was concluded the XELIRI regimen showed similar progression free survival and overall survival with acceptable toxicity compared to the FOLFIRI regime. It has been reported that upfront combination chemotherapy is more toxic, but not more effective than the sequential use of the same cytotoxic drugs in patients with advanced, non-resectable CRC (Ducreux et al., 2011).

1.2.5 PARP Inhibitors

Poly(ADP-ribose) polymerase-1 (PARP1) is a nuclear enzyme heavily involved in base excision repair and non-homologous end joining pathways facilitating DNA repair of both single and double strand breaks (Attia and Al-Enazi, 2014). Activated PARP1 cleaves NAD into nicotinamide and ADP-ribose which is polymerized and covalently coupled to nuclear acceptor proteins. Poly(ADP-ribosyl)-ation has been implicated in the regulation of a diverse array of cellular processes ranging from DNA repair, chromatin organization, transcription, replication to protein degradation (Erdelyi et al., 2005) (**Figure 1.4**).

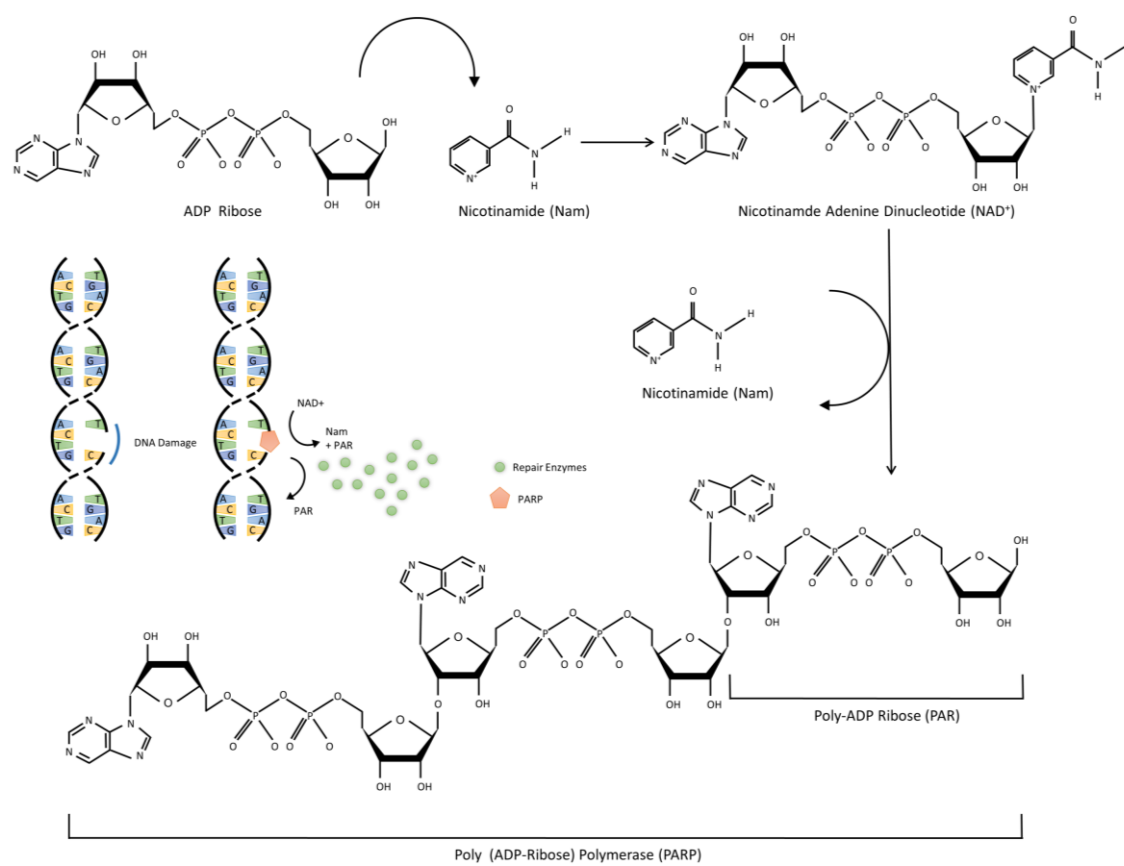
Since the discovery that PARP inhibitors are lethal in cells with DNA repair defects (Curtin, 2014) several PARP inhibitors have entered clinical trials and have shown promising activity in breast, ovarian and other cancers associated with defects in homologous recombination DNA repair. PARP inhibitors have been evaluated in clinical trials as single agents as well as in combination with DNA damaging therapies (Kummar et al., 2012). In animal models, PARP1 knockout has shown to significantly impair DNA repair following damage via radiation (Shall and de Murcia, 2000) and cytotoxic insult (Masutani et al., 2000). Furthermore combining low-dose radiotherapy with PARP inhibition has been shown to enhance anti-tumour

efficacy through potentiating DNA damage (Reiss et al., 2015). In phase I clinical trials enhanced DNA damage has been identified in tumor biopsies and circulating tumor cells following PARP inhibition in patients with chemotherapy-refractory solid tumours already receiving the topoisomerase I inhibitor, topotecan (Kummar et al., 2011). In phase II trials the PARP inhibitor, olaparib demonstrated single agent activity in both breast and ovarian cancer with an overall response rate of approximately 40% in patients with germline mutations in BRCA1/2 (Audeh et al., 2009; Tutt et al., 2009).

Although early cell culture studies concluded that microsatellite instable (MSI) colorectal tumours deficient in double strand break repair showed higher sensitivity to PARP1 inhibition (Vilar et al., 2011), phase II trials using PARP inhibitor olaparib in previously treated MSI and microsatellite stable (MSS) CRC patients failed to show any anti-cancer activity (Leichman et al., 2016). Following treatment with olaparib, median progression free survival for all patients was 1.84 months with no statistically significant differences MSS and MSI groups (Leichman et al., 2016). However, PARP inhibitors have been shown to restore sensitivity of resistant tumours to methylating agents or topoisomerase I inhibitors (Tentori et al., 2005).

Therefore, the use of DNA-damaging chemotherapy and/or radiation therapy in combination with PARP inhibitors should be tested in further studies. Furthermore, PARP inhibitors may also provide protection from dose-limiting side-effects exerted by certain anti-cancer drugs, which trigger oxidative stress and consequent PARP over-activation (Tentori et al., 2005).

Figure 1.4 PARP inhibitors. Poly(ADP-ribose) polymerase (PARP) is a critical enzyme in the repair of DNA strand breaks. PARP-1 uses Nicotinamide Adenine Dinucleotide (NAD⁺), comprised of an ADP Ribose molecule bound to Nicotinamide (Nam), as a substrate to synthesize Poly-ADP Ribose Polymerase (PAR). DNA damage results in the recruitment and binding of PARP-1 to the site of damage, triggering the formation of long branched Poly-ADP Ribose (PAR) chains. PAR promotes recruitment of DNA repair proteins involved in the base excision repair pathway to the site of DNA damage, and facilitates removal of PARP-1 from damage sites, allowing access to other repair proteins. Olaparib is a potent inhibitor of both PARP-1 and PARP-2.



1.3 Gastrointestinal Side-Effects of Chemotherapy

Although chemotherapy has greatly improved overall survival for CRC, cytotoxic side-effects are a significant hurdle greatly impeding the clinical application of otherwise beneficial therapies (Xue et al., 2011;Iwamoto, 2013). Gastrointestinal (GI) side-effects such as nausea, vomiting, ulceration, bloating, constipation and diarrhoea are major obstacles causing delays, adjustments and discontinuation of treatment whilst greatly impacting quality of life in many CRC patients (Benson et al., 2004;Stringer et al., 2007;Denlinger and Barsevick, 2009;Stringer et al., 2009d;Peterson et al., 2011). Although specific chemotherapeutic agents have been correlated with heightened incidence of GI side-effects (**Table 1.1**), incidence as high as 40% in patients receiving standard dose chemotherapy and 100% in patients receiving high dose chemotherapy have been reported (McQuade et al., 2014). Furthermore, the incidence of chronic post-treatment constipation and diarrhoea amongst cancer survivors has been estimated to be as high as 49% with episodes persisting up to 10 years after the cessation of treatment (Schneider et al., 2007;Denlinger and Barsevick, 2009;Kim et al., 2012). The underlying mechanisms of chemotherapy-induced constipation (CIC) and diarrhoea (CID) remain unclear. Although mucositis presenting as inflammation and ulceration of the intestinal epithelium is a significant contributing factor, the pathophysiology of CID and CIC is likely to be complex, involving several overlapping inflammatory, secretory and neural mechanisms.

Table 1.1 Gastrointestinal side-effects of chemotherapy

| Mechanisms | Chemotherapeutic agents | Cancer type | Gastrointestinal Side-Effects |
|-------------------------|--------------------------------|--|--|
| Alkylating Agents | Cisplatin | Lung, Breast, Stomach, Colorectal, Liver | Nausea, Vomiting, Diarrhoea , Constipation (Ilson et al., 1999; Ardizzoni et al., 2007) |
| | Oxaliplatin | Colorectal, Breast, Stomach | Nausea, Vomiting, Diarrhoea , Constipation (Extra et al., 1990; Kim et al., 2003) |
| Antimetabolites | 5-Fluorouracil | Breast, Colorectal, Stomach, Liver | Nausea, Vomiting, Abdominal Pain, Diarrhoea (Douillard et al., 2010; Boussios et al., 2012) |
| | Capecitabine | Colorectal, Breast, Stomach | Nausea, Vomiting, Diarrhoea (Walko and Lindley, 2005; Stathopoulos et al., 2007; Boussios et al., 2012) |
| Topoisomerase inhibitor | Irinotecan | Colorectal, Breast, Stomach, Lung | Nausea, Vomiting, Acute and Delayed Diarrhoea (Hecht, 1998) |

1.3.1 Chemotherapy-Induced Diarrhoea (CID)

Diarrhoea is a frequently under-recognised clinical issue that significantly affects morbidity and mortality of cancer patients worldwide (Maroun et al., 2007). Prevalence and severity of CID vary greatly depending on chemotherapeutic regime administration and dosage. A direct correlation between cumulative dose and severity of CID has been recognised, with high dose regimens associated with heightened incidence of CID (Verstappen et al., 2003). Although the likelihood of CID is typically unpredictable, occurrence of CID has been linked to a variety of patient and treatment-associated risk factors (**Table 1.2**). It has been found that age >65, gender (female) and low functional performance status (ECOG>2) are common patient-associated risk factors as well as associated bowel pathologies and genetic polymorphisms that may affect drug metabolism (Richardson and Dobish, 2007). Certain regimens, especially those containing 5-FU and IRI are associated with rates of CID of up to 80% (Benson et al., 2004; Richardson and Dobish, 2007) with one third of patients experiencing severe (grade 3 or 4) diarrhoea (**Table 1.3**) (Maroun et al., 2007).

CID severely interferes with anti-cancer treatment, resulting in treatment alterations in approximately 60% of patients, dose reductions in 22% of patients, dose delays in 28% of patients and complete termination of treatment in 15% of patients (Arbuckle et al., 2000; Dranitsaris et al., 2005). Persistent and severe chemotherapy-associated diarrhoea is correlated with significant malnutrition and dehydration resulting in concomitant weight loss (cachexia), fatigue, renal failure, haemorrhoids and perianal skin breakdown (Mitchell, 2006; Shafi and Bresalier, 2010). CID related dehydration is linked to early death rates in roughly 5% of patients undergoing anti-cancer treatment (Rothenberg et al., 2001). Further to this, chemotherapeutic administration may also prompt severe intestinal inflammation, bowel wall thickening and ulceration (Kuebler et al., 2007a) contributing to clinical disruptions with potentially life-threatening

ramifications (Rothenberg et al., 2001;Benson et al., 2004;Stein et al., 2010).

For over 30% of CID sufferers, CID interferes with their daily activities (Stein et al., 2010), with detrimental effects on the mental and social health of cancer survivors. Persistent and uncontrollable CID has been linked to anxiety, depression, social isolation and low self-esteem (Viele, 2003), emphasising the importance of both elucidating the underlying mechanisms of CID and improving treatment efficacy (Carelle et al., 2002).

Table 1.2 Risk factors for chemotherapy-induced diarrhoea (modified from (Wadler et al., 1998;Saltz, 2003;Dransitsaris et al., 2005;Richardson and Dobish, 2007)

| | |
|-----------------------------------|---|
| Patient-associated risk factors | Elderly (>65) |
| | Female |
| | Genetic polymorphisms affecting drug metabolism and distribution such as, Gilbert's Syndrome, Crigler-Najjar Syndrome |
| | Biliary obstruction |
| | Associated bowel pathology such as inflammatory or malabsorption |
| Treatment-associated risk factors | Irinotecan & 5-FU |
| | Weekly chemotherapy schedule |
| | Infusional chemotherapy |
| | Bolus 5-FU |
| | Prior history of CID |
| | Prior or concomitant radiotherapy |

Table 1.3 Common toxicity criteria for diarrhoea and constipation grading
(adapted from the National Cancer Institute)

| Toxicity | Diarrhoea | Constipation |
|-----------------|---|---|
| Grade 1 | Increase of < 4 stools per day over baseline | Occasional or intermittent symptoms; occasional use of stool softeners, laxatives, dietary modification, or enema |
| Grade 2 | Increase of 4 - 6 stools per day over baseline | Persistent symptoms with regular use of laxatives or enemas indicated |
| Grade 3 | Increase of > 7 stools per day over baseline. Incontinence. Hospitalization | Symptoms interfering with activities of daily living; obstipation with manual evacuation indicated |
| Grade 4 | Life threatening consequences. Urgent intervention indicated | Life-threatening consequences (e.g., obstruction, toxic megacolon) |
| Grade 5 | Death | Death |

1.3.1.1 Pathophysiology of Chemotherapy-Induced Diarrhoea

Although several chemotherapy regimens have been associated with diarrhoea to varying degrees, most basic research into the mechanisms underlying CID has focused on irinotecan and its active metabolite SN38 (Gibson and Keefe, 2006). As diarrhoea is a well-recognised side-effect of irinotecan treatment, the histological changes that occur throughout the GI tract in response to irinotecan administration have been examined in several animal studies (Araki et al., 1993; Ikuno et al., 1995; Takasuna et al., 1996; Gibson et al., 2003). Pronounced crypt ablation, villus blunting and epithelial atrophy in the small and large intestines have been reported (Logan et al., 2008), resulting in mucosal damage and degeneration being a major theme throughout the literature surrounding CID. Although patients do not routinely have imaging or endoscopy to diagnose the chemotherapy-induced mucosal inflammation (Toucheffeu et al., 2014), CID is still largely believed to be a form, or by-product, of GI mucositis. Mucositis is defined as mucosal injury presenting as inflammation and ulceration, resulting in alterations of intestinal microflora and GI secretion (Stringer, 2009; Stringer et al., 2009a; Stringer et al., 2009b). The basic pathophysiology of mucositis can be broken into 5 sequential phases: i) initiation; ii) up-regulation; iii) signalling and amplification; iv) ulceration and inflammation; and v) healing (Sonis et al., 2004; Lee et al., 2014).

Initiation of mucositis is believed to result from direct or indirect effects of cytotoxic chemotherapeutics on the rapidly dividing epithelial cells in GI tract, triggering apoptosis. This leads to reductions in crypt length and villus area, coupled with activation of nuclear factor-kappa B (NFkB) and subsequent up-regulation of pro-inflammatory cytokines including interleukin 1 (Lawrence, 2009), which contribute to ulceration and inflammation in the mucosal epithelium (Gibson et al., 2003; Stringer et al., 2007; Logan et al., 2008; Stringer et al., 2008; Stringer et al., 2009a; Stringer et al., 2009d). Intestinal microbiota are known to play an integral role in intestinal homeostasis and are now believed to play a key role in the development of mucositis (van Vliet et al., 2010; Toucheffeu et al., 2014).

Recent studies have revealed that chemotherapeutic administration has effects on intestinal microbial composition (Stringer et al., 2009a;Stringer et al., 2009b), and faecal microbiota (Touchefeu et al., 2014).

Much of the research investigating the effects of chemotherapeutic administration on microbiota has focused on the topoisomerase I inhibitor, irinotecan, due to the involvement of microbiota in its metabolism (Stringer, 2013). Upon metabolism in the liver, irinotecan is converted to its active metabolite SN-38 by enzyme carboxylesterase, before being deactivated through glucuronidation by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) to form SN38 glucuronide (SN38-G). However, SN38G may be reactivated to SN38 in the presence of enzyme β -glucuronidase, which may be produced by the intestinal microbiome. Several studies have shown a shift in commensal bacteria, in particular *Bifidobacterium spp.*, towards *Salmonella spp.* and *Escherichia coli* following irinotecan administration (Stringer et al., 2009b). Of the β -glucuronidase-producing bacteria, *Bacteroides spp.* have been shown to decrease following irinotecan treatment, concurrently *Staphylococcus spp.*, *Clostridium spp.* and *E. coli* have been found to be increased, whilst beneficial bacteria, *Lactobacillus spp.* and *Bifidobacterium spp.* were decreased following irinotecan treatment (Stringer et al., 2007). When given in combination with antimetabolite 5-fluorouracil, both *Clostridium cluster XI* and *Enterobacteriaceae* presence were found to be increased following combined irinotecan and 5-fluorouracil treatment, whilst treatment with 5-fluorouracil alone has also been found to increase the presence of *Clostridium spp.* and *Staphylococcus spp.* at 24 h post treatment (Stringer et al., 2009c).

These changes in microbiota are believed to play an important role not only in maintaining intestinal homeostasis and integrity but in the modulation of inflammatory responses through interaction with Toll-like receptors and the nucleotide oligomerisation domain receptors that activate NF κ B (van Vliet et al., 2010). In the healing phase, proliferation and differentiation of the GI epithelium return approximately two weeks post chemotherapy (Sonis et al.,

2004;Lee et al., 2014), but functional changes persist after recovery from morphological changes (Keefe et al., 2000;Rubenstein et al., 2004). The pathophysiology underlying these persistent changes in GI functions includes several overlapping secretory, osmotic, inflammatory and neurogenic mechanisms (McQuade et al., 2014).

Disruption to water and electrolyte balance within the GI tract is a key component in the pathophysiology of all types of diarrhoea. Direct mucosal damage has been suggested as a major contributor to malabsorption and hypersecretion associated with CID (Takasuna et al., 1995;Richardson and Dobish, 2007;Stringer et al., 2007;Stringer et al., 2009b;Stein et al., 2010). Studies using animal models of CID have demonstrated increased apoptosis in the crypts of both the jejunum and colon, resulting in metaplasia of goblet cells and excessive mucous secretion (Ikuno et al., 1995;Gibson et al., 2003). Hyperplasia of the rapidly dividing crypt cells in the epithelium of the gut probably results in heightened proportions of immature secretory cells, leading to increased secretion and decreased absorptive capacity of the villi, thereby contributing to the onset of diarrhoea (Castro-Rodríguez et al., 1997). Retention of non-absorbable compounds within the lumen triggers an osmotic shift of water into the lumen (Castro-Rodríguez et al., 1997;Richardson and Dobish, 2007;Stringer et al., 2007). This reduced absorptive capacity and increased secretion in the small intestines results in increased fluid and solutes in the intestinal lumen and overwhelms the absorptive capacity of the colon resulting in diarrhoea (Gibson and Keefe, 2006).

Secondary to mucosal damage, CID has been associated with mucosal inflammation throughout the GI tract (Logan et al., 2008). Increased expression of cyclooxygenase (COX)-2, associated with increased release of prostaglandin E2 (PGE2), is seen in rat colon following irinotecan administration (Yang et al., 2005). PGE2 stimulates colonic secretion and hyperperistalsis of the gut, whilst inhibiting sodium, potassium and adenosine triphosphatase and triggering excessive chloride secretion, all of which further contribute to the onset of diarrhoea (Kase et al., 1997a;Kase

et al., 1997b; Leahy et al., 2002; Yang et al., 2005). Further, irinotecan stimulates the production of thromboxane A₂, a potent physiological stimulant of chloride and water secretion in the colon (Sakai et al., 1997; Suzuki et al., 2000) as well as tumour necrosis factor- α (TNF- α) a pro-inflammatory cytokine and a primary mediator of immune regulation associated with CID (Yang et al., 2005).

While mucosal damage is clearly important, movement of fluid between the lumen of the intestine and the body fluid compartments is a complex and tightly regulated process involving neural, endocrine, paracrine, and autocrine systems that act via the enteric neurons within the submucosal plexus (Lundgren et al., 2000; Johnson et al., 2012). The role of enteric neurons in the pathophysiology of chemotherapy-induced gastrointestinal dysfunction will be discussed in section 1.4.2 below.

1.3.1.2 Current Treatments for Chemotherapy-Induced Diarrhoea

CID may be classified as uncomplicated (grade 1-2 with no complications) or complicated (grade 3-4 with one or more complicating signs or symptoms), early onset (< 24 hours after administration) or late onset (>24 hours after administration) and as persistent (present for >4 weeks) or non-persistent (present for <4 weeks) according to the National Cancer Institute's Common Terminology Criteria for Adverse Effects grading system (Stein et al., 2010). Although uncomplicated CID can be managed by modification of the diet and administration of standard anti-diarrhoeal drugs such as loperamide, octreotide and tincture of opium, complicated diarrhoea requires aggressive high dose anti-diarrhoeal administration and hospitalisation (McQuade et al., 2014). The recommendations on the management of CID were published in 1998 and updated in 2004 (Wadler et al., 1998; Ferrell and Coyle, 2006), providing guidelines for evaluation and management of CID. These guidelines have not been updated since 2004. Currently the only drugs recommended in the updated treatment guidelines

are opioid derivatives such as loperamide and deodorized tincture of opium (DTO), and octreotide.

Loperamide

Loperamide is a non-analgesic agonist that acts at μ -opioid receptors at the level of the myenteric plexus to decrease intestinal motility (Regnard et al., 2011). High dose loperamide alleviates diarrhoea associated with chemotherapeutic administration (Stein et al., 2010). However, its use leads to a range of side-effects including severe constipation, abdominal pain, dizziness, rashes as well as worsening of already present bloating, nausea and vomiting (Lenfers et al., 1999; Stein et al., 2010). High dose loperamide is reported to increase incidents of paralytic ileus, in association with abdominal distension (Sharma et al., 2005; Richardson and Dobish, 2007). Despite these severe side-effects, loperamide remains the standard first line therapy for CID.

Octreotide

Octreotide is a synthetic somatostatin analogue that promotes absorption by inhibiting specific gut hormones to increase intestinal transit time (Högenauer et al., 2002; Mitchell, 2006) as well as hyperpolarizing enteric secretomotor neurons (Högenauer et al., 2002). Octreotide is administered to treat both complicated diarrhoea and loperamide-refractory diarrhoea and is generally reserved as a second line treatment for patients who are unresponsive to loperamide after 48 hours, despite loperamide dose escalation (Regnard et al., 2011). Although octreotide decreases CID effectively, severe side-effects including slow and/or uneven heartbeat, severe constipation, stomach pain, enlarged thyroid, vomiting, nausea, headache and dizziness occur in over 10% of patients (Bhattacharya et al., 2008).

Deodorised Tincture of Opium

Deodorized tincture of opium (DTO) is another widely used antidiarrheal agent, despite the absence of literature to support its use in CID treatment (Stein et al., 2010). Like loperamide, DTO activates μ -opioid receptors within the GI tract inhibiting intestinal peristalsis, increasing intestinal transit time and promoting fluid reabsorption (Richardson and Dobish, 2007). The efficacy of DTO in treatment of CID has not been reported, but it is a commonly used anti-diarrhoeal drug and may be considered as a second-line therapy for persistent and uncomplicated diarrhoea (Richardson and Dobish, 2007). DTO contains 10mg/ml of morphine and is one of the most potent forms of orally administered morphine available by prescription. DTO induces many side-effects including euphoria, nausea, vomiting, painful/difficult urination, stomach and abdominal pain, seizures and allergic reactions. Further, DTO administration associates with psychological and physical dependence, miosis, respiratory depression (Benson et al., 2004; Richardson and Dobish, 2007) and constipation, with continued/prolonged opioid use linked to severe constipation (Ricardo Buenaventura et al., 2008).

1.3.2 Chemotherapy-Induced Constipation

Constipation is a frequent, and underestimated, complication in patients with advanced cancer (Mancini and Bruera, 1998). As constipation is a subjective sensation, there is difficulty surrounding acceptance of a universal definition, although it is broadly recognised clinically as a mixture of reduced frequency of bowel action and increased stool consistency (Connolly and Larkin, 2012). Constipation occurs in 50-87% of advanced cancer patients (Abernethy et al., 2009). Constipation is the third most common symptom in patients receiving cytotoxic chemotherapy with an overall prevalence of 16%, with 5% classified as severe and 11% classified as moderate (Yamagishi et al., 2009; Anthony, 2010).

The mechanisms underlying CIC are poorly defined with minimal clinical studies existing. Distinguishing true CIC from secondary constipation from drugs given to control other chemotherapy or cancer-induced symptoms (such as anti-emetics for nausea and vomiting and opioids for pain) is a major issue hindering investigation (Gibson and Keefe, 2006). Given the scarcity of literature concerning CIC it is hard to estimate accurate incidence and severity among all chemotherapy-treated cancer sufferers, but specific chemotherapeutic agents such as thalidomide, CISP and vinca alkaloids such as vincristine, vinblastine and vinorelbine induce true CIC in up to 80-90% of patients (Ghobrial and Rajkumar, 2003).

Constipation is not deemed to be of clinical importance until it causes physical risks or impairs quality of life. Constipation can cause a number of significant symptoms. Severely constipated patients experience abdominal distension usually accompanied by severe abrupt episodes of abdominal pain (Falcón et al., 2016). Furthermore, rectal tearing, haemorrhoids and rectal fissures caused by passing hard, dry stool are frequent complications of constipation (Leung et al., 2011). Untreated constipation may progress to obstipation, severe persistent constipation, which can have life threatening complications associated with faecal impaction and bowel obstruction (Leung et al., 2011). Faecal impaction, the presence of unpassable masses of stool, and increases in intraluminal pressure within the bowel can lead to ischaemic necrosis of the mucosa, pain, bleeding and perforation. Faecal impaction is also well recognized as a factor in urinary incontinence in the elderly (MacDonald et al., 1991). Constipation can also cause confusion, increase retroperitoneal or liver pain, trigger rapid onset nausea with or without vomiting in the presence of intestinal blockage and lead to inadequate absorption of oral drugs (Mancini and Bruera, 1998), greatly affecting the tolerability and efficacy of chemotherapeutic administration. There is accumulating evidence that self-reported constipation and functional constipation lead to significant impairment of quality of life, with the implication that this is a serious condition in the majority of people afflicted (Talley, 2003; Dennison et al., 2005), however little work has been undertaken to elucidate prevalence and mechanisms.

1.3.2.1 Pathophysiology of Chemotherapy-Induced Constipation

Normal bowel function requires the coordination of motility, mucosal transport, and defecation reflexes (Mancini and Bruera, 1998). Constipation can be broadly classified into three categories: normal-transit constipation, defecatory disorders and slow-transit constipation (Lembo and Camilleri, 2003). Normal-transit constipation is the most common form, in which frequency of colonic evacuation is normal, yet patients believe they are constipated due to a perceived difficulty with evacuation or the presence of hard stools. Symptoms of normal-transit constipation include bloating and abdominal pain or discomfort, as well as increased psychosocial distress (Ashraf et al., 1996). Constipation resulting from defecatory disorders is most commonly due to dysfunction of the pelvic floor or anal sphincter. Defecatory disorders may result from prolonged avoidance of the pain associated with the passage of a large, hard stool or painful anal fissure or haemorrhoid (Loening-Baucke, 1996). Structural abnormalities, such as rectal intussusception, rectocele, obstructing sigmoidocele, and excessive perineal descent, are less common causes of defecatory disorders (Lembo and Camilleri, 2003). Slow-transit constipation is associated with infrequent urge to defecate, bloating, and abdominal pain or discomfort.

Though little clinical research has been undertaken to elucidate the underlying pathology in CIC, it has been hypothesized that CIC may result from effects of chemotherapy on nerve endings in the gut (Ghobrial and Rajkumar, 2003). The role of the enteric nervous system in the pathophysiology of chemotherapy-induced gastrointestinal dysfunction will be discussed in section 1.4.2 below.

1.3.2.2 Opioid-Induced Constipation

As previously mentioned, a major limitation in the estimation and evaluation of true CIC is the onset of secondary constipation, namely opioid-induced constipation produced by opioid analgesia. Whilst opioid analgesics are the gold standard in pain relief for cancer patients, adverse effects such as opioid-induced bowel dysfunction (OIBD) and opioid-induced constipation (OIC) severely compromise their therapeutic potential (Gonzalez and Halm, 2016). Incidence of OIC ranges from 50%-87% in terminally ill cancer patients and is positively associated with chronic opioid treatment (Abernethy et al., 2009). Opioid receptors are located throughout the central and peripheral nervous system and are involved in pain transmission (Camilleri, 2011). In the GI tract, μ -receptors are widely distributed throughout the ileum, stomach and proximal colon where they contribute to the control of fluid and electrolyte transport as well as motility (McKay et al., 1981; Fickel et al., 1997). Opioid analgesics interfere with GI motility by delaying transit, stimulating non-propulsive motility and altering GI segmentation and tone through their effects on enteric neurons (De Schepper et al., 2004; Wood and Galligan, 2004). These changes coupled with activation of mucosal sensory receptors that trigger a reflex arc that facilitates excessive fluid reabsorption, resulting in OIC (Panchal et al., 2007; Camilleri, 2011). Whilst prophylaxis remains the first-line management option for OIC, followed by administration of laxatives if constipation persists, these interventions alone are frequently ineffective (Gatti and Sabato, 2012). Selective μ -opioid receptor antagonists are emerging as a promising first line treatment for OIC, in particular treatment with methylnaltrexone bromide has been found to improve GI transit in chronically ill patients and has been recommended for use in cancer patients (Gatti and Sabato, 2012). Methylnaltrexone reduces opioid-induced delay in the oral–caecal transit time and induces laxation in both healthy subjects and advanced illness patients (Culpepper-Morgan et al., 1992; Thomas et al., 2008). Similarly, treatment with the peripheral μ -opioid receptor antagonist Alvimopan increases the frequency of spontaneous

bowel movements in non-cancer patients with opioid induced bowel dysfunction (Webster et al., 2008).

1.3.2.3 Current Treatments for Chemotherapy-Induced Constipation

The management of constipation can be divided into general interventions and therapeutic measures. The general interventions involve increasing physical exercise, fluid intake and fibre consumption, availability of comfort, privacy and convenience during defecation as well as elimination of medical factors that may be contributing to constipation (Mancini and Bruera, 1998). Therapeutic interventions for the management of constipation, including CIC involve the administration of both oral and/or rectal bulk-forming, emollient, osmotic/saline, stimulant, and lubricant laxatives (Connolly and Larkin, 2012). Laxative compounds may fall into one of several categories depending on their mechanism of action.

Bulk-Forming Laxatives

Bulk-forming laxatives such as methylcellulose, psyllium, and polycarbophil most closely mimic the physiologic mechanisms involved in promoting GI evacuation. Available as natural or semisynthetic hydrophilic polysaccharides, cellulose derivatives, or polyacrylic resins, bulk forming laxatives work by either dissolving or swelling in the intestines to form a viscous liquid that provides mechanical distension. This facilitates the passage of intestinal contents by stimulating peristalsis and reducing GI transit time. Although typically recommended as initial therapy for most forms of mild constipation (Kirschenbaum, 2001), bulk-forming agents can take up to 72 hours to exert their effects and therefore are not ideal for the initial management of symptomatic constipation in cancer patients (Avila, 2004; Connolly and Larkin, 2012). Bulk forming laxatives require the patients to drink extra fluids as otherwise a viscous mass may form and aggravate a partial bowel obstruction. In addition, significant allergy to these substances has been reported, and their effectiveness in severe constipation is doubtful

(Klaschik et al., 2003). Though they are considered safe, some patients' experience suggests that they may worsen symptoms, causing distension, bloating and abdominal pain (Costilla and Foxx-Orenstein, 2014).

Osmotic Laxatives

Osmotic laxatives such as lactulose, sorbitol, polyethylene glycol compounds, and saline laxatives (magnesium hydroxide), attract and retain fluid within GI tract (Twycross et al., 2012). Osmotic laxatives include salts of poorly absorbable cations (magnesium), anions (phosphate, sulfate) as well as molecules that are not absorbed in the small bowel but are metabolized in the colon (lactulose and sorbitol) and metabolically inert compounds such as polyethylene glycol. The presence of these molecules in the lumen results in water retention to maintain normal osmolarity of the stool (Costilla and Foxx-Orenstein, 2014). The laxative effect of these agents depends on the extent to which they remain in the lumen with the onset between 24 to 72 hours (Xing and Soffer, 2001). Adverse effects such as abdominal pain, flatulence, cramping and distension can arise shortly after ingestion, although side-effects may subside after several days of treatment, higher lactulose doses can induce bloating and colic (Ford and Suarez, 2011; Costilla and Foxx-Orenstein, 2014). Excessive use of osmotic laxatives may result in hypermagnesemia, hyperphosphatemia, hypercalcemia, hyponatremia, hypokalemia and hypoalbuminemia (Xing and Soffer, 2001; Kurniawan and Simadibrata, 2011).

Emollient (Stool Softener) Laxatives

Emollient laxatives, also known as stool softeners, are anionic surfactants increasing wetting efficiency of intestinal fluids and facilitating the mixing of aqueous and fatty substances within the faeces; this softens the faeces allowing them to move more easily through the GI tract (Avila, 2004). Stool softeners are of little value when administered unaccompanied in the treatment of long-term constipation as they do not stimulate peristalsis and evacuation, but concurrent administration with bulk-forming agents and

dietary fibre provides beneficial effect reducing straining (O'Mahony et al., 2001;Avila, 2004). Increased fluid intake is essential during treatment with emollient laxatives to facilitate stool softening and so they are not ideal for chronic constipation in cancer patients. Docusate is the most widely used emollient laxative produced as docusate calcium, docusate sodium, and docusate potassium. The onset of action is 1-2 days after administration, but might be up to 5 days. However, docusates have been found to enhance GI or hepatic uptake of other drugs, increasing the risk of hepatotoxicity (Xing and Soffer, 2001). There is also some evidence that docusates cause significant neuronal loss in the myenteric plexus (Fox et al., 1983) and cause structural changes in the gut mucosa of humans (Xing and Soffer, 2001), but the clinical significance of this remains unclear.

Stimulant Laxatives

Stimulant laxatives such as diphenylmethane derivatives (phenolphthalein, sodium picosulfate, anthranoids (senna and cascara), ricinoleic acid (castor oil), and surface-acting agents directly stimulate myenteric neurons to increase peristalsis resulting in reduced net absorption of water and electrolytes from the intraluminal contents (Twycross et al., 2012). Stimulant laxatives are more potent than bulk-forming and osmotic laxatives and appear to be more effective than enemas in stimulating defecation (Dosh, 2002;Scarlett, 2004). They are amongst the most commonly administered laxatives for opioid-induced constipation (Ruston et al., 2013). Although short-term use is safe, overuse can cause dehydration and long-term ingestion may result in laxative dependence. This dependence, also known as 'laxative bowel', is thought to result from damage to the myenteric plexus and smooth muscle cells in the colon (Xing and Soffer, 2001;Kurniawan and Simadibrata, 2011).

Lubricant Laxatives

Lubricant laxatives emulsify themselves into the faecal mass, coating the faeces and rectum for easier passage whilst retarding colonic water

absorption to simultaneously soften stool (Avila, 2004). Liquid paraffin, also known as mineral oil, is the major lubricant laxative in use although seed oils from croton and arachis are also available (Xing and Soffer, 2001). These laxatives can be administered orally or rectally and are useful for patients who complain of excess straining, but long-term use is associated with malabsorption of fat soluble vitamins and minerals, as well as anal leakage (Costilla and Foxx-Orenstein, 2014). Lubricant laxatives are not routinely recommended for long-term use due to possible inflammatory conditions such as lipoid pneumonia (Schiller, 1999).

Rectal Laxatives

Rectal laxatives such as bisacodyl (stimulant), sodium phosphate (saline), glycerin (osmotic), and mineral oil (lubricant) (Avila, 2004) are generally accepted not to be regularly used for CIC treatment (Fallon and O'Neill, 1997), but may be necessary alongside digital stimulation for treating faecal impaction or constipation associated with neurogenic bowel dysfunction. Rectal suppository of bisacodyl (stimulant) is most commonly utilised when evacuation of soft stools is needed, while glycerin suppositories are more appropriate when a hard stool needs to be softened (Fallon and O'Neill, 1997). Acute severe constipation might require an administration of rectal laxatives by enema, however rectal suppositories or enemas cannot be used in patients with neutropenia and thrombocytopenia (O'Mahony et al., 2001).

1.3.3 Emerging and Potential Treatments for CID and CIC

As current therapies for CID and CIC have limited efficacy and a plethora of adverse effects, a search for and use of novel anti-diarrhoeal and laxative agents is essential to improve quality of life and chemotherapeutic efficacy for cancer patients. Several emerging and already existing therapies used for treatment of other conditions such as diarrhoea predominant irritable bowel syndrome (IBS-D), constipation predominant irritable bowel

syndrome (IBS-C) and chronic idiopathic diarrhoea and constipation could be employed for the treatment of CID and CIC.

Chloride Channel Inhibition and Activation

Chloride is an essential ion in intestinal secretion and absorption. Secretory diarrhoea, such as that experienced in IRI-treated patients, results from a combination of excessive secretion and reduced absorption in the intestinal lumen (Thiagarajah and Verkman, 2012). Excessive fluid secretion is driven by active chloride secretion, followed by secondary movement of water and sodium into the intestine. Although there is a lack of selective potent inhibitors of voltage gated chloride channels, inhibition of calcium-activated chloride channels throughout the intestines successfully reduced secretion of chloride into the intestinal lumen (Thiagarajah and Verkman, 2013; Thiagarajah et al., 2015). In a mouse model of rotavirus-induced severe secretory diarrhoea, inhibition of calcium-activated chloride channels with a red wine extract reduced intestinal fluid secretion, diminishing the symptoms of diarrhoea (Ko et al., 2014).

Conversely, chloride channel activation has been used in the management of chronic idiopathic constipation and constipation related to irritable bowel syndrome (IBS-C). Lubiprostone is a bicyclic fatty acid derived from prostaglandin E1 that specifically activates chloride channels in the intestine, whilst having no effect on smooth muscle contraction (Jun, 2013). The underlying mechanism of lubiprostone involves stimulation of electrogenic chloride secretion through activation of chloride channel type-2 (Lacy and Levy, 2007) and cystic fibrosis transmembrane conductance regulator chloride channels (Bijvelds et al., 2009) in the apical membrane of intestinal epithelial cells. Activation of these epithelial channels results in active secretion of chloride into the intestinal lumen followed by a passive secretion of electrolytes and water increasing the liquidity of the luminal contents (Jun, 2013). Resulting luminal distension from increased intestinal fluid content promotes GI motility and increases intestinal and colonic transit. In healthy volunteers, daily lubiprostone delays gastric emptying,

increases fasting gastric volume, reduces maximum tolerated gastric volume and accelerates small bowel and colon transit (Camilleri et al., 2006). In randomised trials involving patients with IBS-C, lubiprostone twice daily reduced abdominal pain and increased complete spontaneous bowel movement and improved stool consistency, straining, and bloating (Schey and Rao, 2011). Currently, oral lubiprostone is approved for IBS-C at 8 µg twice daily and CIC at doses of 24µg twice daily, but approval for CIC is limited to only women who have not responded to laxatives (Davis and Gamier, 2015). At present there are no studies investigating the efficacy of lubiprostone for CID.

Cannabinoid Receptor Inhibition and Activation

Cannabinoids mediate their effects via binding to two main G-protein coupled receptors, CB₁ and CB₂, widely expressed in the GI tract (Abalo et al., 2012). Although the activity of the endocannabinoid system varies between species and different regions of the GI tract within the same species, activation of CB₁ receptors coupled to cholinergic motor neurons has been found to inhibit excitatory neuromuscular transmission in human colonic circular muscle (Hinds et al., 2006) and inhibit colonic propulsion in mice and rat (Pinto et al., 2002; Abalo et al., 2015). In recent human trials, dronabinol, a non-selective cannabinoid receptor agonist, was found to inhibit colonic motility in both healthy subjects (Esfandyari et al., 2006; Esfandyari et al., 2007) and patients with IBS-related diarrhoea (IBS-D) (Wong et al., 2011). Conversely, a CB₁ receptor inverse agonist, taranabant, has been shown to improve symptoms related to slow GI motility and abdominal pain when administered *in vivo* in mice (Fichna et al., 2013). Taranabant increased the number of bowel movements after systemic and oral administration and significantly increased faecal pellet output in mice with constipation induced by ipratropium (Fichna et al., 2013). It has been demonstrated that a low dose of a non-selective cannabinoid agonist WIN55,212-2 reduced the severity of 5-fluorouracil-induced diarrhea in rats (Abalo et al., 2016).

Guanylate Cyclase C Activation

Guanylate cyclase C is the principal receptor for heat-stable enterotoxins and plays a major role in *E. coli*-induced secretory diarrhoea (Camilleri, 2010). Enterotoxins and endogenous peptides bind to guanylate cyclase C and stimulate the production of intracellular cyclic guanosine monophosphate (cGMP). Increased levels of cGMP activate the secretion of chloride ions through the cystic fibrosis transmembrane conductance regulator. Linaclotide is a minimally absorbed 14-aminoacid peptide that selectively stimulates intestinal epithelial cell Guanylate cyclase C receptors, resulting in increased intracellular and extracellular cGMP leading to accelerated stool transit and laxation (Harris and Crowell, 2007). In phase II and III placebo-controlled studies in chronically constipated and IBS-C patients, linaclotide was found to accelerate colonic transit and improve abdominal pain and symptoms of constipation (Andresen et al., 2007; Johnston et al., 2008; Johnston et al., 2009; Lembo et al., 2010). Linaclotide is particularly interesting in that it is both a laxative and analgesic, reducing visceral hypersensitivity with very few drug interactions, it is presently licensed for chronic idiopathic constipation and IBS-C in the USA (Davis and Gamier, 2015), but no trials on CIC have been reported to date.

Probiotics, Antibiotics and β -Glucuronidase Inhibitors

With the recognition that intestinal microbiota play key roles in the pathophysiology of mucositis and development of CID/CIC, both antibiotics and probiotics have emerged as promising therapeutic options. Administration of probiotics has been shown to prevent CID in both 5-fluorouracil and irinotecan-treated animals (Bültzingslöwen et al., 2003; Bowen et al., 2007). Similarly, a combination of *Lactobacillus rhamnosus* and fibre has been found to reduce the severity of grade 3/4 5-fluorouracil/leucovorin-induced diarrhoea by 15% in a randomized study of patients treated for colorectal cancer (Österlund et al., 2007). Interestingly,

administration of oral antibiotics such as fluoroquinolone has also been recommended for aggressive treatment of CID (Benson et al., 2004; Maroun et al., 2007).

The selective inhibition of bacterial β -glucuronidase has recently been shown to alleviate drug-induced gastrointestinal toxicity in mice (Wallace et al., 2015). A low-potency β -glucuronidase inhibitor showed promise in 2004 in reducing the GI toxicity associated with irinotecan in rats (Fittkau et al., 2004). Similarly, oral administration of potent bacterial β -glucuronidase inhibitors has been found to reduce the severity of irinotecan-induced toxicity (Wallace et al., 2010). In clinical trials, Kampo medicine Hangeshashinto (TJ-14) which contains baicalin, a β -glucuronidase inhibitor, has been found to successfully reduce both the incidence and duration of chemotherapy-induced oral mucositis in CRC patients when compared to placebo (Matsuda et al., 2015). In non-small-cell lung cancer patients Hangeshashinto alleviated irinotecan-induced diarrhoea (Mori et al., 2003). Compared with control patients, the TJ-14-treated patients showed a significant improvement in both diarrhoea grade, as well as a reduced frequency of grade 3 and 4 diarrhoea (Mori et al., 2003).

Thus, CID and CIC are amongst the most common chemotherapy-induced GI toxicities, heavily contributing to treatment delays, dose reductions and in some cases cessation of anti-cancer treatment, greatly effecting management and clinical outcomes. Current treatments for CID and CIC are limited and come with a profuse amount of concomitant symptoms; however novel therapies present a promising avenue of treatment for CID and CIC. Identification of potential targets and the development of novel treatments alleviating chemotherapy-induced toxicity are essential to improve clinical outcomes and quality of life amongst cancer sufferers. One such target is the enteric nervous system innervating gastrointestinal tract and controlling its functions.

1.4 Neural Control of Gastrointestinal Functions

The GI tract is innervated by the intrinsic ENS together with fibres from extrinsic sympathetic, parasympathetic (vagus nerve and pelvic nerve) and sensory afferent neurons (Phillips and Powley, 2007). Both extrinsic and intrinsic innervations play important roles in the motor activity of the GI tract. Damage to the ENS has been linked to a great number of GI pathologies.

1.4.1 The Enteric Nervous System

Sometimes referred to as “the second brain” due to its ability to function autonomously of the central nervous system, the ENS is the intrinsic innervation of the gastrointestinal tract, comprised of ganglia, primary interganglionic fibre tracts as well as secondary and tertiary fibres which project to many of the effector systems of the gut including muscle cells, glands and blood vessels (Hansen, 2003;Furness, 2012). The ENS can be divided into 2 major ganglionated plexi, the myenteric (Auerbach's) and submucosal (Meissner's), which are responsible for controlling gut functions including absorption, secretion, motility and vascular tone. Furthermore, within a given plexus enteric neurons can be classified on the basis of their morphology, neurochemistry, biophysical properties, projections and connectivity (Costa and Brookes, 2008).

1.4.1.1 The Submucosal Plexus

Situated superficially to the mucosa, the submucosal plexus is located between the circular muscle and muscularis mucosa layers of the mucosa and receives inputs from the myenteric plexus as well as the sympathetic and parasympathetic nerve fibres. The neurons in the submucosal plexus innervate the mucosal epithelium and submucosal arterioles to control and maintain water and electrolyte balance, secretion and vascular tone (Mourad et al., 2003;Bornstein et al., 2012;Furness, 2012).

Movement of fluid between the intestinal lumen and blood circulation is a complex and tightly regulated process involving neural, endocrine, paracrine, and autocrine systems (Bornstein et al., 2012). Fluid is absorbed from the lumen containing nutrients via ion-coupled transporters, and returned through secretomotor reflexes. Water and electrolytes drawn from both the circulation and the absorbed fluids are moved from the interstitium of the lamina propria to the lumen. This reflexive control is exerted namely through sympathetic secretomotor and vasodilator pathways (Furness, 2012), however neural control of secretion and absorption of water and electrolytes occurs on multiple interacting levels.

Submucosal secretomotor neurons are directly modulated by circuitry within the myenteric plexus and via long neural reflex pathways that pass through the prevertebral sympathetic ganglia, as well as by parasympathetic and sympathetic pathways coming from the central nervous system (Bornstein et al., 2012). Myenteric interneurons form a longitudinally organized recurrent excitatory network (Neal and Bornstein, 2007;2008) and the 5-hydroxytryptamine (5-HT)/choline acetyltransferase (ChAT) neurons in this network probably also innervate submucosal ganglia. Similarly, myenteric intrinsic sensory neurons (ISNs) provide input to submucosal ISNs (Furness, 2012) and submucosal ISNs project to the myenteric plexus (Kirchgessner et al., 1992) where they influence myenteric ISNs (Smith et al., 1991) suggesting cross talk between the two ISN networks in any localized region of the small intestine (Bornstein et al., 2012).

The secretory reflex is composed of enterochromaffin (EC) cells, ISNs, various interneurons and secretomotor neurons (Christofi, 2008). 5-HT released from EC cells in the mucosa acts on 5-HT₃, 5-HT₄, and/or 5-HT_{1P} receptors on ISNs to activate a secretory reflex (Vanner and Macnaughton, 2004). ISNs detect sensory information from the luminal environment in the form of action potentials carried in their primary afferent process. Input from ISNs converges in the submucosal plexus to activate neuropeptide Y (NPY), vasoactive intestinal peptide (VIP) and choline acetyltransferase (ChAT) immunoreactive (IR) secretomotor and/or interneurons (Vanner

and Macnaughton, 2004). Activation of adenylyl cyclase and resulting increases in intracellular cyclic AMP drives the opening of cystic fibrosis transmembrane conductance regulator and active chloride secretion (Bornstein et al., 2012).

1.4.1.2 The Myenteric Plexus

The myenteric plexus located between the circular and longitudinal muscles of the muscularis externa functions to provide motor innervation to both muscle layers of the gut (Furness, 2012). Myenteric ganglia share similar properties to the CNS including presence of glia, interneurons, small extracellular spaces, dense synaptic neuropil, isolation from blood vessels and multiple synaptic mechanisms and neurotransmitters. Ganglia vary in shape and size depending on function, and may contain anywhere between 5 to 200 neuronal cell bodies (Furness et al., 2014).

Three main classes of myenteric neurons govern the complex motor reflex pathways. These include populations of intrinsic sensory (or primary afferent) neurons that respond to changes in muscle length or tension, mucosal deformation and/or chemical stimuli such as fatty acids and amino acids within the intestinal lumen. There are orally directed (ascending) and anally directed (descending) interneurons, as well as excitatory motor neurons supplying the circular muscle and a separate population of excitatory motor neurons supplying the longitudinal muscle and populations of inhibitory motor neurons supplying each of these muscle layers (Bornstein et al., 2002;Furness et al., 2004;Bornstein, 2006;Gwynne and Bornstein, 2007a). Extrinsic nerves provide terminals largely to the intrinsic ganglionated plexuses rather than directly to the muscle layers of the gut, except in the sphincters and in the striated muscle of the oesophagus (Furness, 2012). Therefore, the extrinsic reflex pathways coordinate the activities of different regions of the GI tract largely by modifying the activity of enteric neurons representing a final common level of control of gastrointestinal function (Bornstein et al., 2002).

1.4.1.3 Myenteric Control of Motor Function

Motor function in the small intestine and colon is controlled by smooth muscle cells, nervous tissues and interstitial cells of Cajal (ICC) (Hasler, 1999). In many species, different patterns of activity occur in fed and fasted states. Early radiometric studies identified several distinct types of propulsive behaviour in the presence of food *in vivo* (Bornstein et al., 2002). These can be broadly described as segmentation, peristalsis and peristaltic rushes. Segmentation may be defined as mixing behaviour and involves rhythmic stationary contractions that repeatedly divide and redivide a mass of intestinal content. In contrast, peristalsis and peristaltic rushes propel the intestinal content in an anal direction, with the difference between them being a matter of degree, duration and velocity. A fourth style of propulsive activity, antiperistalsis has also been identified as propulsion of content in an oral direction (Bornstein et al., 2002).

Smooth muscle cells form an electrical syncytium within the gut and are innervated, directly or indirectly through ICC, by neurons (De Giorgio, 2015). The integration of inputs from neurons and ICC to smooth muscle cells in the GI tract allows expression of various motor patterns including phasic contractile activity (peristaltic and segmenting contractions) and tonic contractile activity (Gwynne et al., 2004;Dinning et al., 2009;Huizinga and Lammers, 2009;Kuizenga et al., 2015).

Peristaltic Contractions

Peristalsis can be defined as gastrointestinal motor patterns involving partial or total occlusion of the lumen that move content in the anal direction (Huizinga and Lammers, 2009). Several complementary and cooperating mechanisms are involved in the generation of peristalsis. The synaptic conversion of motor and interneurons in a single polysynaptic reflex, termed the peristaltic reflex, is only one specific form of peristalsis, orchestrated by the enteric nervous system in response to a bolus where contraction is evoked oral to the bolus and relaxation is observed anal to the bolus. This

reflex has been studied extensively experimentally, and the directional neural pathways (ascending excitatory reflexes that evoke contractions above a bolus; descending inhibitory reflexes which cause relaxations below) which elicit this response have been firmly established (Bayliss and Starling, 1899).

The longitudinal muscle and circular muscle layers of the intestines exhibit rhythmic, phasic contractions that form the basis for peristaltic contractions. These are generated by electrical slow wave activity that originates in the myenteric network of ICC (Lammers, 2005; Sanders et al., 2006; Lee et al., 2007). Muscle depolarization to allow calcium channels to open is the trigger for substantial contractile activity. All peristaltic movements involve contraction of the circular muscle layer, accomplished by means of influx of calcium into smooth muscle cells through voltage-sensitive calcium channels and calcium release from intracellular stores (Huizinga and Lammers, 2009). ICC act to provide rhythmic depolarization of gut musculature, bringing the muscle cells periodically close to threshold for this influx of calcium (Huizinga and Lammers, 2009). Although frequency and propagation direction of phasic contractions is controlled by slow waves, by themselves, ICC-mediated slow waves produce insufficient depolarization to initiate contraction (Hasler, 1999). The three main ways in which further depolarization can be evoked to bring the membrane potential above threshold for calcium influx and hence contraction are; muscle distension, excitatory neurotransmission, or inhibition of inhibitory neurotransmission (Huizinga and Lammers, 2009)

Recording in the longitudinal or circular muscle during firing of the motor neurons reveals depolarising responses of the muscle cells, called excitatory junction potentials (EJPs) (Spencer and Smith, 2001). Recording during firing of inhibitory motor neurons reveals hyperpolarising responses of the muscle cells, called inhibitory junction potentials (IJPs) (Spencer and Smith, 2001). Hence a hard-wired enteric neural circuitry is in place to cause faecal pellet propulsion (Huizinga and Lammers, 2009).

Synaptic transmission in the ascending excitatory reflex pathway is primarily via fast excitatory postsynaptic potentials (EPSPs) largely mediated by acetylcholine acting on nicotinic acetylcholine receptors, but a residual contraction remains in the presence of muscarinic antagonists and this is abolished by tachykinin antagonists (Gwynne and Bornstein, 2007a). Thus, the entire excitatory response is accounted for by acetylcholine and tachykinins (Bornstein et al., 2004), these neurons are immunoreactive for ChAT.

Inhibitory neurons utilize multiple transmitters with the relative importance of each varying with the region of the digestive tract and species (Bornstein et al., 2004). Adenosine triphosphate (ATP), nitric oxide (NO), vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase activating peptide (PACAP) have all been identified as mediators of inhibitory neuromuscular transmission (Lecci et al., 2002). VIP and PACAP are co-localized with nitric oxide synthase (NOS) within inhibitory motor neurones. Transmitters at other synapses in this pathway are yet to be recognized. Suppression of spontaneous output associated with upstream EJPs and downstream IJPs following longitudinal stretch is known as the occult reflex (Dickson et al., 2007).

Although there are minor differences between species in that some of the motor neurones contain additional neurochemical markers (e.g. enkephalin and GABA), this basic pattern is conserved across most species including the guinea-pig, mouse, rat and human (Sang and Young, 1996; Porter et al., 1997; Lomax and Furness, 2000). The above mentioned variations do not appear to have postsynaptic effects or play a significant role in the contraction and relaxation of smooth muscle (Bornstein et al., 2004)

Segmenting Contractions

Segmenting activity, like the peristaltic reflex, is recurrent, however does not result in propulsion of contents, but rather mixing (Huizinga and Chen, 2014). Unlike propulsive patterns in which downstream blocks of the polysynaptic circuit become activated in sequence, segmentation is the

result of coordinated ENS activity. Each stationary constriction is associated with a large EJP mediated by muscarinic receptors that leads to a single action potential at the site of the constriction and time-locked IJPs outside the constricted region (Gwynne and Bornstein, 2007b). This leads to substantial depolarizations of the circular muscle via muscarinic receptors. Each depolarization typically evokes only a single smooth muscle action potential, which contrasts with the depolarizations associated with the propulsive contractions evoked by distension, each of which triggers two or more action potentials (Gwynne and Bornstein, 2007b). On either side of the localized activity of excitatory motor neurons, there is a region in which firing of inhibitory motor neurons is enhanced, manifesting as large IJPs adjacent to the constricted region and time locked to the constrictions. Inhibitory motor neurons are essential for generation of the segmentation motility pattern, activation of inhibitory motor neurons in the receiving segments alongside inactivation of inhibitory motor neurons and activating excitatory motor neurons in the propulsive segments provides the underlying pattern of segmenting activity. The functional electrical syncytial properties of intestinal smooth muscle demand this kind of inhibitory neuronal behaviour due to the fact that the circular muscle in the wall of the receiving segment is part of the same electrical syncytium as the adjacent propulsive segment (Lee et al., 2007). Inhibition prevents electrical excitation and the associated contraction spreading from the contracting propulsive segment into the receiving segment. Because both the excitatory and the inhibitory motor neurons are located within the myenteric plexus (Bornstein et al., 2002), major elements of the neural circuit mediating segmentation are located within this plexus (Gwynne and Bornstein, 2007b).

This role of ICCs in the generation of segmenting contractions is contentious. It has been suggested that the rhythmic contractions characteristic of segmentation are regulated by slow waves generated by ICCs with ENS activity serving a permissive role (Hasler, 1999; Thuneberg and Peters, 2001), although it has been found that segmentation in the

guinea-pig small intestine is independent of ICC-mediated intestinal slow waves (Gwynne and Bornstein, 2007b).

Rate of movement of a meal through the stomach and along the intestine has been correlated with nutritional density. It has been found that chemically inert meals move along the intestine more rapidly than meals with significant nutritive content, with the relative proportions of fat, carbohydrate and protein being significant determining factors. Patterns of contractions within the intestine also vary according to the chemical composition of meals (Bornstein et al., 2002; Gwynne et al., 2004). Nutrient rich meals induce a larger proportion of segmenting contractions than non-nutritive meals (Borgström and Arborelius Jr, 1974; Schemann and Ehrlein, 1986). Although the rate of peristalsis remains unchanged, the length over which these contractions propagate is substantially reduced by increased nutrient density. Thus, segmentation predominates in the presence of nutrients, while peristalsis predominates when chyme is nutrient free (Bornstein et al., 2002).

Motility in the Isolated Colon

Specific motor patterns have been described in *ex vivo* experiments of the isolated colon. When an animal is fasted a complex wave of activity propagates repeatedly, but slowly, along the intestine from the stomach to the caecum. The behaviour is variously known as the migrating motor complex (MMC), migrating myoelectric complex, or the interdigestive motor cycle (Bornstein et al., 2002). The MMC essentially consists of an anally propagating wave of strong excitation of muscle that alternates with a longer period of very low activity or possibly inhibition of the muscle. These two phases (Phase III and Phase I, respectively) are separated by periods of irregular muscular activity (Phases II and IV). MMCs are widely identified with the gastric antrum, the duodenum, jejunum, ileum and colon, however colonic MMCs (CMMCs) are independent of those in the small intestine.

Spontaneous colonic migrating motor complexes (CMMCs), defined as propagating contractions directed from the proximal to the distal end of the colon which travelled more than 50% of the colon length (Spencer and Bywater, 2002)(Spencer and Bywater, 2002;Roberts et al., 2007;Roberts et al., 2008) are ubiquitous to all strains of mice (Spencer, 2001). Several other motor patterns have been identified in isolated colon including giant migrating contractions (GMCs), short contractions (SCs) and fragmented contractions (FCs). GMCs have been defined as large brief contractions that propagate rapidly and continue for a substantial distance. GMCs are seen in the ileum, the caecum and the colon and are clearly distinct from MMCs, but are similar to the peristaltic rushes seen in the fed state (Bornstein et al., 2002). Contractions that propagated less than 50% of the colonic length are considered to be SCs and incomplete non-propagating phasic contractions occurring concurrently in different parts of the colon rather than propagating over the length of the colon are termed FCs. SCs and FCs have been identified in the isolated colon of Balb/c mice (McQuade et al., 2016a;McQuade et al., 2016b).

The relative proportions of segmenting and propulsive contractions differ in various pathological conditions such as Crohn's disease where segmentation is reduced and propulsive contractions are increased even when the disease is inactive (Jebb et al., 1994;Annese et al., 1995) and idiopathic constipation where delayed colonic transit is associated with decreased peristaltic motility and increased segmentation (Whitehead et al., 1991). Deficiencies in the nerve circuits as well as pathological excitation of the ENS have been shown to cause a variety of gastrointestinal diseases (Hansen, 2003)

1.4.2 The Role of the ENS in Pathology Including Chemotherapy-induced GI Dysfunction

Changes in enteric neurons are implicated in gastrointestinal dysfunction associated with a plethora of disorders including achalasia, Hirschsprung's disease, intestinal neuronal dysplasia, Chagas disease, irritable bowel syndrome and severe idiopathic slow transit constipation (De Giorgio et al., 2000;Törnblom et al., 2002;Wingate et al., 2002;De Giorgio and Camilleri, 2004;De Giorgio et al., 2004a;Bassotti and Villanacci, 2006;Chandrasekharan et al., 2011;Furness, 2012). Subtle changes to the ENS, not evident in conventional histological examination, have been suggested as a potential underlying mechanism for abnormal colonic motor function leading to constipation (Bassotti and Villanacci, 2011). For instance, alterations in the number of myenteric neurons expressing the excitatory neurotransmitter substance P, as well as abnormalities in the inhibitory neurotransmitters, vasoactive intestinal peptide and nitric oxide, and a reduction in the number of ICCs have been observed in patients with slow-transit constipation (Cortesini et al., 1995;Tzavella et al., 1996;He et al., 2000). However, the effects of chemotherapeutics on enteric neurons and GI dysfunction have been largely overlooked until recently.

Although neurotoxicity caused by different classes of chemotherapeutic drugs differs to a significant extent, neuronal degeneration and neuropathy are emerging as key players in chemotherapy-induced gastrointestinal dysfunction (Vera et al., 2011;Wafai et al., 2013;McQuade et al., 2016a;Pini et al., 2016). Several key studies have shown that repeated chemotherapeutic administration results in significant enteric neuronal loss which is correlated with downstream effects on colonic motility and GI transit (Vera et al., 2011;Wafai et al., 2013;Pini et al., 2016). CISP administration has been found to significantly reduce the number of myenteric neurons per ganglion in the gastric fundus and distal colon (Vera et al., 2011;Pini et al., 2016). These changes correlate with reduced upper gastrointestinal transit and obliteration of colonic contractile activity (Vera et al., 2011;Pini et al., 2016). Similarly, OXL administration induces significant neuronal loss in the

myenteric plexus of the distal colon, which is correlated with reduced colonic contractile activity (Wafai et al., 2013). Interestingly, alongside myenteric neuronal loss, alterations in the number and proportion of NOS-immunoreactive neurons were seen following both OXL and CISP treatment (Vera et al., 2011;Wafai et al., 2013;Pini et al., 2016). Preferential changes in NOS neurons have been identified in various enteric neuropathies including achalasia, diabetic gastroparesis, Chagas disease, Hirschsprung's disease and ischemia/reperfusion injury (Rivera et al., 2011a). It has been suggested that the involvement of NOS neurons in enteric neuropathies is due to potentially damaging effects of the free radical, nitric oxide (NO) (Martínez-Ruiz et al., 2011;Rivera et al., 2011a). Due to the relationship between NO production from NOS and reactive oxygen species (ROS), generation of excessive NO is linked to oxidative stress.

1.5 Oxidative Stress Associated with Neuropathy

Oxidative stress arises when the generation of ROS exceeds the capacity of cellular antioxidant systems to remove it, resulting in redox imbalance and heightened free radical production (Valko et al., 2007). Depending on circumstances, regulated oxidative stress can initiate diverse cellular responses involved in cellular protection, mitochondrial fission and autophagy of abnormal mitochondria and cells to protect spreading the damage to the neighbouring mitochondria and cells (Zorov et al., 2005). Conversely, unregulated oxidative stress can result in severe cellular damage, unwanted cell death, and consequently whole organ and organism failure (Zorov et al., 2012;Zorov et al., 2014). Therefore, adaptive redox stresses, such as those occurring under the process of a programmed removal of damaged biological systems including mitochondria and other cellular components ("physiological"), must be differentiated from maladaptive unwanted ("pathological") oxidative damage.

Under normal physiological conditions, net ROS production accounts for approximately 2% of the total oxygen consumed by mitochondria, whilst ROS production in mitochondria with disabled antioxidant systems

fluctuates from 0.25 to 11% depending on the animal species and respiration rates (Zorov et al., 2014). The most common ROS are hydroxyl ($\text{OH}\cdot$), superoxide ($\text{O}_2^{\cdot-}$) and nitric oxide ($\text{NO}\cdot$), other molecules such as hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO^-) although not technically free radicals, are involved in the production of free radicals through various chemical reactions and are, therefore, implicated in oxidative stress pathways (Uttara et al., 2009).

NO is catalysed from L-arginine by NOS in the presence of O_2 and NADPH. Although NO itself is not highly toxic, it is frequently considered cytotoxic due to its indirect role in $\text{O}_2^{\cdot-}$ production. In the presence of phagocyte oxidase (NADPH oxidase) the necessary cofactors for NO production, O_2 and NADPH undergo electron transfer resulting in the production of $\text{O}_2^{\cdot-}$ (Lin and Beal, 2006). This reaction provides the primary source of $\text{O}_2^{\cdot-}$, however $\text{O}_2^{\cdot-}$ may also be produced as a by-product of mitochondrial respiration (Valko et al., 2007). $\text{O}_2^{\cdot-}$ is rapidly removed by superoxide dismutase (SOD), a scavenging enzyme which acts to catalyse the dismutation of $\text{O}_2^{\cdot-}$, alternately adding or removing an electron from the molecule to generate one of two less damaging species, oxygen (O_2) or H_2O_2 (Bertolotto and Massone, 2012). Whilst the O_2 produced is recycled into other cellular processes, H_2O_2 may undergo several reactions to produce any of hypochlorite (HOCl), O_2 , H_2O or $\text{OH}\cdot$. Though both NO and $\text{O}_2^{\cdot-}$ are rapidly diffused and removed, respectively, when synthesized in close proximity, they will combine spontaneously to form ONOO^- by a diffusion-limited reaction (Huie and Padmaja, 1993). No enzyme is required for the formation of ONOO^- , moreover NO is the only known biological molecule that is produced in sufficient concentrations and reacts fast enough to outcompete endogenous levels of SOD with $\text{O}_2^{\cdot-}$ (Pacher et al., 2007). ONOO^- promotes biological effects via different types of reactions (Radi et al., 2001), one such reaction being the modification of protein tyrosines to generate nitrotyrosines.

Oxidative stress is responsible for neuronal damage in several neuropathies including diabetic neuropathy, acrylamide induced neuropathy and

Charcot–Marie neuropathy (Saifi et al., 2003; Vincent et al., 2004; Chandrasekharan et al., 2011; Areti et al., 2014). Further, oxidative stress and corresponding mitochondrial dysfunction are key players in the mediation of chemotherapy-induced peripheral nerve damage (Zheng et al., 2011). Oxidative stress mediated neurodegeneration may be executed by way of bio-energetic failure, depletion of antioxidant defences, biomolecular damage, microtubule disruption, ion channel activation, neuroinflammation, or neuronal death through apoptosis (McDonald and Windebank, 2002; Salvemini et al., 2011; Ta et al., 2013; Areti et al., 2014). Recent studies have shown that oxidative stress-induced apoptosis in neurons correlates with mitochondrial DNA base excision repair imbalance and mitochondrial dysfunction (Harrison et al., 2005).

1.5.1 Mitochondrial Damage

ROS can cause a variety of DNA damage, including single and double strand breaks and DNA base modifications. Therefore, efficient repair of ROS-induced DNA damage is important for preventing mutations and maintaining the stability of the mitochondrial genome (Hakem, 2008). Mitochondrial DNA (mtDNA) in particular is prone to oxidative damage compared with its nuclear counterpart, in part due to its location close to endogenously generated ROS (Yakes and Van Houten, 1997), and to the fact that mtDNA lacks introns and undergoes transcription at a high rate, resulting in a high probability of oxidative modification of DNA bases in the coding region (Glowacki et al., 2013).

Due to the mutagenic nature of many ROS-induced lesions, there is a higher mutation rate in mtDNA than in nuclear DNA (nDNA) and repair of these lesions may be crucial for the fate of mitochondria. No nucleotide excision repair (NER) pathway, essential for the stability of the nuclear genome, has been observed in mitochondria. Mismatch repair (MMR) and base excision repair (BER) have been reported to operate in mtDNA (Glowacki et al., 2013). Effective repair of oxidatively damaged mtDNA is essential, due to

its proximity to the respiration chain and significantly higher levels of accumulated oxidative damage than its nuclear counterpart (Todorov and Todorov, 2009). Moreover, the mitochondria contain genes of some of the respiratory chain components, thus, defective mtDNA that is translated into defective mitochondrial components, can further aggravate mitochondrial dysfunction by accelerating aberrant ROS production, inefficient ATP generation and ROS-induced self-mutagenesis. Compounding this dilemma, mitochondrial damage decreases the level of oxidative phosphorylation whilst increasing ROS production (Glowacki et al., 2013). This can then result in oxidative DNA and protein damage and, in consequence, the start of a vicious cycle of events.

Under conditions of mitochondrial calcium overload and oxidative stress, a non-specific pore, the mitochondrial permeability transition pore (mPTP), opens in the inner mitochondrial membrane (Halestrap, 2009). Evidence suggests that brief mPTP openings play an important physiological role in maintaining healthy mitochondrial homeostasis (Zorov et al., 2014). Activation causes intra- and inter-mitochondrial oxidative changes resulting in mitochondrial ROS release. This cycle of mitochondrial ROS formation and release is known as ROS-induced ROS release (RIRR) (Zorov et al., 2000). Whilst brief and reversible mPTP opening-associated ROS release serves an apparent housekeeping function through the discharge of accumulated ROS, longer mPTP openings triggered by higher ROS levels may release a large ROS burst leading to destruction of mitochondria, and if propagated from mitochondrion to mitochondrion, damage of the cell itself (Zorov et al., 2014). Opening of the mPTP is also associated with mitochondrial and cytosolic NAD⁺ depletion (Di Lisa et al., 2001). Although RIRR may serve a functional physiological role by removal of unwanted cells or damaged mitochondria, under pathological conditions it may contribute to elimination of vital and essential mitochondria and cells.

Several experimental studies in animal models have suggested that mitochondrial dysfunction contributes to chemotherapy-induced neuropathic symptoms, with histological and microscopic observation of

peripheral nerve sections of chemotherapy-treated animals showing swollen and vacuolated mitochondria (Areti et al., 2014). Whilst little research has been undertaken to examine mitochondrial function or dysfunction in chemotherapy-treated neuronal preparations, reduced phosphorylating capacity resulting in decreased ATP production in the mitochondria has been demonstrated in nerves from OXL-treated rats (Zheng et al., 2011).

It is well known that ROS production induces elevation of DNA strand breaks, which activate the nuclear PARP enzyme (Bardos et al., 2003), moreover poly(ADP-ribosylation) compartmentalised to the mitochondria can be converted from a homeostatic process to a mechanism of cell death when oxidative stress is accompanied by energy depletion (Du et al., 2003). In models of mitochondrial dysfunction or metabolic impairment, increasing NAD⁺ availability through PARP inhibition or NAD⁺ precursors have been shown to improve mitochondrial function and enhance oxidative metabolism (Bai et al., 2011; Pirinen et al., 2014; Felici et al., 2015; Lehmann et al., 2016).

1.6 PARP Inhibition as a Neuroprotective Treatment

PARP1 overactivation resulting in cellular NAD⁺ exhaustion and subsequent ATP depletion has been shown to contribute to tissue injury in diabetes, myocardial or cerebral ischemia reperfusion and inflammation. In particular, PARP overactivation has been linked to a plethora of neurodegenerative and neuropathic disorders including Parkinson's disease, Alzheimer's disease, Huntington's disease, diabetic neuropathy and chemotherapy-induced peripheral neuropathy.

Accumulation of single strand and double strand DNA breaks following treatment with DNA targeting chemotherapeutics such as OXL, 5-FU and IRI has been found to result in severe PARP over-activation. PARP inhibition and PARP1 knockout has demonstrated neuroprotective properties in models of cerebral ischemia, Huntington's disease and diabetic neuropathy (Kaundal et al., 2006; Lupachyk et al., 2011; Cardinale

et al., 2015). PARP inhibition has also been shown to directly protect electron transport chain complexes from inactivation induced by oxidative stress (Halmosi et al., 2001). Although PARP inhibition is yet to be investigated in gastrointestinal pathologies, PARP inhibition has been tested in animal models of CIPN and diabetic neuropathy with promising results (Negi et al., 2010;Lupachyk et al., 2011). Moreover, attenuation of symptoms of CIPN by the usage of peroxynitrite scavengers and PARP inhibitors further supports a role for nitrosative/oxidative stress in chemotherapy-induced neuropathy (Janes et al., 2013;Ta et al., 2013). In animal models of vincristine, CISP and OXL-induced peripheral neuropathy PARP inhibition successfully reduces symptoms of hot and cold hyperalgesia and mechanical allodynia in chemotherapy-treated mice (Ta et al., 2013) and mechanical allodynia in chemotherapy-treated rats (Brederson et al., 2012).

A novel PARP1 inhibitor, BGP-15, has recently been shown to reduce CISP and taxol-induced nephrotoxicity (Racz et al., 2002) and peripheral neuropathy without compromising anti-tumor activity (Bardos et al., 2003). Although the exact mechanism underlying the neuroprotective activity of BGP-15 remains unclear it has been suggested that it may be related to the modulation of PARP activity and protection of mitochondria from oxidative damages (Szabados et al., 2000).

1.7 Summary

CRC is a leading cause of morbidity and mortality worldwide. Although clinically effective, many of the chemotherapeutic agents used in the treatment of CRC are associated with severe side-effects including peripheral sensory neuropathy and GI toxicity. Chemotherapy-induced GI toxicities, in particular CID and CIC, heavily contribute to treatment delays, dose reductions and in some cases cessation of anti-CRC treatment, greatly affecting disease management and clinical outcomes. Most drugs currently in clinical use to alleviate CID and CIC cause adverse effects themselves and in many cases are of limited efficacy, therefore, the search for novel targets and therapies is crucial. The traditional view is that GI side-effects of anti-cancer drugs are due to mucosal damage. Although this undoubtedly plays a significant role in the acute symptoms associated with chemotherapeutic treatment, the persistence of GI symptoms long after treatment suggests that chemotherapy induces damage to an adjacent non-proliferative system such as the ENS. The ENS is the primary source of innervation to the GI tract, yet limited work has been undertaken to investigate its role in the pathophysiology of chemotherapy-induced GI toxicity and dysfunction. The mechanisms underlying enteric neuropathy associated with anti-cancer chemotherapy have not been elucidated. Chemotherapy-induced sensory peripheral neuropathy has been intensively researched; several prospective mechanisms including inflammation and oxidative stress have been proposed. Utilisation of antioxidants, ROS scavengers and PARP inhibitors in animal models of sensory peripheral neuropathy has yielded promising results, however these have not been explored to alleviate chemotherapy-induced GI dysfunction.

1.8 Hypothesis and Aims

I hypothesise that chronic treatment with chemotherapeutic agents, OXL, 5-FU and IRI, is associated with oxidative stress and/or inflammation-induced enteric neuropathy leading to GI dysfunction. I also hypothesise that co-treatment with PARP1 inhibitor, BGP-15, can alleviate chemotherapy-induced oxidative stress and/or inflammation underlying enteric neuropathy, thereby restoring GI functions.

To test these hypotheses, the following specific aims were set:

1. To determine the effects of *in vivo* treatment with chemotherapeutic drugs, OXL, 5-FU and IRI on enteric neurons in the mouse.
2. To characterise changes in intestinal functions caused by these chemotherapeutic agents including GI transit and motility associated with CID and CIC.
3. To investigate the role of oxidative stress and inflammation in chemotherapy-induced death and damage of enteric neurons.
4. To test the therapeutic potential of PARP1 inhibitor BGP-15 co-treatment in alleviating enteric neuropathy and GI dysfunction caused by OXL.

CHAPTER TWO: ROLE OF OXIDATIVE STRESS IN OXALIPLATIN-INDUCED ENTERIC NEUROPATHY AND COLONIC DYSMOTILITY IN MICE

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RM McQuade, SE Carbone, V Stojanovska, A Rahman, RM Gwynne, AM Robinson, CA Goodman, JC Bornstein, K Nurgali (2016) Role of oxidative stress in oxaliplatin-induced enteric neuropathy and colonic dysmotility in mice. *British Journal of Pharmacology*, Vol 173(24), p3502-3521.

2.1 Summary

Oxaliplatin is a platinum-based chemotherapeutic drug used as a first-line therapy for colorectal cancer. However, its use is associated with severe gastrointestinal side-effects resulting in dose limitations and/or cessation of treatment. In this study, we tested whether oxidative stress, caused by chronic oxaliplatin treatment, induces enteric neuronal damage and colonic dysmotility. Oxaliplatin (3 mg/kg/d) was administered *in vivo* to Balb/c mice intraperitoneally three times a week. The distal colon was collected at day 14 of treatment. Immunohistochemistry was performed in wholemount preparations of submucosal and myenteric ganglia. Neuromuscular transmission was studied by intracellular electrophysiology. Circular muscle tone was studied by force transducers. Colon propulsive activity studied in organ bath experiments and faeces were collected to measure water content. Chronic *in vivo* oxaliplatin treatment resulted in increased formation of reactive oxygen species (O_2^-), nitration of proteins, mitochondrial membrane depolarisation resulting in the release of cytochrome c, loss of neurons, increased iNOS expression and apoptosis in both the submucosal and myenteric plexuses of the colon. Oxaliplatin treatment enhanced nitric oxide (NO)-mediated inhibitory junction potentials and altered the response of circular muscles to the NO donor, sodium nitroprusside. It also reduced the frequency of colonic migrating motor complexes and decreased circular muscle tone, effects reversed by the NO synthase inhibitor, N ω -Nitro-L-arginine. Our study is the first to provide evidence that oxidative stress is a key player in enteric neuropathy and colonic dysmotility leading to symptoms of chronic constipation observed in oxaliplatin-treated mice.

Abbreviations

| | |
|--------------|-------------------------------------|
| ATP | adenosine triphosphate |
| CMMC | colonic migrating motor complex |
| DRG | dorsal root ganglia |
| EJPs | excitatory junction potentials |
| ENS | enteric nervous system |
| FC | fragmented contraction |
| fIJPs | fast inhibitory junction potentials |
| GFAP | glial fibrillary acidic protein |
| iNOS | inducible nitric oxide synthase |
| IR | immunoreactive |
| L-NNA | n ω -nitro-l-arginine |
| nNOS | neuronal nitric oxide synthase |
| NO | nitric oxide |
| SC | short contraction |
| sIJPs | slow inhibitory junction potentials |
| SNP | sodium nitroprusside |

2.2 Introduction

Oxaliplatin, usually administered together with 5-fluorouracil and leucovorin, is a third generation platinum-based agent used as the first line of treatment for tumours resistant to the first and second generation platinum-based agents, cisplatin and carboplatin (Raymond et al., 1998). Combinations containing oxaliplatin have shown great therapeutic potential, improving progression-free and overall survival rates of patients with colorectal cancer (André et al., 2004;De Gramont et al., 2007).

Oxaliplatin exerts its cytotoxic effects via formation of platinum-DNA adducts that trigger immobilization of the mitotic cell cycle and stimulate apoptosis of dividing cells (Graham et al., 2000;Goodisman et al., 2006). At clinically recommended doses, oxaliplatin is reported to be less toxic for the auditory, hematologic and renal systems than cisplatin and carboplatin (Raymond et al., 1998). However, oxaliplatin-induced neurotoxicity differs from that induced by other platinum compounds, triggering both acute and delayed peripheral neuropathies as well as gastrointestinal dysfunctions (Stojanovska et al., 2014).

Gastrointestinal side-effects are a predominant cause of dose limitation, presenting a constant challenge for efficient and tolerable treatment of cancer (Verstappen et al., 2003;McQuade et al., 2014). About 40% of patients receiving standard dose chemotherapy and nearly all patients receiving high dose chemotherapy exhibit pain, ulceration, bloating, vomiting, diarrhoea and/or constipation throughout the course of treatment (McQuade et al., 2014). Platinum-based agents, specifically are linked to a heightened incidence of gastrointestinal side-effects with up to 90% of patients experiencing vomiting, nausea and/or diarrhoea during the course of treatment (Sharma et al., 2005). Addition of platinum-based chemotherapeutics to combination regimes (such as FOLFOX – a combination of 5-fluorouracil, leucovorin and oxaliplatin) increases the incidence of chronic diarrhoea and treatment-related death (Souglakos et

al., 2006). Gastrointestinal side-effects can persist up to 10 years after the treatment has ceased (Denlinger and Barsevick, 2009).

Gastrointestinal functions are controlled by the enteric nervous system (ENS) embedded in the wall of the gastrointestinal tract (Furness, 2012). Damage to the ENS underlies gastrointestinal dysfunction in many pathophysiological conditions (Furness, 2012). To date, few studies have investigated the effects of platinum-based chemotherapeutics on enteric neurons in animal models (Vera et al., 2011;Wafai et al., 2013). These studies reported significant reductions in the number of myenteric neurons in the colon and stomach, alongside altered expression of neuronal nitric oxide synthase (nNOS) following *in vivo* treatment with cisplatin (Vera et al., 2011;Pini et al., 2016) and oxaliplatin (Wafai et al., 2013). These changes in the ENS are correlated with reductions in gastrointestinal transit and colonic propulsive activity in oxaliplatin-treated animals, which may be related to symptoms of constipation or diarrhoea.

Nitric oxide (NO), produced by the activation of nitric oxide synthase (NOS) enzyme is a highly reactive and widely distributed transmitter found throughout both the central and peripheral nervous systems. In the gastrointestinal tract, NO is a well-established mediator of vasodilation and gastrointestinal relaxation (Takahashi, 2003;Bornstein et al., 2004). Two types of NOS participate in normal physiological responses, neuronal NOS (nNOS) localized to neurons, and endothelial NOS (eNOS) localised to the epithelium. Increased expression of inducible NOS (iNOS) occurs during times of cellular stress. Neurons containing nNOS are the primary source of NO in the ENS and represent approximately 30% of the neuronal population in the myenteric plexus of the mouse small intestine and colon (Qu et al., 2008;Wafai et al., 2013). In enteric ganglia nNOS is expressed by descending interneurons and by inhibitory motor neurons supplying the intestinal smooth muscle (Lecci et al., 2002). Moreover, a myogenic nNOS isoform is expressed by gastrointestinal smooth muscle cells (Daniel et al., 1994). NO released from both neurons and smooth muscle is essential for sphincter relaxation and generation of complex gastrointestinal motor

patterns, which contribute to propulsion during digestion (Sarna et al., 1993; Roberts et al., 2007; Roberts et al., 2008). Altered expression of nNOS in the ENS has been linked to several pathological conditions, and increased levels of nNOS in the ENS can be indicative of oxidative stress (Rivera et al., 2011a). . The impact of oxidative stress on post mitotic cells, including enteric neurons, can be cumulative resulting in neuronal loss and deterioration of neuronal function impairing gastrointestinal motility patterns (Chandrasekharan et al., 2011). The role of oxidative stress in chemotherapy-induced gastrointestinal dysfunction has not been explored. This study investigated the role of oxidative stress in enteric neuronal damage and colonic dysmotility caused by *in vivo* oxaliplatin treatment.

2.3 Methods

2.3.1 Ethical Approval

All procedures were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animal studies are reported in compliance with the ARRIVE guidelines (McGrath and Lilley, 2015).

2.3.2 Animals

Male Balb/c mice aged 6-8 weeks (18 – 25 g) supplied from the Animal Resources Centre (Perth, Australia) were used for all experiments. Mice had free access to food and water and were kept under a 12 hour light/dark cycle in a well-ventilated room at an approximate temperature of 22°C. Mice acclimatised for a minimum of 5 days and a maximum of 7 days prior to the commencement of *in vivo* intraperitoneal injections. A total of 70 mice were used for this study.

2.3.3 *In vivo* Oxaliplatin Injections

Mice were randomly assigned into two groups: oxaliplatin-treated and sham-treated. Mice received intraperitoneal injections of oxaliplatin (Tocris Bioscience, UK) 3 mg/kg/dose 3 times a week via a 26 gauge needle. Oxaliplatin was dissolved in sterile water in order to make 10^{-2} M L⁻¹ stock solutions and refrigerated at -20°C. The stock was then defrosted and further diluted with sterile water to make 10^{-3} M L⁻¹ solutions for injections. The oxaliplatin dose was calculated to be equivalent to standard human dose per body surface area (Renn et al., 2011). Sham-treated mice received sterile water via intraperitoneal injection 3 times a week via a 26 gauge needle. The volume injected did not exceed 200 µL per injection. Mice were euthanised via cervical dislocation 14 days after the first injection. Colon was collected for *in vitro* experiments.

2.3.4 Assessment of Mitochondrial Superoxide Production

MitoSOX™ Red M36008 (Invitrogen, Australia), was used to visualise mitochondrially-derived superoxide in wholemount preparations of submucosal and myenteric ganglia of the distal colon. Freshly excised distal colon preparations were dissected to expose submucosal and myenteric ganglia. Preparations were incubated in oxygenated physiological saline with MitoSOX™ Red M36008 (5 µM) in a gently shaking incubator Unimax 1010 (Heidolph Instruments, Germany) at a constant temperature of 37°C for 40 min. Tissues were washed (2 x 30 min) with oxygenated physiological saline (composition in mM: NaCl 118, KCl 4.6, CaCl₂ 3.5, MgSO₄ 1.2, NaH₂PO₄ 1, NaHCO₃ 25, d-Glucose 11; bubbled with 95%O₂ and 5% CO₂) and fixed in 4% paraformaldehyde overnight at 4°C. The following day tissues were washed (2 x 30 min) with physiological saline and mounted on glass slides with DAKO fluorescent mounting medium for imaging. All images were captured at identical acquisition exposure-time conditions, calibrated to standardised minimum baseline fluorescence, converted to binary and changes in fluorescence from baseline were measured in arbitrary units (arb. units) using Image J software (NIH, MD, USA). The

corrected total fluorescence was calculated as previously described (Burgess et al., 2010) in 32 5x5µm² boxes within myenteric ganglia from each preparation to exclude fluorescence outside the ganglia.

2.3.5 Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential changes in cells may be detected with the use of cationic, lipophilic JC-10 dye. In normal cells, JC-10 concentrates in the mitochondrial matrix where it forms red fluorescent aggregates (JC aggregates). In contrast, apoptotic cells stain in green fluorescent colour (JC monomeric form) due to the JC-10-labelled release of cytochrome c diffusing out of the mitochondria as a result of mitochondrial depolarisation and increased permeability. JC-10 fluorescent mitochondrial membrane potential microplate assay kit (Abcam, MA, USA) was used to detect mitochondrial membrane potential changes and the release of cytochrome c from damaged mitochondria in the myenteric ganglia of the distal colon. Freshly excised distal colon preparations from sham and oxaliplatin-treated mice were bathed in oxygenated physiological saline and dissected to expose the myenteric ganglia. Immediately following dissection, preparations were incubated for 20 min with 500 µL of JC-10 dye solution (buffer A) in a gently shaking incubator Unimax 1010 (Heidolph Instruments, Germany) at a constant temperature of 37°C. After 20 min, 500 µL of buffer B solution was added to tissue preparations and allowed to incubate for another 20 min in a gently shaking incubator at a constant temperature of 37°C. Immediately following final incubation, tissues were mounted on glass slides with DAKO fluorescent mounting medium for imaging under a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan).

2.3.6 Immunohistochemistry

Collected tissues (distal and proximal colon) were placed in oxygenated phosphate-buffered saline (PBS) (pH 7.2) containing nicardipine (3 µM) (Sigma-Aldrich, Australia) for 20 min to inhibit smooth muscle contraction. Samples were cut open along the mesenteric border, cleared of their

contents, maximally stretched and dissected mucosa down to expose either the submucosal plexus (distal colon) or myenteric plexus (distal and proximal colon). Tissues were then fixed with Zamboni's fixative (2% formaldehyde, 0.2% picric acid) overnight at 4°C. Preparations were cleared of fixative by washing 3 times for 10 min with Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Australia) followed by 3 x 10 min washes with PBS. Fixed tissues were stored at 4°C in PBS for a maximum of 5 days.

Wholemount preparations were incubated with 10% normal donkey serum (Chemicon, USA) for 1 hour at room temperature. Tissues were then washed (2 x 5 mins) with PBS and incubated with primary antibodies against β -Tubulin class III (TuJ1) (chicken, 1:1000, Abcam, MA, USA), Nitrotyrosine (rabbit, 1:1000, Millipore, CA, USA), neuronal nitric oxide synthase (nNOS) (goat, 1:500, Abcam, MA, USA), and cleaved Caspase-3 (rabbit, 1:500, Cell Signalling Technologies, MA, USA) overnight at 4°C. Tissues were then washed in PBS (3 x 10 min) before incubation with species-specific secondary antibodies labelled with different fluorophores: donkey anti-chicken Alexa 594 (1:200, Jackson Immuno research Laboratories, PA, USA), donkey anti-goat Alexa 488 (1:200, Jackson Immuno research Laboratories, PA, USA) and donkey anti-rabbit Alexa 488 and 647 (1:200, Jackson Immuno research Laboratories, PA, USA) for 2 hours at room temperature. Double immunohistochemical labelling was performed using mixtures of antibodies raised in different species and using species specific secondary antibodies labelled with different fluorophores. Tissues were given a further 3 x 10min washes with PBS, followed by a 2min incubation with the fluorescent nucleic acid stain 4'-6-diamidino-2-phenylindole (DAPI) (14 nM) (Invitrogen, Australia). Wholemount preparations were given 3 final 10 min washes in PBS and then mounted on glass slides using fluorescent mounting medium (DAKO, Australia). Wholemount preparations were observed under a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan), 8 randomly chosen images from each preparation were captured with a 20 x objective and processed using NIS Elements software (Nikon, Japan). The numbers of β -Tubulin-immunoreactive (IR) neurons, nNOS-IR neurons and neurons displaying translocation of nitrated proteins to the nuclei were quantified in both submucosal and myenteric ganglia within a 2

mm² area of each distal colon preparation. Proximal colon preparations were used to quantify the numbers of β -Tubulin-IR and nNOS-IR neurons. Images were then calibrated to standardised minimum baseline fluorescence, converted to binary and changes in Caspase-3 fluorescence were detected using Image J software (NIH, MD, USA). Quantitative analyses were conducted blindly.

2.3.7 Histology

The distal colon was harvested and placed in a 10% formalin solution overnight and then transferred into 70% ethanol the following day. Paraffin embedded colon sections were cut 5 μ m thick and de-waxed in a 60°C oven for 30 minutes. To examine the morphological changes to the colon, a standard Hematoxylin and Eosin staining protocol was followed (Nurgali et al., 2011; Robinson et al., 2014). Ten sections per preparation were analysed. All images were analysed blindly.

2.3.8 Imaging

Three dimensional (z-series) images of wholemount preparations were taken using a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan). Fluorophores were visualised using excitation filters for Alexa 594 Red (excitation wavelength 559 nm), Alexa 488 (excitation wavelength 473 nm), and Alexa 405 (excitation wavelength 405 nm). Z-series images were taken at step size of 1.75 μ m (1600 x 1200 pixels).

2.3.9 Western Blotting

The whole colon was harvested, cut along the mesenteric border and pinned mucosa side up to a silicone-based petri dish containing physiological saline. The colon was flushed of its contents, and the mucosa, submucosa and the circular muscle layers were dissected and discarded. The remaining longitudinal muscle-myenteric plexus (LMMP) wholemount preparations were then snap frozen using liquid nitrogen.

Frozen tissues were homogenised with a Polytron homogeniser (Kinematica AG, Lucerne, Switzerland) for 20 seconds in ice-cold WB buffer (40 mM Tris, pH 7.5; 1 mM EDTA; 5 mM EGTA; 0.5% Triton X-100; 25 mM β -glycerophosphate; 25 mM NaF; 1 mM Na₃VO₄; 10 μ g/ml leupeptin; and 1 mM PMSF), and the whole homogenate was used for Western blot analysis. Sample protein concentrations were determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and equivalent amounts of protein from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on SDS-PAGE acrylamide gels. Following electrophoretic separation, proteins were transferred to a PVDF membrane, blocked with 5% powdered milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h followed by an overnight incubation at 4°C with rabbit anti-iNOS primary antibody (D6B6S, Cell Signaling Technology, Danvers, MA, USA) dissolved in TBST containing 1% BSA. The following day membranes were washed for 30 min in TBST and then probed with a peroxidase-conjugated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Following 30 min of washing in TBST, the blots were developed with a DARQ CCD camera mounted to a Fusion FX imaging system (Vilber Lourmat, Germany) using ECL Prime reagent (Amersham, Piscataway, NJ, USA). Densitometric measurements of the protein of interest were carried out using Fusion CAPT Advance software (Vilber Lourmat, Germany). Membranes were then stained with Coomassie Blue, scanned and total protein loaded quantified using Image J software. The signal intensity of the protein of interest was normalised to the signal for total protein loaded.

2.3.10 Intracellular Recordings

Segments of the distal colon proximal to the pelvic brim were collected from day 14 sham and oxaliplatin-treated mice and placed in physiological saline bubbled with 95% O₂ and 5% CO₂ at room temperature. The physiological saline contained the L-type Ca²⁺ channel blocker nicardipine (3 μ M) (Sigma-Aldrich, Australia) to limit contractions. The distal colon was opened along

the mesenteric border and pinned in a Sylgard-lined (Dow Corning, USA) Petri dish. The mucosa and submucosa were removed revealing the circular muscle layer. A 20 mm long, full circumference segment was transferred into a Sylgard-lined recording chamber and pinned with 50 μ m gold-plated tungsten pins. The recording chamber was placed on the stage of an inverted microscope fitted with fluorescent optics (Zeiss Axiovert 200), and was continuously superfused with physiological saline (3 ml/min⁻¹) pre-heated to yield a bath temperature of 35°C. Following 2 hour recovery from dissection (Carbone et al., 2012), circular smooth muscle cells were impaled with conventional intracellular borosilicate glass capillary electrodes filled with 5% 5,6-carboxyfluorescein in 20 mM Tris buffer (pH 7.0) in 1 M KCl. Electrode resistances ranged from 60-120 M Ω . Recordings were made using an Axoclamp 2B amplifier (Axon Instruments, USA), digitised at 1-10 kHz via a Digidata 1440A interface (Molecular Devices, USA) and stored using PClamp 10.0 (Molecular Devices) on a PC computer. Carboxyfluorescein-labelled cells were identified as circular muscle cells *in situ* from their morphology (Carbone et al., 2012). Intracellular hyperpolarising current pulses (duration 500 ms, intensity 100-500 pA) were used to determine input resistance (R_{in}). A tungsten electrode (10–50 mm tip diameter, placed 1 mm circumferential to the recording microelectrode) was connected to an ISO-Flex stimulator controlled by a Master-8 pulse generator (AMPI, Israel). Single pulse stimuli (20 V, 0.4 ms duration) and short trains of high frequency pulses (20 V, 3 pulses, 40 ms interval, 0.4 ms duration) were used to activate nerve fibres. Junction potential responses were recorded in the impaled smooth muscle cells. Responses in 3-4 cells were averaged per test condition, in each animal. Data were analysed using AxoGraph 10 software.

2.3.11 Contraction Force

Freshly excised distal colon was cut into 3 mm rings, cleaned of connective tissue and fat and then placed in a custom built organ-bath chamber containing physiological saline oxygenated with 95% O₂ and 5% CO₂ and maintained at a constant temperature of 37°C and pH of 7.4. Colonic rings were mounted between two small metal hooks attached to force displacement transducers (Zultek Engineering, Australia), and stretched to a resting tension of 0.2 g. After 30 min, rings were returned to resting tension and allowed to stabilise for 2 hours with physiological saline changed every 20 min (Habiyakare et al., 2014). Following stabilisation, 10 µM sodium nitroprusside (SNP) was added to organ bath. Baseline values were obtained by averaging 60 s of data 5 min prior to drug application; maximum relaxation was calculated as absolute change from the baseline values.

2.3.12 Colonic Motility Experiments

The entire colon was removed from day 14 sham and oxaliplatin-treated mice and set up in organ-bath chambers to record motor patterns *in vitro* (Wafai et al., 2013). Briefly, the colon was placed into warmed (35°C), oxygenated physiological saline (composition stated above, section 2.3.4) until the faecal pellets were expelled. The empty colon was cannulated at both ends and arranged horizontally in organ-bath chambers. The proximal end of the colon was connected to a reservoir containing oxygenated physiological saline to maintain intraluminal pressure. The distal end was attached to an outflow tube that provided a maximum of 2 cm H₂O back-pressure. Organ baths were continuously superfused with oxygenated physiological saline solution and preparations were left to equilibrate for 30 min. Contractile activity of each segment was recorded with a Logitech Quickcam Pro camera positioned 7–8 cm above the preparation. Videos (2x20 min) of each test condition were captured and saved in avi format using VirtualDub software (version 1.9.11).

Colonic migrating motor complexes (CMMCs) were defined as propagating contractions directed from the proximal to the distal end of the colon which travelled more than 50% of the colon length (Roberts et al., 2007; Roberts et al., 2008). Contractions that propagated less than 50% of the length of the colon were considered to be short contractions. Another form of incomplete contraction was identified as fragmented contractions occurring simultaneously at different parts of the colon rather than propagating over the length of the colon. Recordings were used to construct spatiotemporal maps using in-house edge detection software (Gwynne et al., 2004). Spatiotemporal maps plot the diameter of the colon at all points during the recording allowing contractile motor patterns to be analysed with Matlab software (version 12).

2.3.13 Drugs Used

[(1R,2S,4S,5S)-4-[2-iodo-6-(methylamino)purin-9-yl]-2-phosphonooxy-1-bicyclo [3.1.0]hexanyl]methyl dihydrogen phosphate (MRS2500) (Tocris, UK), [(1R,5S)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl]3-hydroxy-2-phenylpropanoate (atropine), 2-carbamoyloxyethyl-trimethylazanium (carbachol), Nω-Nitro-L-arginine (L-NNA), O5-methyl O3-[2-(methyl-(phenylmethyl)amino)ethyl] 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (nicardipine) and sodium nitroprusside (all from Sigma-Aldrich, Australia) were prepared as stock solutions and diluted in physiological saline daily before addition to preparations.

2.3.14 Faecal Water Content and Colonic Faecal Content

Wet weight of faecal pellets was measured immediately upon pellet expulsion. Pellets were then dehydrated for 72 hours at room temperature prior to measurement of the dry weight. Water content was calculated as the difference between the wet weight and dry weight. Total number of faecal pellets along the entire length of the colon was counted in freshly excised intact colons from day 14 sham and oxaliplatin-treated mice.

2.3.15 Data and Statistical Analysis

Sample size was calculated based on our previous studies on the enteric neuropathy and intestinal dysmotility associated with chemotherapy (Wafai et al., 2013;McQuade et al., 2016a). To detect a 30% change at a power 0.8 and $\alpha = 0.05$ with 10% SD, the effect size should be minimum $n=5$ animals per group as calculated by the GPOWER program. Data were assessed using two-way ANOVA, Welch's two-tailed t test and Student's two-tailed t test. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., CA, USA). Data are presented as mean \pm standard error of the mean (SEM). Value differences were considered statistically significant at $P < 0.05$. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

2.4 Results

2.4.1 Loss of enteric neurons, increase in subpopulations of nNOS-immunoreactive neurons and increase in iNOS expression following oxaliplatin treatment

To investigate changes to the total number of myenteric neurons, wholemount preparations of the distal and proximal colon were labelled with β -Tubulin antibody to count neurons within a 2mm^2 area (**Figure 2.1**). Repeated *in vivo* administration of oxaliplatin-induced myenteric neuronal loss in both the proximal (3607 ± 64 neurons/area, $P < 0.05$) and distal (721 ± 8 neurons/area, $P < 0.05$) colon when compared to sham (proximal: 4294 ± 84 neurons/area; distal: 1261 ± 34 neurons/area) ($n=6$ mice/group, Figure 2A). Significant neuronal loss was also observed in the submucosal plexus in the distal colon from oxaliplatin-treated mice (90 ± 8 neurons/area, $P < 0.05$) when compared to sham (129 ± 10 neurons/area) ($n=6$ mice/group, **Figure 2.2A'**). To determine if oxaliplatin administration was associated with changes in subpopulations of myenteric neurons inhibitory muscle motor and

interneurons immunoreactive for nNOS were analysed. Fewer nNOS-IR neurons were observed in the myenteric plexus of the both the proximal (736 ± 9 neurons/area, $P<0.05$) and distal (344 ± 6 neurons/area, $P<0.05$) colon following oxaliplatin administration when compared to sham (proximal: 860 ± 40 neurons/area; distal: 510 ± 12 neurons/area) ($n=6$ mice/group, **Figure 2.2B**), but not in the submucosal plexus (sham: 51 ± 3 neurons/area; oxaliplatin 52 ± 13 neurons/area). The proportion of nNOS-IR neurons was, however, significantly increased in the myenteric (sham: $40\pm0.2\%$; oxaliplatin: $48\pm1.0\%$, $P<0.05$) and submucosal plexus of the distal colon (Sham: $39\pm0.9\%$; oxaliplatin: $56\pm1.5\%$, $P<0.05$), but not the myenteric plexus of the proximal colon (sham: $20\pm0.6\%$; oxaliplatin: $23\pm1.8\%$) from oxaliplatin-treated mice when compared to sham-treated mice (**Figure 2.2C, C'**).

Figure 2.1 Wholemout preparations of myenteric neurons in the proximal and distal colon following 14 days of *in vivo* oxaliplatin treatment. Myenteric neurons labelled with anti- β -Tubulin III antibody (Tub III, red) counterstained with DAPI (blue) which labels neuronal nuclei within the ganglion (arrow) and smooth muscle cell nuclei outside the ganglion (arrowhead) **(C')**, nNOS-IR neurons (green). Scale bar =20 μ m.

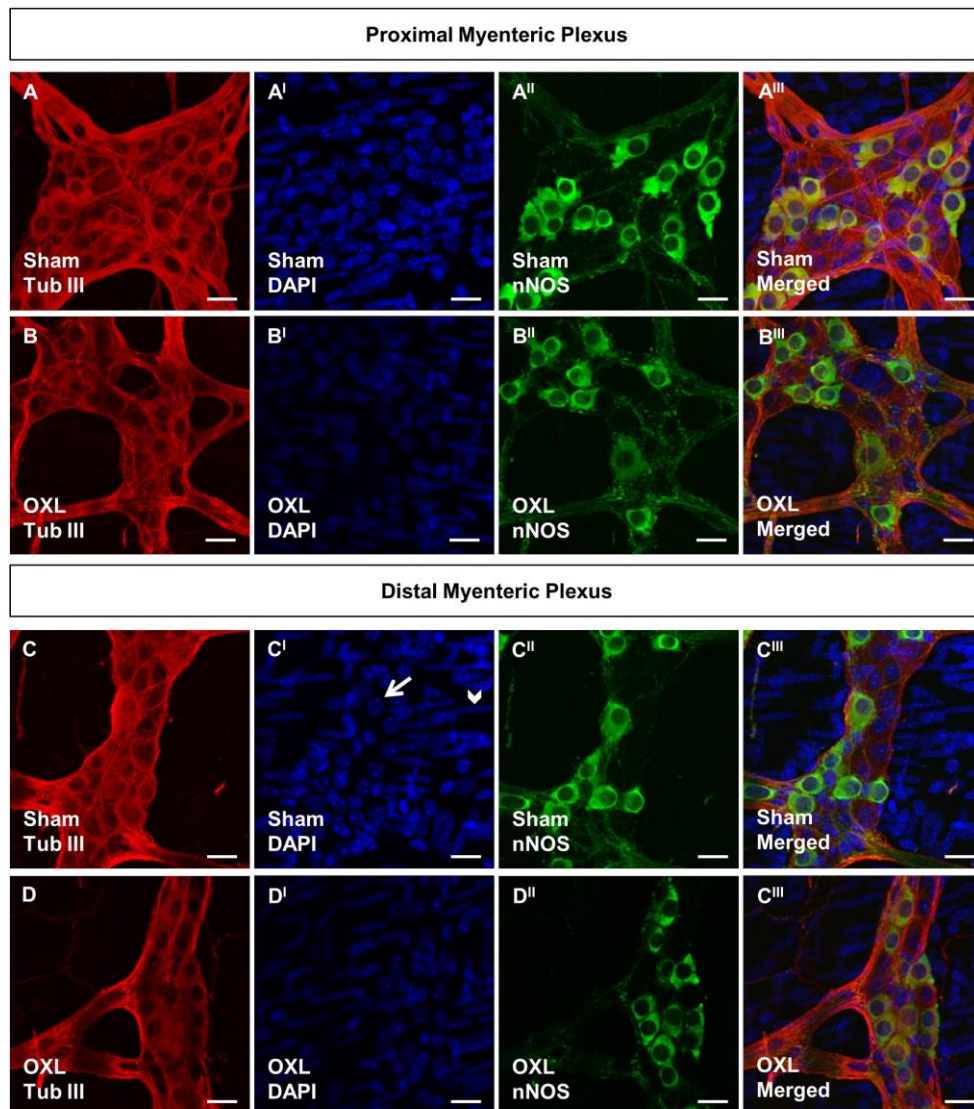
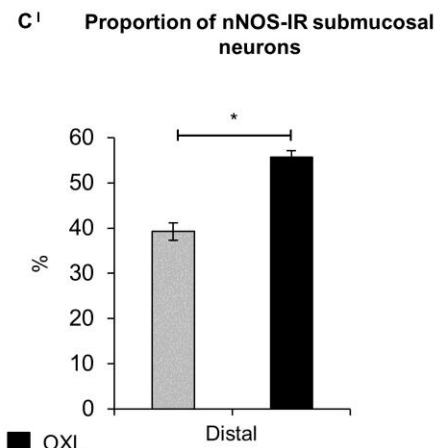
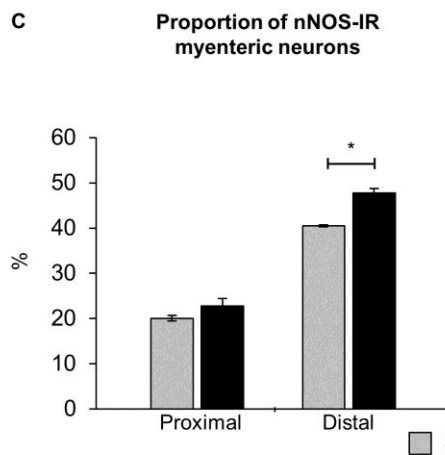
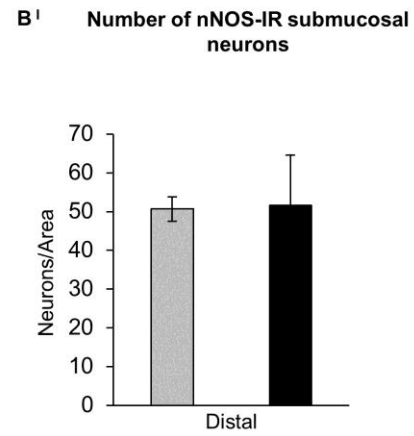
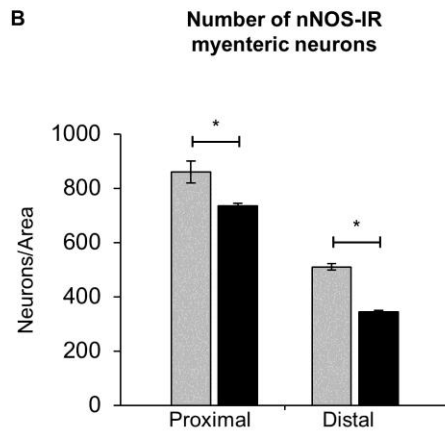
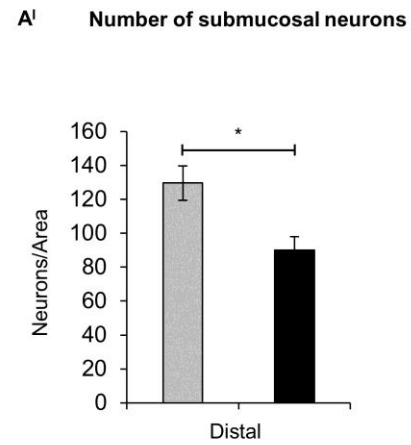
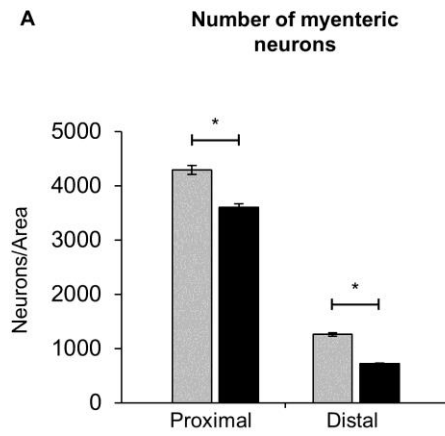


Figure 2.2 Effect of *in vivo* oxaliplatin treatment on total number of neurons and average number and proportion of nNOS-IR enteric neurons. Average number of myenteric neurons in the proximal and distal colon and submucosal neurons was counted per 2mm² in the distal colon from day 14 sham and oxaliplatin-treated mice (**A-A'**). Average number of nNOS-IR neurons (**B-B'**) in myenteric and submucosal ganglia counted within 2mm² area. Proportion of nNOS-IR neurons to the total number of myenteric and submucosal neurons (**C-C'**). Grey column: sham-treated, black column: oxaliplatin-treated mice. Data presented as mean \pm S.E.M. **P*<0.05, n=6 mice/group/time point.



2.4.2 Reactive oxygen species and protein nitration in the submucosal and myenteric ganglia of the colon following *in vivo* oxaliplatin treatment

To evaluate production of reactive oxygen species (ROS) following long-term oxaliplatin treatment, distal colon samples were probed with a fluorescent mitochondrial superoxide marker MitoSOX™ Red M36008 (**Figure 2.3**). Increased MitoSOX fluorescence was found in both the submucosal (24 ± 2 arbitrary units, relative to background, $P < 0.05$) (**Figure 2.4A-A', B-B'**) and myenteric (38 ± 3 arbitrary units, $P < 0.05$) (**Figure 2.4C-C', D-D'**) plexuses of the distal colon from oxaliplatin-treated mice compared to sham-treated animals (submucosal: 18 ± 2 arbitrary units; myenteric: 28 ± 3 arbitrary units) ($n = 6$ mice/group, **Figure 2.4E**).

Western blot analysis revealed a 40% increase in the expression of iNOS in LMMP preparations from the colon of oxaliplatin-treated mice ($n = 5$ mice/group, **Figure 2.5F**).

An antibody against nitrotyrosine was used to label nitrated proteins within the submucosal (**Figure 2.6A-B''**) and myenteric (**Figure 2.6C-D''**) plexuses. The number of neurons per 2mm^2 displaying translocation of nitrated proteins to the nuclei was higher in both the submucosal (36 ± 14 , $P < 0.05$) and myenteric (36 ± 15 , $P < 0.05$) plexuses of the colon from oxaliplatin-treated ($n = 6$ animals/group) compared to sham-treated (submucosal: 0.2 ± 0.1 ; myenteric: 0.1 ± 0.02 , $n = 6$ animals/group) (**Figure 2.7**).

Figure 2.3 Mitochondrial superoxide in the colonic submucosal and myenteric ganglia. Fluorescent and binary images of wholemound preparations of submucosal (**A-A'**, **B-B'**) and myenteric (**C-C'**, **D-D'**) ganglia labelled with MitoSOXTM Red in the colons from day 14 sham and oxaliplatin-treated mice. Scale bar = 50µm.

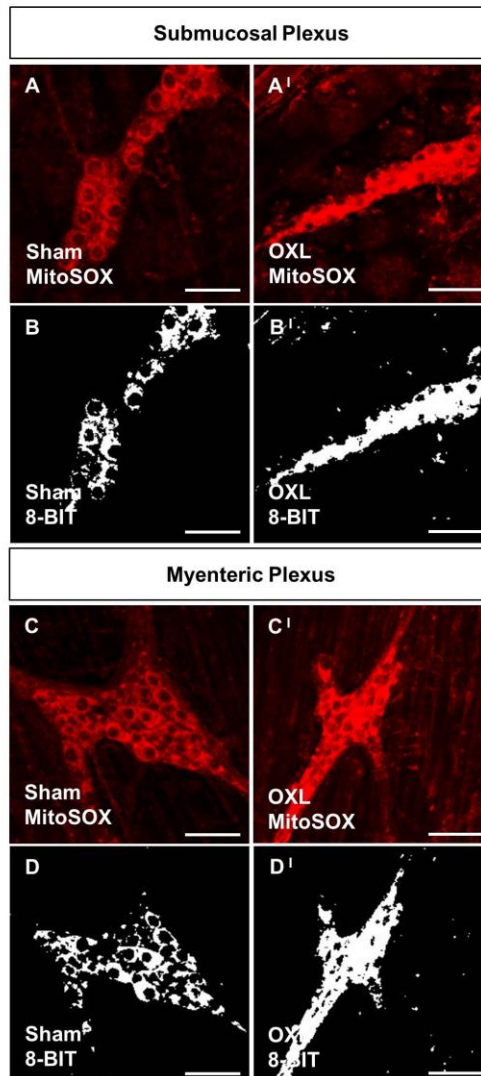


Figure 2.4 MitoSOX Fluorescence. Quantification of the levels of mitochondrial superoxide production visualised by fluorescent probe in submucosal and myenteric ganglia in colonic preparations from day 14 sham and oxaliplatin-treated animals. Data presented as mean \pm S.E.M. * $P < 0.05$, $n = 5$ mice/group.

Changes in MitoSOX fluorescence

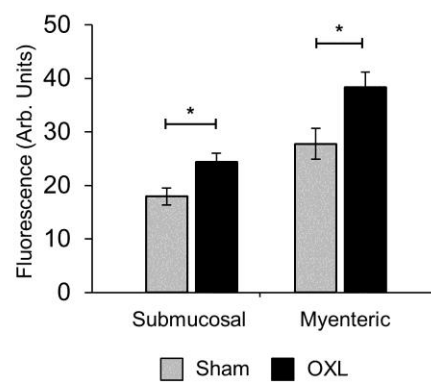
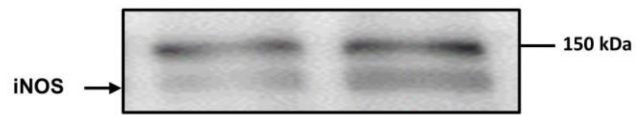


Figure 2.5 iNOS protein expression in the colon. Representative images and quantification of the Western blot analysis for inducible nitric oxide synthase (iNOS) in longitudinal muscle-myenteric plexus tissue from day 14 sham and oxaliplatin-treated mice. iNOS protein was normalised to total protein values obtained from the Coomassie Blue membrane staining (see Methods section). All values are expressed as a percentage of the values obtained from sham-treated mice. Data presented as mean \pm S.E.M. * P <0.05, n=6 mice/group



iNOS protein expression

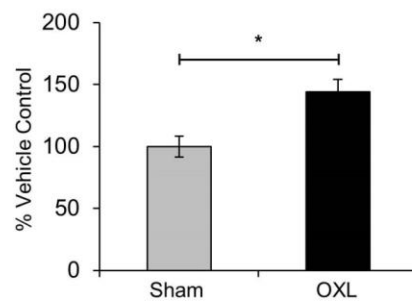


Figure 2.6 Translocation of nitrotyrosine to the nuclei of submucosal and myenteric neurons. Wholemound preparations of colonic submucosal ganglia from day 14 sham **(A-A'')** and oxaliplatin-treated **(B-B'')** mice and myenteric ganglia from sham **(C-C'')** and oxaliplatin-treated **(D-D'')** mice. Scale bar = 50µm. Neurons and ganglia were labelled with anti-β-Tubulin III antibody (Tub III, red). Nitrotyrosine within the ganglia was labelled with anti-nitrotyrosine antibody (NT, green).

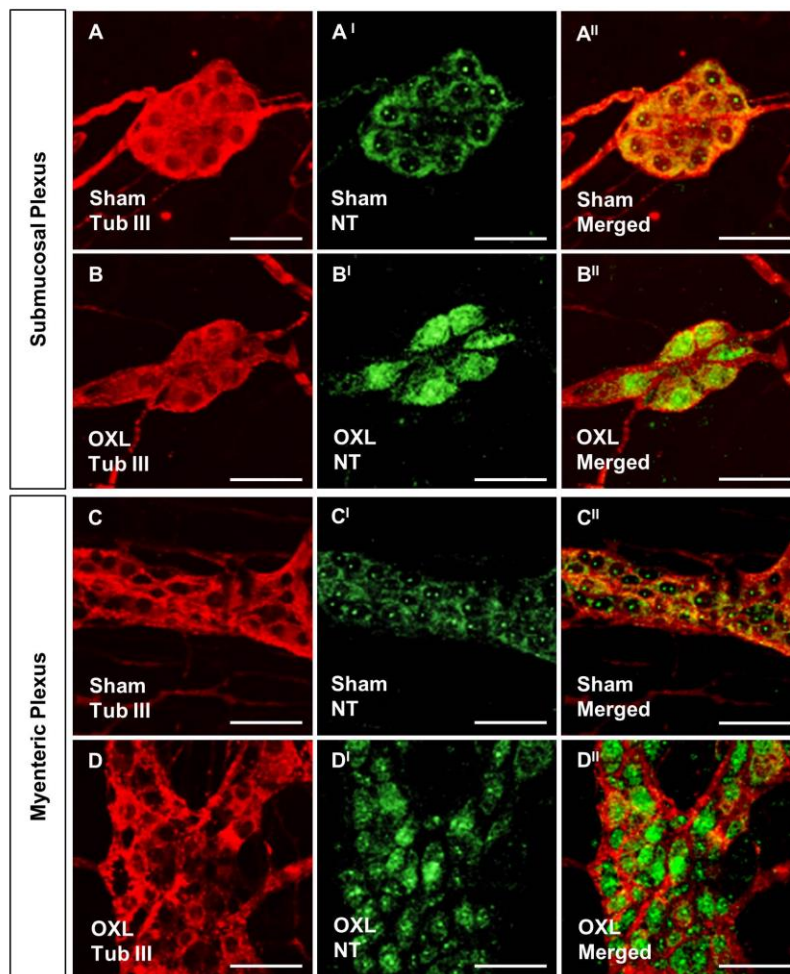
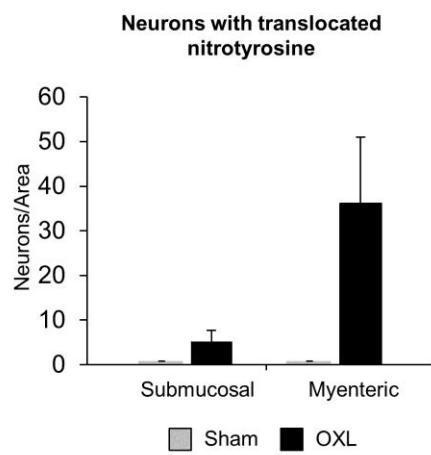


Figure 2.7 Translocation of nitrotyrosine to the nuclei of submucosal and myenteric neurons. Total number of submucosal and myenteric neurons containing translocated nitrotyrosine was counted per 2mm² in the distal colon from day 14 sham and oxaliplatin-treated mice. Data presented as mean \pm S.E.M **P*<0.05 (n=6 mice/group).



2.4.3 Apoptosis of submucosal and myenteric neurons in the colon

Diffusion of cytochrome *c* out of the mitochondria as a result of mitochondrial membrane depolarisation and increased permeability was measured via fluorescence (green) of monomeric JC-10. Increased monomeric JC-10 fluorescence was found in both submucosal (26 ± 3 arbitrary units, relative to background, $P < 0.0$) (**Figure 2.8A-A', B-B'**) and myenteric (51 ± 3 arbitrary units, $P < 0.05$) (**Figure 2.8C-C', D-D'**) plexuses of the distal colon from oxaliplatin-treated mice compared to sham-treated animals (submucosal: 3 ± 0.9 arbitrary units; myenteric: 14 ± 2 arbitrary units) (**Figure 2.9**, $n=3$ mice/group,). Both tissues from both sham and oxaliplatin-treated mice experienced the same dissection and therefore the same level of cellular stress associated with dissection. Cellular stress associated with dissection can induce short-term mitochondrial depolarisation, resulting in modest increases in JC-10 fluorescence in tissues from both sham and oxaliplatin-treated mice. However, the level of JC-10 fluorescence in tissue from oxaliplatin-treated mice was significantly higher (**Figure 2.9**) due to long-term mitochondrial depolarisation associated with neurotoxicity.

Caspase-3 immunoreactivity was absent in both the submucosal and myenteric ganglia in the colon preparations from sham-treated mice, but was observed in both the submucosal and myenteric ganglia in the colon preparations from oxaliplatin-treated mice indicating neuronal apoptosis (**Figure 2.10**, $n=6$ mice/group). Caspase-3 labelling appeared both within the neuronal cell body as well as smaller, irregular patches of apoptotic debris that did not co-localise with β -Tubulin III immunoreactivity.

Figure 2.8 Changes in neuronal mitochondrial membrane potential indicative of cytochrome *c* release in submucosal and myenteric plexuses. Fluorescent and binary wholemount preparations of submucosal (**A-A'**, **B-B'**) and myenteric (**C-C'**, **D-D'**) ganglia labelled with JC-10 dye in the colons from day 14 sham and oxaliplatin-treated mice. Green fluorescent colour (JC monomeric form) is due to the JC-10-labelled release of cytochrome *c* diffusing out of the mitochondria as a result of mitochondrial depolarisation and increased permeability. Scale bar = 50µm.

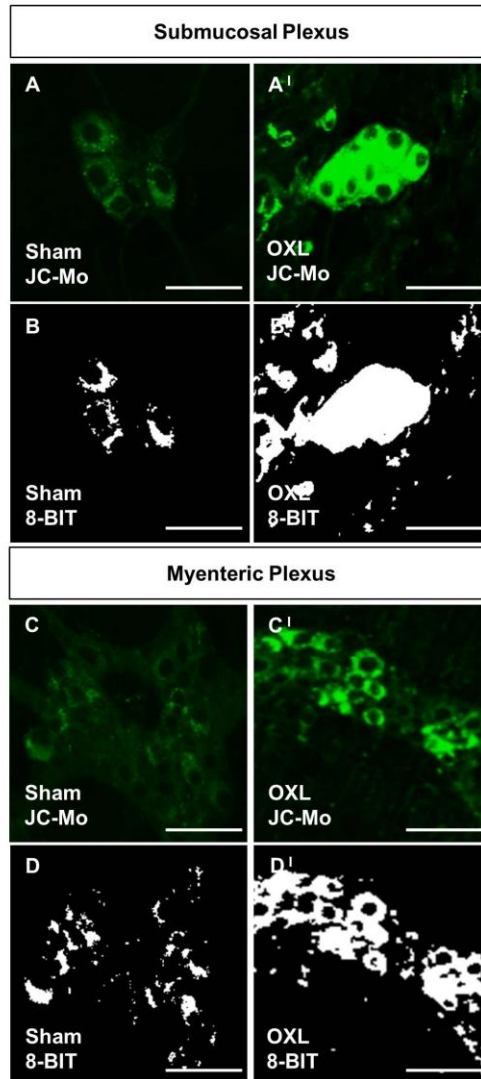


Figure 2.9 JC-10 Fluorescence. Quantification of the levels of monomeric JC-10 production visualised by fluorescent probe in submucosal and myenteric ganglia in colonic preparations from sham and oxaliplatin-treated animals. * $P < 0.05$ (n=6 mice/group).

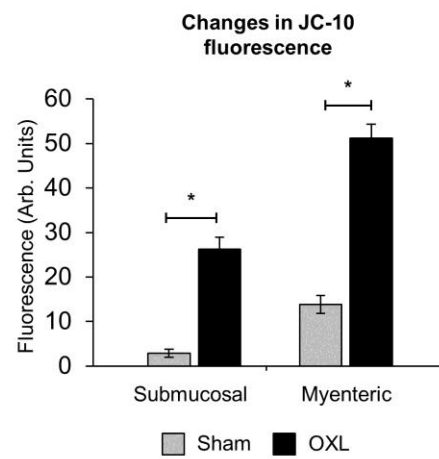
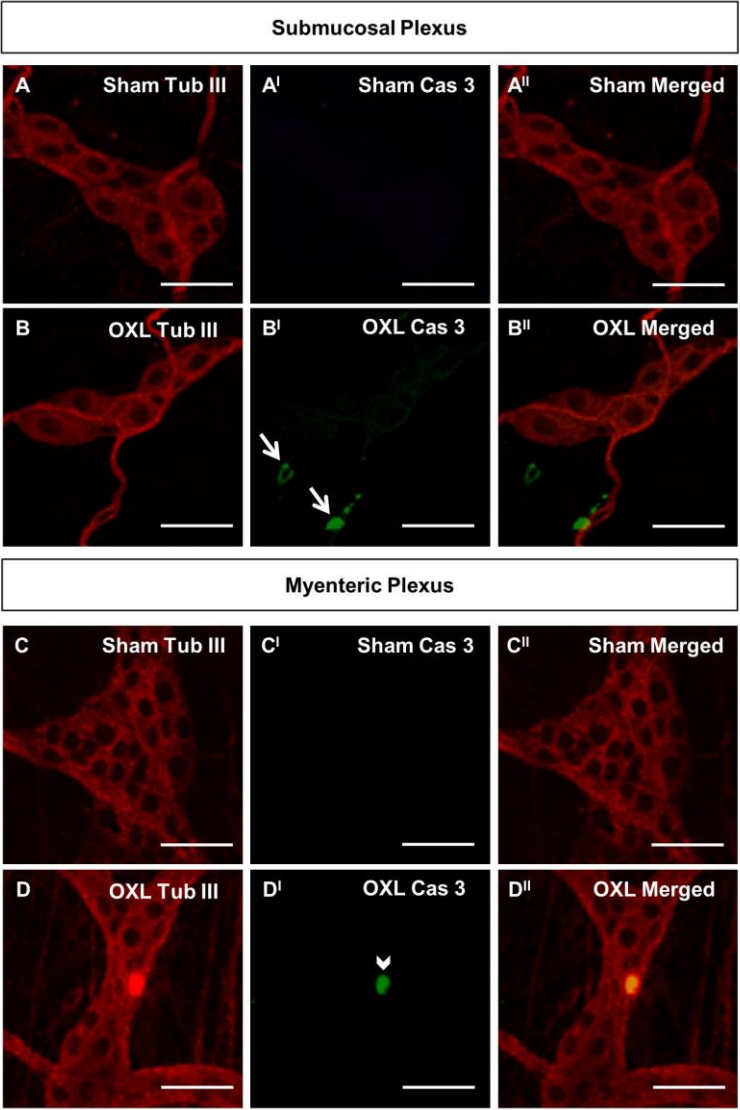


Figure 2.10 Detection of cleaved caspase-3 in submucosal and myenteric ganglia. Wholemount preparations of colonic submucosal ganglia from sham (**A-A''**) and oxaliplatin-treated (**B-B''**) mice and myenteric ganglia from sham (**C-C''**) and oxaliplatin-treated (**D-D''**) mice (n=6 mice/group). Neurons and ganglia were labelled with anti- β -Tubulin III antibody (Tub III, red). Neurons undergoing apoptosis were labelled with antibody against cleaved caspase-3 (Cas3, green). Scale bar = 50 μ m.



2.4.4 Effects of oxaliplatin treatment on neuromuscular transmission in the colon

Neuromuscular transmission in the distal colon was investigated using intracellular electrophysiology. Electrical stimulation of nerve fibres innervating the circular muscle in distal colon segments evoked junction potentials in single smooth muscle cells that were defined as fast inhibitory junction potentials (fIJPs), slow inhibitory junction potentials (sIJPs) and excitatory junction potentials (EJPs) using pharmacological blockers.

In control physiological saline, the resting membrane potential (RMP) of smooth muscle cells from sham-treated mice averaged -38 ± 1.0 mV ($n=6$ mice) and did not differ from that of smooth muscle cells from oxaliplatin-treated mice (-39 ± 1.0 mV, $n=6$ mice). RMPs in both groups were unaffected by the selective P2Y₁ receptor antagonist MRS2500 (1 μ M) (sham: -39 ± 1.4 mV, oxaliplatin: -37 ± 1.3 mV), L-NNA (1 mM) (sham: -39 ± 1.3 mV, oxaliplatin: -38 ± 0.9 mV) or the muscarinic antagonist, atropine (1 μ M) (sham: -38 ± 1.4 mV, oxaliplatin: -41 ± 3.3 mV). The input resistance of smooth muscle cells from sham-treated mice averaged 8.3 ± 0.9 M Ω ($n=6$ mice) in control physiological saline, this did not differ significantly from the input resistance of smooth muscle cells from oxaliplatin-treated mice (8.7 ± 1.0 M Ω , $n=6$ mice).

Single pulse and compound stimuli (trains of 20V, 40 ms interval, 0.4 ms pulse duration) evoked fIJPs in colonic smooth muscle cells (**Figure 2.11A, A'**). The amplitudes of fIJPs recorded in smooth muscle cells from the distal colon of sham-treated mice following a 20V compound stimulus (-39 ± 3 mV, $n=6$ mice) did not differ significantly from those recorded from the distal colon of oxaliplatin-treated mice (-38 ± 2 mV, $n=6$ mice) (**Figure 2.11B**). The duration of fIJPs (width at half amplitude) was not different between (sham: 397 ± 17 ms) and oxaliplatin-treated (422 ± 27 ms) groups. The amplitudes of fIJPs increased with increasing stimulus strength, but no significant differences were found in responses between sham and oxaliplatin-treated

mice (**Figure 2.11C**). Fast IJPs were mediated by a purine as they were inhibited by MRS2500 (1 μ M) (n=6 mice/group).

Short compound stimuli (trains of 20V, 3 pulses, 40 ms interval, 0.4 ms pulse duration) applied in the presence of MRS2500 evoked sIJPs (**Figure 2.11D**), whose amplitudes were greater in smooth muscle cells from the distal colon of mice treated with oxaliplatin (-6.1 ± 0.5 mV, $P < 0.05$) than from sham-treated mice (-4.6 ± 0.5 mV) (n=6 mice/group) (**Figure 2.11E**). The sIJPs were mediated by NO, as they were abolished by addition of L-NNA (1mM) (n=6 mice/group).

Short compound stimuli (trains of 20V, 3 pulses, 40 ms interval, 0.4 ms pulse duration) of nerve fibres in the presence of both MRS2500 and L-NNA, inhibiting fast and slow IJPs respectively, evoked EJPs (n=6 mice/group) (**Figure 2.12A**). EJPs were cholinergic as subsequent addition of atropine (1 μ M) effectively inhibited them (**Figure 2.12B**). The EJPs recorded in smooth muscle cells from the distal colon of sham-treated mice (6 ± 0.4 mV) did not differ from those of oxaliplatin-treated mice (6 ± 0.7 mV) (n=6 mice/group) (**Figure 2.12C**). Atropine reduced the amplitude of responses in sham-treated mice from 6 ± 0.4 to 0.8 ± 0.3 mV ($P < 0.05$) and from 6 ± 0.7 to 0.8 ± 0.2 mV in oxaliplatin-treated mice ($P < 0.05$, n=6 animals/group) (**Figure 2.12C**).

Figure 2.11 Intracellular recordings of fast and slow inhibitory junction potentials from colonic smooth muscle cells. Single pulse electrical stimulus (40 V, 0.4 ms duration) and high frequency compound stimulus (20 V, 0.04 ms interval, 0.4 ms duration) evoked fast inhibitory junction potentials (fIJPs) in smooth muscle cells from day 14 sham **(A)** and oxaliplatin-treated **(A')** mice (* stimulus artefact). **(B)** The mean amplitude of fIJPs in response to compound stimuli from smooth muscle cells of sham and oxaliplatin-treated mice (n = 4 mice/group). **(C)** Amplitudes of fIJPs to increasing strength of the electrical stimuli (2-60 V) in smooth muscle cells of sham (grey line) and oxaliplatin-treated (black line) mice. **(D)** Blocking fIJPs with a selective antagonist of P2Y₁ receptors, MRS2500 (1 μ M) revealed slow inhibitory junction potentials (sIJPs) in smooth muscle cells from both sham (grey line) and oxaliplatin-treated (black line) mice. **(E)** Comparison of the amplitude of sIJPs in smooth muscle cells from sham and oxaliplatin-treated mice. Data presented as mean \pm S.E.M. **P*<0.05 (n=6 mice/group).

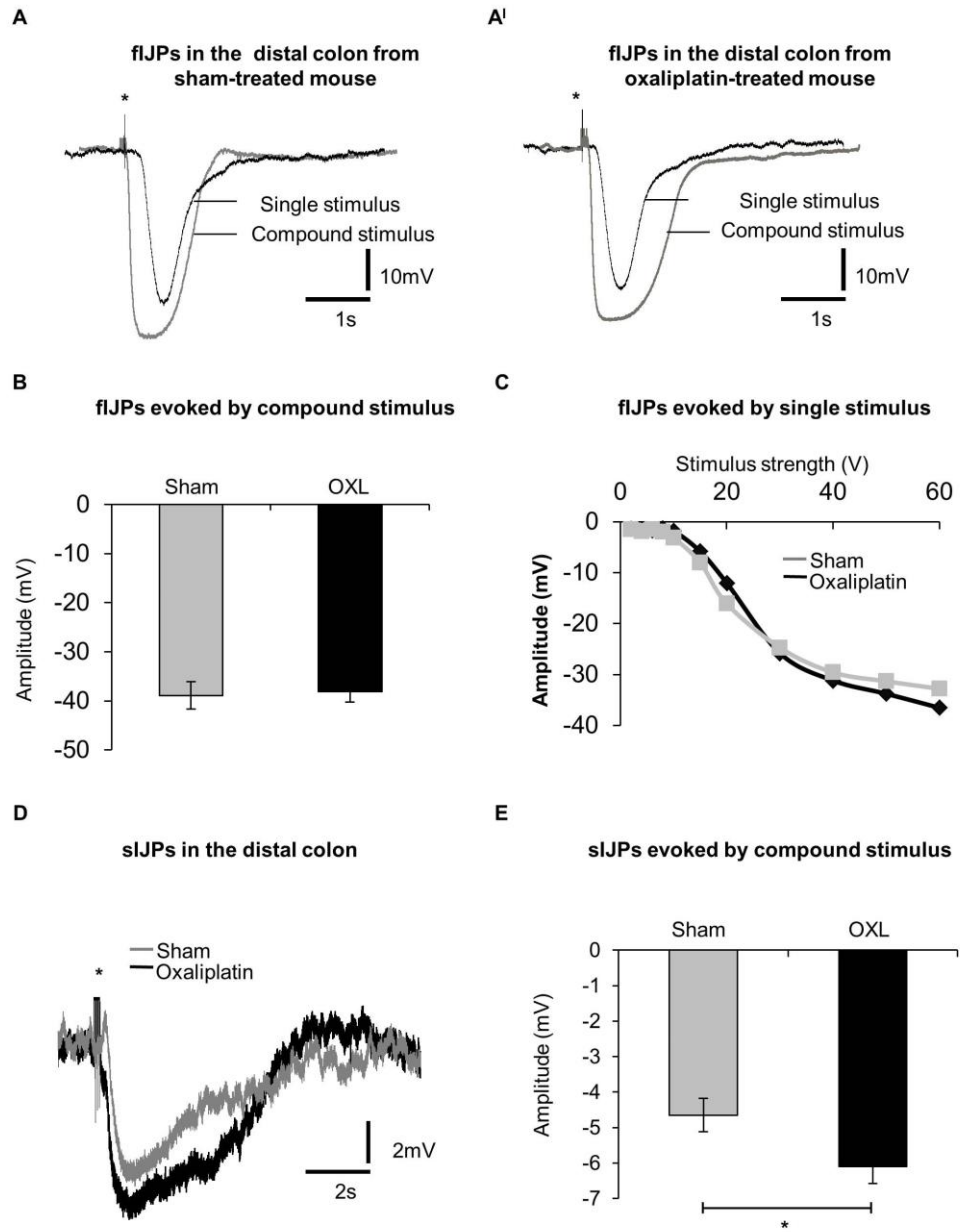
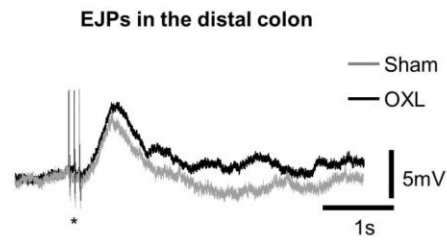
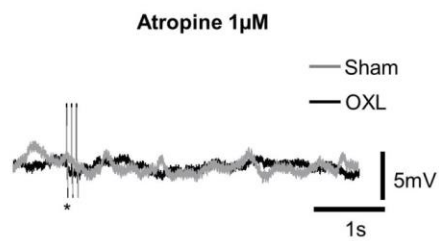


Figure 2.12 Intracellular recordings of excitatory junction potentials from colonic smooth muscle cells. (A) Excitatory junction potentials (EJPs) in response to high frequency stimulation (20 V, 0.04 ms interval, 0.4 ms duration) were revealed in smooth muscle cells from sham (grey trace) and oxaliplatin-treated (black trace) mice in the presence of MRS2500 (1 μ M) and L-NNA (1mM). (B) Subsequent addition of atropine reduced the amplitude of the EJPs (* stimulus artefact). (C) The mean amplitude of EJPs in smooth muscle cells from sham-treated and oxaliplatin-treated mice before and after addition of atropine (1 μ M). * P <0.05 (n=6/group).

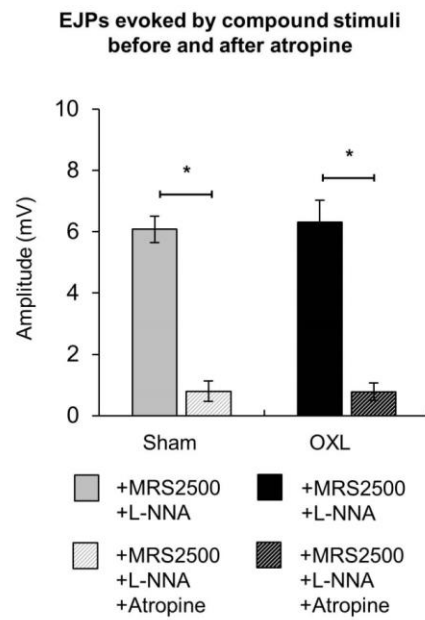
A



B



C

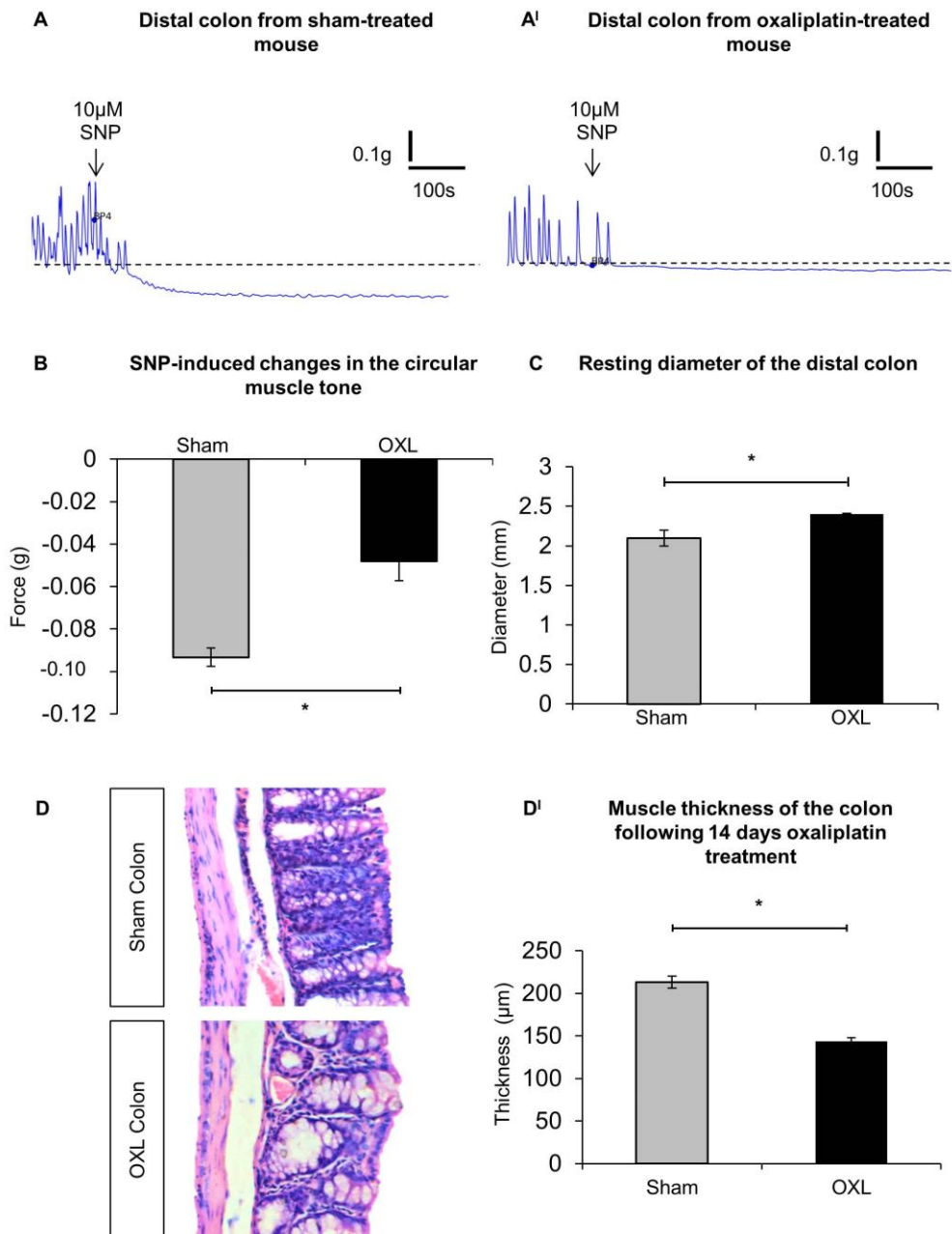


2.4.5 Effects of oxaliplatin treatment on colonic smooth muscles

Smooth muscle tone of the distal colon was studied in organ bath experiments using force transducers. We measured the force produced by 3mm wide circular muscle rings. Application of SNP (10 μ M) to organ baths containing distal colon segments resulted in a decrease in circular muscle tone, i.e. relaxation, in tissues from both sham (**Figure 2.13A**) and oxaliplatin-treated (**Figure 2.13A'**) mice. The reduction in tension produced by SNP was greater in the colon segments from sham-treated mice (from 0.27 ± 0.07 g to -0.09 ± 0.005 g) than in segments from oxaliplatin-treated mice (from 0.21 ± 0.07 g to -0.04 ± 0.01 g, $P < 0.05$) ($n = 6$ mice/group, **Figure 2.13B**). Thus, the relaxation force in response to SNP was reduced in colonic circular muscles from oxaliplatin-treated mice.

The resting colon diameter measured in organ-bath experiments between contractions was larger in preparations from mice treated with oxaliplatin (2.4 ± 0.1 mm) compared to sham-treated mice (2.1 ± 0.1 mm) ($n = 7$ animals/group, $P = 0.05$) (**Figure 2.13C**). Histological analysis of colon sections revealed thinning of the colonic muscle layer in oxaliplatin-treated mice when compared to sham (**Figure 2.13D**). Quantitative analysis confirmed that this reduction was statistically significant (sham: 213 ± 7 μ m; oxaliplatin-treated: 144 ± 4 μ m, $P < 0.05$, $n = 6$ mice/group, 10 sections per preparation from each animal) (**Figure 2.13D'**).

Figure 2.13 Effects of oxaliplatin treatment on colonic smooth muscles. (A-A') Smooth muscle relaxation following application of the nitric oxide donor, sodium nitroprusside (10 μ M) to the colon from day 14 sham **(A)** and oxaliplatin-treated **(A')** mice. **(B)** Comparison of the maximum relaxation produced by circular muscles in response to sodium nitroprusside in colonic preparations from sham and oxaliplatin-treated mice quantified as an absolute change in the force transduction from the basal values. * P <0.05 (n=6 mice/group). **(C)** Resting diameter of the distal colon from sham and oxaliplatin-treated mice. * P =0.05 (n=7 mice/group). **(D)** Gross morphological changes in the colon following repeated *in vivo* oxaliplatin administration. Colonic crypt length was shorter in oxaliplatin-treated mice and muscle thickness was reduced in comparison to the sham-treated animals. **(D')** Statistical analysis of the muscle layer thickness in the colon preparations from sham and oxaliplatin-treated mice. Data presented as mean \pm S.E.M. * P <0.05 (6 mice/group, 10 sections per preparation from each animal).



2.4.6 Colonic motility

Colonic motor patterns:

Analysis of the total number of contractions included all types of motor patterns in the colon (CMMCs, short and fragmented contractions) (n=10 mice/group, **Figure 2.14A**). The total number and rate of rise of contractions were reduced in the colons from oxaliplatin-treated animals compared to sham-treated mice ($P<0.05$ for both, **Table 2.1**). The addition of L-NNA did not alter the overall number of contractions in either group (**Figure 2.14B**, **Table 2.1**). Further analysis revealed differences in occurrence of various types of colonic motor patterns in the oxaliplatin-treated group with a significant decrease in the proportion of CMMCs ($P<0.05$, **Figure 2.14C**) and increase in the proportion of fragmented contractions ($P<0.05$, **Figure 2.14E**). Application of L-NNA restored the proportion of CMMCs in oxaliplatin-treated colons to the levels comparable with sham-treated colons (**Figure 2.14C**), but had no significant effect on the proportion of either short or fragmented contractions (**Figure 2.14D, E**). The frequency, speed of propagation and length of contractions were measured for each specific type of motor activity.

Colonic migrating motor complexes:

In vivo treatment with oxaliplatin was associated with a lower CMMC frequency than in sham-treated preparations ($P<0.05$). Application of L-NNA significantly increased the frequency of CMMCs in colons from oxaliplatin-treated ($P<0.05$), but not sham-treated mice (**Figure 2.15A**, **Table 2.1**). The rate of rise of CMMCs was comparable between sham-treated and oxaliplatin-treated mice, but increased CMMC rate of rise following addition of L-NNA was noted in the colons from sham ($P<0.05$), but not oxaliplatin-treated animals (**Figure 2.15B**, **Table 2.1**).

Short contractions:

Treatment with oxaliplatin resulted in a reduced frequency of short contractions in the colon (n=10/group, $P<0.05$, **Figure 2.15C, Table 2.1**). L-NNA had no effect on the frequency of short contractions in either group. The rate of rise of short contractions was two-fold slower in colons from oxaliplatin-treated mice ($P<0.05$, **Figure 2.15D, Table 2.1**). L-NNA significantly enhanced the propagation speed of short contractions in the sham ($P<0.05$), but not the oxaliplatin-treated group. The frequency of short anterograde contractions in the distal region of the colon was significantly reduced in oxaliplatin-treated animals compared to sham-treated mice ($P<0.05$, **Figure 2.15E, Table 2.1**). Short contractions initiated in the distal colon were also found to travel in a retrograde direction. These retrograde short contractions occurred at a significantly reduced frequency in colons from oxaliplatin-treated mice compared to sham-treated mice ($P<0.05$, **Figure 2.15F, Table 2.1**). L-NNA had no effects on anterograde or retrograde short distal contractions in either group.

Fragmented contractions:

These were defined as incomplete contractions occurring simultaneously rather than propagating over the length of the colon (**Figure 2.16A**). The frequency of fragmented contractions was significantly higher in oxaliplatin-treated than in sham-treated mice (n=10 mice/group, $P<0.05$, **Figure 2.16B, Table 2.1**). Application of L-NNA did not affect the frequency of fragmented contractions in either oxaliplatin-treated or sham-treated mice.

Table 2.1 Parameters of different types of colonic contractions

| | | Sham-treated (n=10 mice) | Oxaliplatin-treated (n=10 mice) |
|---|---------|------------------------------------|--|
| All Contractions | | | |
| Frequency (contractions/10 min) | Control | 33.8±2.4 | 21.7±2.9* |
| | L-NNA | 31.6±3.9 | 21.8±3.9 |
| Speed (mm/s) | Control | 1.8±0.1 | 1.3±0.2 |
| | L-NNA | 3.4±0.5 | 2.1±0.4 |
| CMMCs | | | |
| Proportion (%) | Control | 27.8±3.5 | 9.4±3.7* |
| | L-NNA | 32.4±5.9 | 31.0±6.4^ |
| Frequency (contractions/10 min) | Control | 9.0±0.9 | 2.5±1.0* |
| | L-NNA | 9.8±2.2 | 6.0±1.2^ |
| Speed (mm/s) | Control | 2.2±0.3 | 2.0±0.5 |
| | L-NNA | 3.5±0.8* | 1.9±0.5 |
| Short Contractions | | | |
| Proportion (%) | Control | 66.2±2.4 | 58.5±6.9 |
| | L-NNA | 55.5±3.9* | 42.2±6.2 |
| Frequency (contractions/10 min) | Control | 22.7±2.4 | 13.0±2.0* |
| | L-NNA | 17.6±2.3 | 9.8±1.9 |
| Speed (mm/s) | Control | 1.6±0.1 | 0.8±0.1* |
| | L-NNA | 2.8±0.6* | 1.2±0.2 |
| Distal anterograde short contraction frequency (per/10mins) | Control | 14.5±1.5 | 6.4±1.5* |
| | L-NNA | 10.3±1.5 | 4.8±1.4 |
| Distal retrograde short contraction frequency (per/10mins) | Control | 2.0±0.6 | 0.1±0.1* |
| | L-NNA | 1.0±0.3 | 0.3±0.1 |
| Fragmented Contractions | | | |
| Proportion (%) | Control | 6.0±2.1 | 32.1±6.1* |
| | L-NNA | 12.1±3.1 | 26.8±5.2 |
| Frequency (contractions/10 min) | Control | 2.1±0.7 | 6.2±1.2* |
| | L-NNA | 4.2±1.2 | 6.0±1.1 |

* $P < 0.05$ compared to sham-control; ^ $P < 0.05$ compared to oxaliplatin-control

Figure 2.14 Total number of contractions and proportion of different types of contractile activity in the colon before and after application of L-NNA. (A) Examples of spatiotemporal maps generated from digital video recordings of colonic motility from day 14 sham and oxaliplatin-treated mice before (control) and after addition of L-NNA. Each contraction can be seen as a reduction in the gut width (red), while relaxation as an increase in the gut width (blue). Colonic migrating motor complexes (CMMCs) propagate >50% of the colon length, short contractions (SCs) propagate <50% of the colon length and fragmented contractions (FCs) are interrupted by period(s) of relaxation during contraction. (B) The total number of contractions including all types of contractile activity in the colons from sham and oxaliplatin-treated mice. The proportion of CMMCs (C), short contractions (D) and fragmented contractions (E) to the total number of contractions was calculated in maps from sham-treated and oxaliplatin-treated mice before and after addition of L-NNA. Data are presented as an average of contractions per 10 min from a total of 40 min of video recording at baseline intraluminal pressure prior to and after the addition of L-NNA. Data presented as mean \pm S.E.M. * P <0.05, (n=10 mice/group).

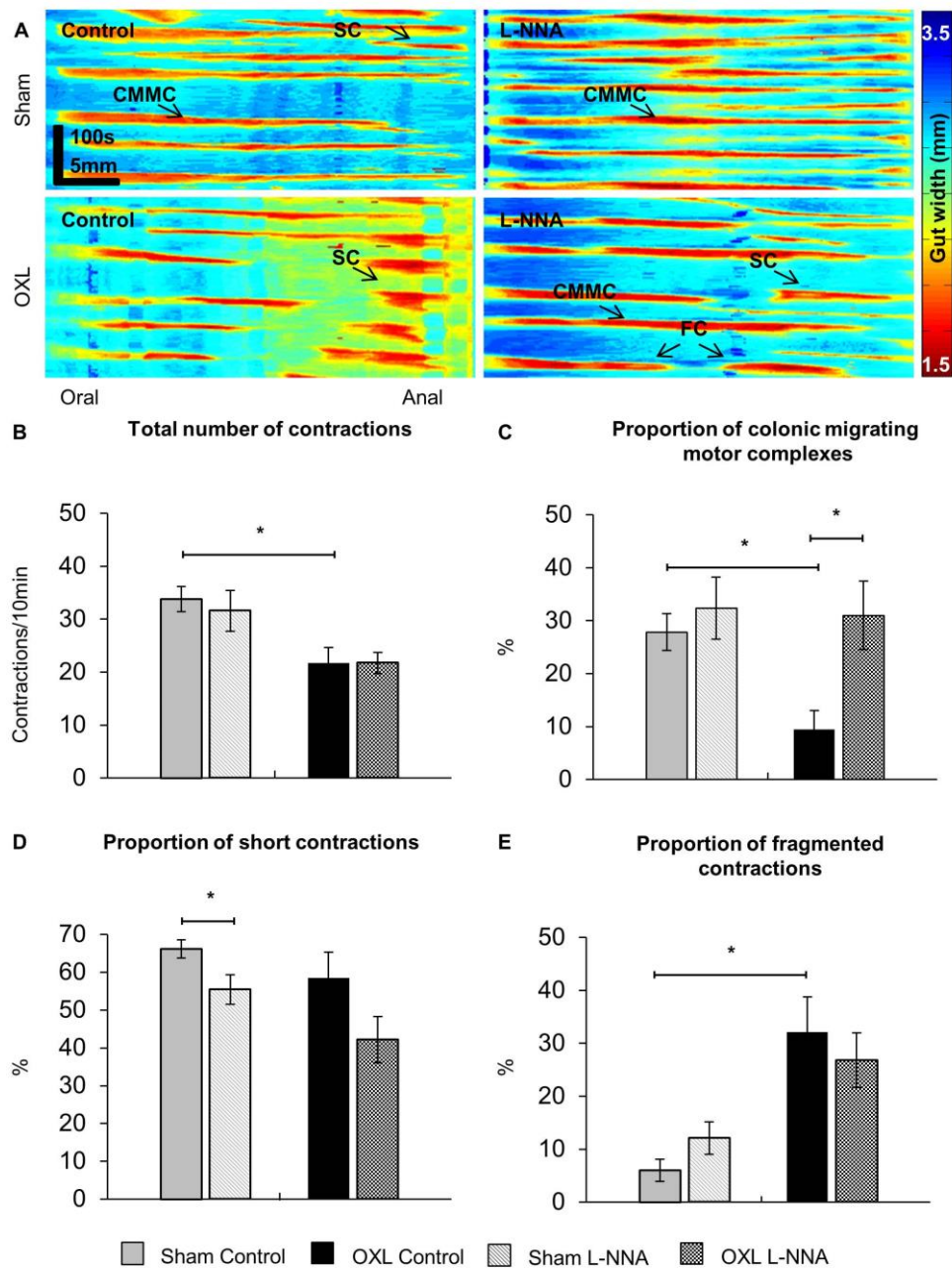


Figure 2.15 Effects of L-NNA on colonic migrating motor complexes and short contractions. (A) The frequencies of CMMCs quantified in spatiotemporal maps from sham-treated and oxaliplatin-treated mice before and after L-NNA application. (B) Speed of CMMCs in the colons from sham and oxaliplatin-treated mice in both test conditions. * $P < 0.05$ (n=10 mice/group). (C) The frequencies of short contractions were quantified in spatiotemporal maps from sham and oxaliplatin-treated mice before and after L-NNA application. (D) Speed of all short contractions analysed before and after L-NNA application. Changes in the frequency of distal anterograde (E) and retrograde (F) short contractions in the colons from sham and oxaliplatin-treated mice in both test conditions. Data presented as mean \pm S.E.M. * $P < 0.05$, (n=10 mice/group).

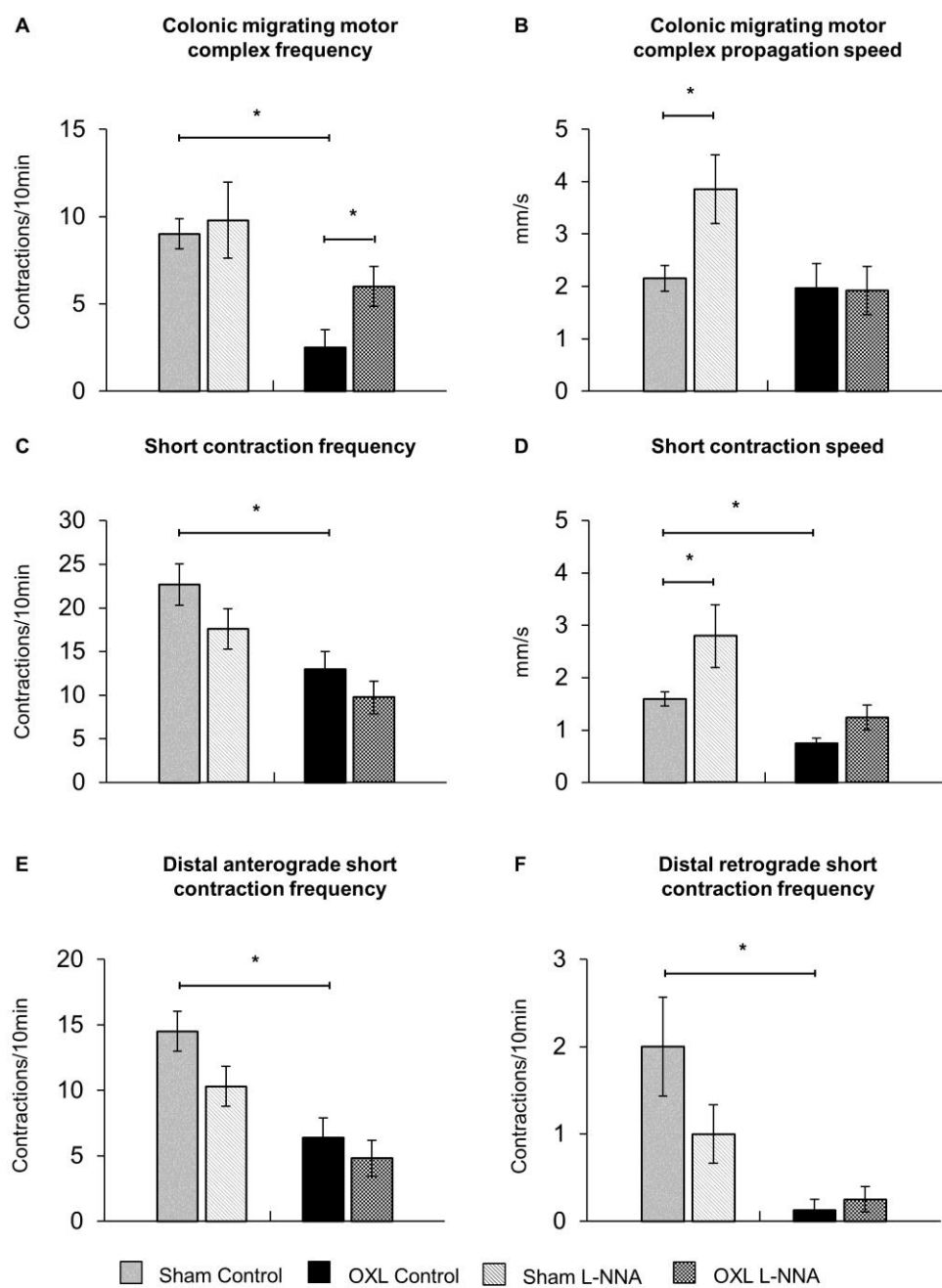
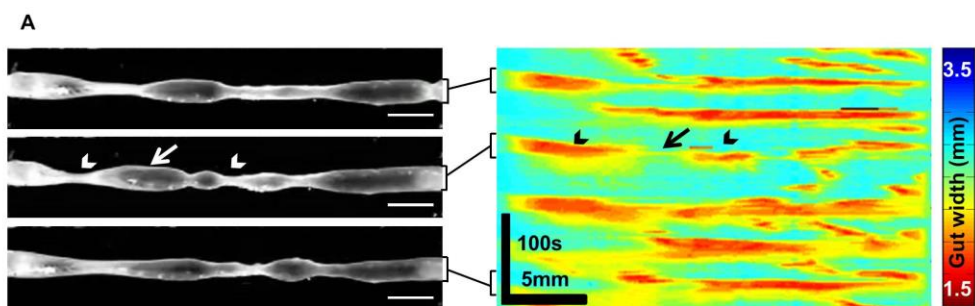
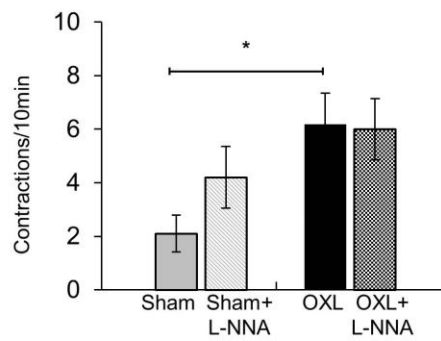


Figure 2.16 Effects of L-NNA on fragmented contractions. (A) Fragmented contractions were defined as interrupted contractions consisting of period(s) of relaxation (arrow) and simultaneously occurring contractions (arrowheads). The frequency (**B**) of fragmented contractions in the colon from sham and oxaliplatin-treated mice before and after L-NNA application. Data presented as mean \pm S.E.M. * $P < 0.05$ (n=10 mice/group).



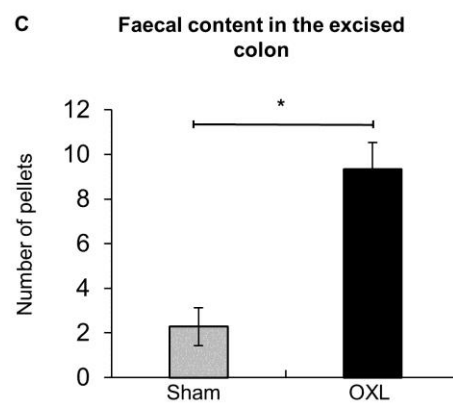
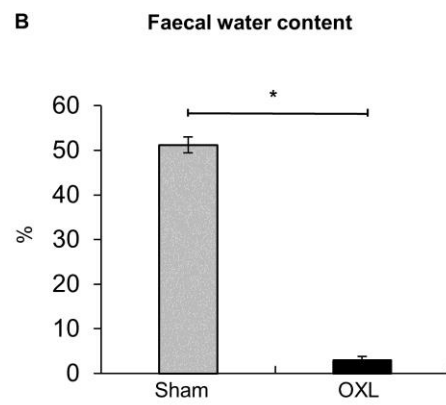
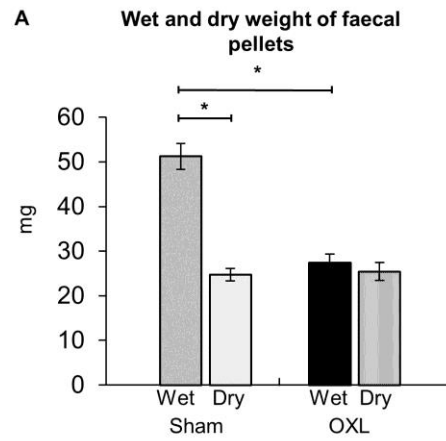
B Fragmented contraction frequency



2.4.7 Colonic faecal content

To define the clinical symptoms resulting from the altered patterns of colonic motor activity, fresh faecal pellets were collected from both sham and oxaliplatin-treated mice and faecal water content was calculated as the difference between wet and dry pellet weight (**Figure 2.17A-C**). The fresh pellets (wet weight) from oxaliplatin-treated mice (27.4 ± 2.0 mg) weighed significantly less than those of sham-treated mice (51.2 ± 2.9 mg, $P < 0.05$) ($n = 10$ mice/group) (**Figure 2.17A**). After dehydration, the dry weight of pellets from sham-treated mice was 24.7 ± 1.4 mg, and 25.4 ± 2.0 mg from oxaliplatin-treated mice (**Figure 2.17A**). Thus, the water content was significantly lower in pellets collected from oxaliplatin-treated mice (0.9 ± 0.3 mg, $2.95 \pm 0.8\%$) than in those collected from sham-treated mice (25.7 ± 1.9 mg, $51.2 \pm 1.8\%$, $P < 0.05$) ($n = 10$ mice/group) (**Figure 2.17B**). Total number of pellets within the entire length of excised colons from oxaliplatin-treated mice (9.3 ± 1.2) was significantly higher than the number in sham-treated mice (2.1 ± 0.8 , $P < 0.05$) ($n = 10$ mice/group) (**Figure 2.17C**).

Figure 2.17 Effects of oxaliplatin of colonic faecal content. (A) Wet weight of faecal pellets measured immediately upon pellet expulsion; dry weight of faecal pellets measured after 72 hours of dehydration at room temperature. (B) Faecal water content calculated as the difference between the wet weight and dry weight. (C) Total number of faecal pellets along the entire length of the colon counted in freshly excised intact colons. Data presented as mean \pm S.E.M. *P<0.05 (n=10 mice/group).



2.5 Discussion

This study is the first to examine the role of oxidative stress *in vivo* on enteric neurons of the colon that may be involved in mechanisms underlying colonic dysmotility associated with oxaliplatin treatment. The results show that mitochondrial superoxide and nitrated protein levels are significantly increased in the myenteric and submucosal ganglia after oxaliplatin treatment. Mitochondrial depolarisation and increased mitochondrial permeability leading to release of cytochrome *c* from mitochondria were increased in myenteric ganglia in the colon from oxaliplatin-treated mice. This presumably led to increased expression of an “executioner” caspase-3 in both the submucosal and myenteric ganglia in the colon preparations from oxaliplatin-treated mice. Oxidative stress and neuronal apoptosis resulted in functional changes in the colon (increased nitrgic neuromuscular transmission, decreased smooth muscle tone, changes in the patterns and parameters of colonic motility) leading to symptoms of constipation in mice after long-term treatment with oxaliplatin.

2.5.1 Oxaliplatin-Induced Oxidative Stress and Neuronal Apoptosis

Oxidative stress in enteric neurons following oxaliplatin treatment was evidenced by increased mitochondrial superoxide and translocation of nitrated proteins in submucosal and myenteric plexuses as well as the increase in expression of iNOS observed in our study. Mitochondria are responsible for most of the ROS burden under both normal and pathological conditions. The increased level of mitochondrial superoxide observed in enteric neurons is consistent with findings showing increased mitochondrial ROS in neuronal cultures from dorsal root ganglia (DRG) of oxaliplatin-treated rats (Kelley et al., 2014). Inhibition of mitochondrial electron transport chain complexes I and III involved in mitochondrial ROS formation improves sensory hyperalgesia in a rat model of peripheral neuropathy induced by another platinum-based chemotherapeutic drug, cisplatin (Joseph and Levine, 2009). Mitotoxicity affecting mitochondrial respiration and ATP production were found in sciatic nerve from oxaliplatin-treated rats

and linked to oxaliplatin-induced peripheral neuropathy (Zheng et al., 2011), which could be prevented by peroxynitrite decomposition catalyst (Janes et al., 2013). Thus, reduction of oxidative stress-related biomarkers in peripheral neurons may be a promising avenue for alleviation of chemotherapy-induced neuropathies.

Oxaliplatin-induced mitochondrial damage has also been proposed as a potential mechanism leading to activation of NOS (Areti et al., 2014). The direct toxicity of NO can be greatly enhanced by reacting with superoxide to form peroxynitrite, which modifies tyrosines in proteins to create nitrotyrosines. Nitration of structural proteins can have major pathological consequences, but can also be an indicator of oxidative stress (Beckman and Koppenol, 1996). We identified translocation of nitrated proteins in both submucosal and myenteric plexuses of the colon from oxaliplatin-treated mice. Translocation of nitrotyrosine has been previously found in myenteric neurons following ischemia and reperfusion in mice (Rivera et al., 2011b) and induction of colitis in rats (Zingarelli et al., 2003) both of which were correlated with increases in NO and ROS. The increased level of iNOS observed in the longitudinal muscle-myenteric plexus preparations in our study probably leads to increased production of NO contributing to the accumulation of nitrotyrosine in enteric neurons. Although nitration can be used as an indirect measure of peroxynitrite, accumulation of nitrotyrosine itself is correlated with increased susceptibility to NO-induced apoptosis (Savidge, 2011). Accumulation of nitrotyrosine has been linked to protein misfolding, mitochondrial dysfunction and neuronal degeneration (Nakamura and Lipton, 2008). Increased ROS and concurrent oxidative stress are linked to damage in various cellular components, and neuronal death (Wei et al., 2000).

One of many by-products of oxaliplatin metabolism is oxalate and increases in oxalate levels trigger intracellular Ca^{2+} influx (Webster et al., 2005). Increased cytoplasmic Ca^{2+} activates nNOS, potentially leading to excessive NO production as well as activating the release of cytochrome c (Yagihashi et al., 2000). Release of JC-10-labelled cytochrome c diffusing

out of the mitochondria as a result of mitochondrial depolarisation and increased permeability was observed in both submucosal and myenteric neurons in our study. The increase in superoxide and NO production may be a direct consequence of the loss of mitochondrial cytochrome c (Düssmann et al., 2003). The rate of superoxide production is linked to the magnitude of mitochondrial membrane potential and mitochondrial permeability (Brand et al., 2004). The magnitude of mitochondrial depolarisation dictates cytochrome c release and hence the activation of apoptosis. Modest mitochondrial depolarisation is transient and reversible, and has been found to be protective against moderate tissue injury (Schweizer and Richter, 1994;Gottlieb et al., 2003). Apoptotic cells exhibit distinctive biochemical features, including the presence of specific cleaved proteins (Hengartner, 2000). Caspase-3 is a key effector or “executioner” caspase, cleaving various substrates to cause the morphological and biochemical changes seen in apoptotic cells (Slee et al., 2001;Elmore, 2007;McIlwain et al., 2013). Our results are consistent with previous studies using caspase-3 antibody which found both intracellular immunoreactivity and detected extracellular aggregates of apoptotic debris outside neuronal cells (Pasinelli et al., 2000). Due to the fact that the half-life of activated caspase-3 is 8 hours, it is difficult to detect significant amounts of activated caspase 3 within enteric ganglia. It is possible that the activation of caspase-3 might have occurred at the earlier stages of oxaliplatin treatment resulting into neuronal apoptosis; this would explain both neuronal loss and presence of apoptotic debris observed in our study at day 14. Increased expression of caspase-3 observed in our study is indicative of oxaliplatin-induced apoptosis which resulted in 43% loss of myenteric neurons in the distal colon, 16% of myenteric neurons in the proximal colon and 30% of submucosal neurons in the distal colon. Similarly, oxaliplatin treatment induced 12% loss of myenteric and 21% submucosal neurons in the mouse ileum at day 14 (Robinson et al., 2016). Although oxaliplatin causes neuronal death in all studied intestinal regions, it seems that neurons in the distal colon are more susceptible to oxaliplatin-induced damage than neurons in the proximal colon and ileum. There may be many reasons for these regional differences, but it should be noted that the proportion of the

nNOS-IR neurons was significantly increased in the distal, but not proximal colon, consistent with a role for NO in oxaliplatin-induced enteric neuronal loss. Moreover, repeated *in vivo* oxaliplatin administration induces decreases in glial fibrillary acidic protein (GFAP)-immunoreactive enteric glial, contrasting with increases in s100 β -immunoreactive enteric glial cells in both the myenteric and submucosal ganglia at day 14 after commencement of treatment (Robinson et al., 2016).

2.5.2 Changes in Neuromuscular Transmission and Smooth Muscle Tone

NO released from nNOS expressing inhibitory motor neurons supplying the intestinal smooth muscles is an integral inhibitory neurotransmitter in the gastrointestinal tract (Lecci et al., 2002;Takahashi, 2003). nNOS produces basal levels of NO to maintain the hyperpolarised state of the circular muscle cells leading to physiological tonic inhibition of the intestine (Bult et al., 1990;Daniel et al., 1994). Excessive NO production and consequent nitrosylation has been linked to inhibition of dynamin-related protein 1, resulting in synaptic impairment and disruption to synaptic transmission (Savidge, 2011). In our study, intracellular recordings from colonic circular muscles revealed changes in neuromuscular transmission with an increase in amplitude of NO-mediated sIJP potentials in the oxaliplatin-treated group. No changes in ATP-mediated fIJP or ACh-mediated EJPS were observed. These results differ from findings in inflamed guinea-pig colon where oxidative stress leads to reduced purinergic neuromuscular transmission (Roberts et al., 2013). This may be due to differences in mechanisms of oxaliplatin-induced neuronal damage from inflammation-induced enteric neuropathy. Oxaliplatin exerts its cytotoxic effects via direct binding to nuclear and mitochondrial DNA and formation of platinum-DNA adducts (Graham et al., 2000;Goodisman et al., 2006). Direct mitochondrial damage leads to excessive NO production and activation of apoptotic cascades as discussed above.

Direct oxaliplatin toxicity and increased NO signalling impact functions of colonic circular muscles. The relaxation evoked by the NO donor, sodium nitroprusside, was reduced by about 50% in the distal colon from oxaliplatin-treated mice. The baseline diameter of the distal colon was greater in oxaliplatin-treated animals than in sham-treated mice suggesting a reduction in the colonic muscle tone after oxaliplatin treatment. This was reversed by application of L-NNA inhibiting all NOS isoforms including iNOS, which was increased in longitudinal muscle-myenteric neuron preparations after oxaliplatin treatment. The reduced smooth muscle tone as well the reduced response to the NO donor could be a result of the relatively higher inhibitory activity compared to the excitatory due to the increased proportion of nNOS neurons observed in oxaliplatin-treated mice. However, given that SNP donates NO through mechanisms involving sulfhydryl-containing compounds glutathione and cysteine, resulting in the formation of disulfides and S-nitrosothiols, NO, and cyanide ions, it is possible that the rate of donation and release of NO may be affected by oxaliplatin. The relationship between SNP induced NO donation and oxaliplatin needs to be further investigated. The reduction in muscle thickness that we observed after oxaliplatin treatment may also contribute to a reduction in muscle tone. Decreased basal tone and, thus, a larger resting diameter of the distal colon compromised the ability of smooth muscles to further relax. Similar findings have been observed following intestinal inflammation where smooth muscle relaxation in response to a NO donor was decreased (Van Bergeijk et al., 1998; Rajagopal et al., 2014). This compromised ability to relax was in part attributed to iNOS-mediated protein nitrosylation (Rajagopal et al., 2014).

2.5.3 Oxaliplatin-Induced Changes in Colonic Motility

To investigate the role of NO in colonic dysmotility resulting from oxaliplatin treatment, we performed experiments with and without application of the nitric oxide synthase inhibitor, L-NNA, and analysed phases of contraction in various parts of the colon, overall colonic motor patterns and parameters of specific types of motor activity.

Alterations in the basal tone of the colon and augmented nitrgenic transmission may be attributed to increased NO release providing enhanced inhibitory input to the smooth muscle and altering colonic contractile patterns. Analysis of spatiotemporal maps revealed reductions in the proportion and frequency of CMMCs as well as in frequency and speed of short contractions in the distal colon from oxaliplatin-treated mice. Anterograde and retrograde short contractions were observed in the distal colon of both sham and oxaliplatin-treated mice. Both types of short contractions were significantly reduced in the distal colon after oxaliplatin treatment. In contrast, the proportion and frequency of fragmented contractions were greater in colons from oxaliplatin-treated mice. These findings are consistent with previous studies demonstrating inhibition of CMMCs in the colon of oxaliplatin-treated mice (Wafai et al., 2013) and inhibition of intestinal transit in cisplatin-treated rats (Vera et al., 2011). Morphological and functional changes of interstitial cells of Cajal may also be involved in oxaliplatin-induced dysmotility, these require further investigation.

Previous studies using NOS inhibitors have established a role of NO in the pathophysiology of gastrointestinal functional disorders (Rivera et al., 2011a). NO is a primary regulator of complex intestinal motor patterns and increased NO release can disrupt CMMCs (Sarna et al., 1993;Rodriguez-Membrilla et al., 1995;Castro et al., 2012). In our study, application of the NOS inhibitor, L-NNA restored the proportion and frequency of CMMCs in oxaliplatin-treated mice. NOS inhibition increased the rate of rise of all types of contractions (CMMCs, short and fragmented) in the colons from sham-treated, but not oxaliplatin-treated mice. This is consistent with previous studies reporting increased speed of contraction induced by L-NNA in control mice (Rodriguez-Membrilla et al., 1995). Inhibition of NOS by L-NNA did not affect short and fragmented contractions in colons from oxaliplatin-treated mice. This suggests that the augmented proportion and frequency of fragmented contractions and reduced frequency of short contractions observed in oxaliplatin-treated mice are not due to increased NO release, but can be attributed to either disruption of enteric neural

pathways or a direct effect on colonic smooth muscle function. Colonic dysmotility was associated with reduction in faecal water content and increase in number of pellets in the colon providing evidence that oxaliplatin-treated mice had constipation. These symptoms were correlated with a failure to gain weight which was not dependant on food or water consumption (please see Chapter 5).

2.6 Conclusion

Oxaliplatin treatment induces severe gastrointestinal side-effects such as nausea, vomiting, constipation and diarrhea which account for dose limitations and/or cessation of treatment. These symptoms can persist up to 10 years after the treatment has been ceased (Denlinger and Barsevick, 2009). Oxidative stress has been suggested as a potential mechanism underlying platinum neurotoxicity (Di Cesare Mannelli et al., 2012). Our study is the first providing evidence that oxidative stress is a key player in enteric neuronal death, changes in intestinal smooth muscle tone and neuromuscular transmission underlying colonic dysmotility and leading to chronic constipation associated with oxaliplatin treatment. Further studies elucidating mechanisms of oxaliplatin-induced oxidative stress are warranted in order to develop strategies to alleviate gastrointestinal side-effects of chemotherapy and achieve more effective anti-cancer treatment.

CHAPTER THREE: ENTERIC NEUROPATHY AND INCREASED CHOLINERGIC PHENOTYPE UNDERLIE LONG-TERM GASTROINTESTINAL DYSFUNCTION INDUCED BY IRINOTECAN TREATMENT IN MICE

The material presented in this chapter has been submitted for publication and has been reproduced here with minor alterations:

RM McQuade, V Stojanovska, E Donald, R Abalo, JC Bornstein, K Nurgali (2017) Enteric neuropathy and increased cholinergic phenotype underlie long-term gastrointestinal dysfunction induced by irinotecan treatment in mice. Submitted to *Neurotherapeutics*.

3.1 Summary

Gastrointestinal dysfunction is a common side-effect of chemotherapy leading to dose reductions and treatment delays. These side-effects may persist up to 10 years post-treatment. A topoisomerase I inhibitor irinotecan (IRI), commonly used for the treatment of colorectal cancer, is associated with severe acute and delayed-onset diarrhoea. The long-term effects of IRI may be due to damage to enteric neurons innervating the gastrointestinal tract and controlling its functions. The effects of IRI on gastrointestinal transit, colonic motility and myenteric neurons were studied. Balb/c mice received intraperitoneal injections of IRI (30 mg/kg-1) 3 times a week for 14 days, sham-treated mice received sterile water (vehicle) injections. In vivo analysis of gastrointestinal transit via serial x-ray imaging, faecal water content, assessment of gross morphological damage and immunohistochemical analysis of myenteric neurons were performed at 3, 7 and 14 days following first injection and at 7 days post-treatment. Ex vivo colonic motility was analysed at 14 days following first injection and 7 days post-treatment. Mucosal damage and inflammation were found following both short and long-term treatment with IRI. IRI-induced neuronal loss and increases in the number and proportion of ChAT-IR neurons and the density of VACHT-IR fibres, associated with changes in colonic motility, gastrointestinal transit and faecal water content. These changes persisted in post-treatment mice. Our findings suggest that enteric neuronal loss coupled with increased expression of ChAT and VaChT-IR neurons may be underlying factors in the development of IRI-induced gastrointestinal dysfunction.

Abbreviations

| | |
|--------------------------------|--|
| 5-FU | 5-fluorouracil |
| ChAT | choline acetyltransferase |
| CHT1 | high-affinity choline transporter 1 |
| CID | chemotherapy-induced diarrhoea |
| CMMC | colonic migrating motor complex |
| CRC | colorectal cancer |
| CTL1 | choline transporter-like 1 |
| DMSO | dimethyl sulfoxide |
| ENS | enteric nervous system |
| FC | fragmented contraction |
| FOLFIRI | -fluorouracil, irinotecan and leucovorin |
| H&E | haematoxylin and eosin |
| IFN-γ | Interferon γ |
| IL-1β | interleukin 1 β |
| IR | Immunoreactive |
| IRI | Irinotecan |
| LV | leucovorin |
| NF-κB | nuclear factor- κ B |
| OCT1 | organic cation transporter 1 |
| OCT2 | organic cation transporter 2 |
| OCT3 | organic cation transporter 3 |
| OCTs | organic cation transporters |
| PBS | Phosphate buffered saline |
| PGP9.5 | Protein Gene Product 9.5 |
| SC | Short contraction |
| TNFα | tumour necrosis factor α |
| Top I | topoisomerase I |
| VACHT | vesicular acetylcholine transporter |

3.2 Introduction

A combination of irinotecan (IRI) with 5-fluorouracil (5-FU) and leucovorin (LV) (FOLFIRI) is one of the most common and effective therapies administered to CRC patients (Conti et al., 1996; Saltz et al., 2000). IRI is a semi-synthetic analogue of the naturally occurring quinoline alkaloid, camptothecin, and exerts its cytotoxicity via inhibition of topoisomerase I (Top I) triggering S-phase specific cell death (Xu and Villalona-Calero, 2002). Top I is essential for transcription and acts to cut, relax and reanneal DNA strands. IRI's active metabolite SN-38 binds to Top I and its DNA complex resulting in the formation of a stable ternary structure that prevents DNA re-ligation and promotes DNA damage and apoptosis (Xu and Villalona-Calero, 2002).

Clinical trials in patients with metastatic CRC have demonstrated a significant survival advantage for FOLFIRI compared with 5-FU/LV alone (Douillard et al., 2000; Saltz et al., 2000), but major dose limiting toxicities such as neutropenia and chronic diarrhoea diminish the clinical efficacy of FOLFIRI treatment (Armand, 1996; Rothenberg et al., 1996; Weekes et al., 2009). Although neutropenia is manageable, IRI-induced diarrhoea is typically severe, resulting in hospitalisations, dose-reductions and delays, and termination of treatment in many cases (Swami et al., 2013). Though the prevalence and severity of IRI-induced diarrhoea varies greatly based on regime specifics such as dosage and adjuvant therapies, rates as high as 80% have been reported (Rothenberg et al., 1996).

IRI induces both acute and delayed-onset type diarrhoea. Acute diarrhoea experienced within the first 24 hours following IRI administration, occurs in 60-80% of patients (Gibson and Stringer, 2009). Delayed-onset diarrhoea arises at least 24 hours after IRI administration and occurs in approximately 80% of patients (Saliba et al., 1998). Chemotherapy-induced diarrhoea (CID) been linked to early death rates of up to 5% in patients receiving IRI in combination with 5-FU/LV (Rothenberg et al., 2001).

A number of different underlying mechanisms for the gastrointestinal side-effects of IRI treatment have been proposed including luminal accumulation and reactivation of SN-38, sustained disruption to intestinal microflora and continual mucosal damage (Javle et al., 2007;Gibson and Stringer, 2009;Stringer et al., 2009b;McQuade et al., 2014). Although the acute diarrhoea is thought to be primarily secretory with attenuation of symptoms by administration of atropine, suggesting involvement of cholinergic secretomotor neurons, the underlying mechanism of delayed onset IRI-induced diarrhoea is unclear (Gibson et al., 2003;Gibson and Stringer, 2009). Thus, the persistent recurring and long-term symptoms associated with IRI treatment may be a result of damage to the ENS.

In this study we investigated the effects of short and long-term IRI treatment on: i) the histological architecture of the colonic mucosa; ii) the total number of myenteric neurons and excitatory neurons controlling colonic motility, as well as the effects of long-term IRI-treatment and post-treatment effects on iii) spontaneous *ex vivo* colonic motility; iv) gastrointestinal transit time and emptying and v) faecal weight and water content.

3.3 Methods

3.3.1 Ethical Approval

All procedures were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

3.3.2 Animals

Male Balb/c mice aged 6-8 weeks (18–25g) supplied from the Animal Resources Centre (Perth, Australia) were used for the experiments. Mice had free access to food and water and were kept under a 12-hour light/dark cycle in a well-ventilated room at an approximate temperature of 22°C. Mice acclimatised for a minimum of 5 days prior to the commencement of *in vivo* intraperitoneal injections. A total of 40 mice were used for this study.

3.3.3 *In vivo* Irinotecan Injections

Mice received intraperitoneal injections of IRI (30 mg/kg⁻¹) (Sigma-Aldrich, Australia), 3 times a week via a 26 gauge needle. IRI was dissolved in sterile water to make 10⁻¹ M L⁻¹ stock solutions refrigerated at -20°C. The stock was then defrosted and diluted with sterile water to make 10⁻² M L⁻¹ solutions for intraperitoneal injections via a 26 gauge needle. The dose of IRI was calculated to reach a cumulative dose equivalent to a standard human dose in combination therapy, 180 mg/m² per body surface area (Reagan-Shaw et al., 2008; Köhne et al., 2012). Sham-treated mice received sterile water via intraperitoneal injection 3 times a week. The injected volumes were calculated to the body weight; the maximum volume of injected IRI or vehicle did not exceed 200 µL per injection. Mice were euthanized via cervical dislocation at 3 (2 treatments), 7 (3 treatments) and 14 (6 treatments) days after the first injection. Post-treatment group mice were euthanized 7 days after the final injection (6 treatments). Colons from all groups were collected for *in vitro* experiments.

3.3.4 Histology

To examine the morphological changes to the colon, standard Haematoxylin and Eosin (H&E) staining protocol was followed (McQuade et al., 2016a) as described in Chapter 2, section 2.3.7. Briefly, the colon was harvested and placed in a 10% formalin solution overnight and then transferred into 70% ethanol the following day. Paraffin embedded colon sections were cut 5µm

thick and de-waxed in a 60°C oven for 30 minutes. Ten sections per preparation were analysed. All images were analysed blindly.

3.3.5 Immunohistochemistry in Wholemout Preparations

Segments of the distal colon (2-3 cm) were placed in oxygenated phosphate-buffered saline (PBS) (pH 7.2) containing nicardipine (3 μ M) (Sigma-Aldrich, Australia) for 20 mins to inhibit smooth muscle contractions (n=5 mice/group). Samples were cut open along the mesenteric border, cleared of their contents, maximally stretched and pinned mucosa down. Tissues were fixed with Zamboni's fixative (2% formaldehyde, 0.2% picric acid) overnight at 4°C. Preparations were cleared of fixative by washing 3 x 10 min with DMSO (Sigma-Aldrich, Australia) followed by 3 x 10 min washes with PBS. Once washed, tissues were dissected mucosa up to expose the myenteric plexus. Fixed tissues were stored at 4°C in PBS for a maximum of 5 days.

Wholemout preparations were incubated with 10% normal donkey serum (Chemicon, USA) for 1 hour at room temperature. Tissues were then washed (2 x 5 mins) with PBS and incubated with primary antibodies against Protein Gene Product 9.5 (PGP9.5) (chicken, 1:500, Abcam, MA, USA), choline acetyltransferase (ChAT) (goat, 1:500, Abcam, MA, USA) or vesicular acetylcholine transporter (VACHT) (goat, 1:500, Abcam, MA, USA) overnight at 4°C. Tissues were then washed in PBS (3 x 10 min) before incubation with species-specific secondary antibodies labelled with different fluorophores: donkey anti-chicken Alexa 594 (1:200, Jackson ImmunoResearch Laboratories, PA, USA) and donkey anti-goat Alexa 488 (1:200, Jackson ImmunoResearch Laboratories, PA, USA) for 2 hours at room temperature. Wholemout preparations were given 3 x 10 min final washes in PBS and then mounted on glass slides using fluorescent mounting medium (DAKO, Australia).

3.3.6 Immunohistochemistry in Cross Sections

Colon samples were cut open along the mesenteric border, cleared of their contents, and pinned mucosa up without stretching (n=5 mice/group). Tissues were fixed with Zamboni's fixative overnight at 4°C. Preparations were cleared of fixative by washing 3 x 10 min with DMSO (Sigma-Aldrich, Australia) followed by 3 x 10 min washes with PBS. After washing, tissues were embedded in 100% OCT and frozen using liquid nitrogen (LN₂) and isopentane (2-methyl butane) and stored in -80°C freezer. Tissues were cut at 20µm section thickness using a Leica CM1950 cryostat (Leica Biosystems, Germany), adhered to slides and allowed to rest for 30 minutes at room temperature before processing.

Cross section preparations were incubated with 10% normal donkey serum (Chemicon, USA) for 1 hour at room temperature. Tissues were then washed (2 x 5 mins) with PBS and incubated with primary antibodies against CD45 (Rat, 1:500, BioLegend, Australia), overnight at 4°C. Sections were then washed in PBS (3 x 10 min) before incubation with secondary antibodies labelled with fluorophore donkey anti-rat Alexa 488 (1:200, Jackson ImmunoResearch Laboratories, PA, USA) for 2 hours at room temperature. The sections were given 3 x 10 min final washes in PBS and then cover slipped using fluorescence mounting medium (DAKO, Australia).

3.3.7 Imaging

Imaging was carried out on wholemount preparations and cross sections under a Nikon Eclipse Ti laser scanning microscope as described in Chapter 2, section 2.3.8. Eight randomly chosen three dimensional (z-series) images from each preparation were captured with a 20 x objective and processed using NIS Elements software (Nikon, Japan). Fluorophores were visualized using excitation filters for Alexa 594 Red (excitation wavelength 559nm), Alexa 488 (excitation wavelength 473nm), and Alexa 405 (excitation wavelength 405nm). Z-series images were taken at step size of 1.75 µm (1600 x 1200 pixels). The number of PGP9.5 and ChAT immunoreactive (IR) neurons was quantified in the myenteric ganglia and CD45-IR cells in

cross sections were quantified within a 2 mm² area of each preparation. Quantitative analyses were conducted blindly.

3.3.8 Colonic Motility Experiments

The entire colon was removed from day 14 and post-treatment sham and IRI-treated mice (n=5 mice/group) and set up in organ-bath chambers to record motor patterns *in vitro* (Wafai et al., 2013), as described in Chapter 2, section 2.3.12. Briefly, the colon was placed into warmed (35°C), oxygenated physiological saline until the faecal pellets were expelled. The empty colon was cannulated at both ends and arranged horizontally in an organ-bath chamber. The proximal end of the colon was connected to a reservoir containing oxygenated physiological saline to maintain intraluminal pressure. The distal end was attached to an outflow tube that provided a maximum of 2 cm H₂O back-pressure. Organ baths were continuously superfused with oxygenated physiological saline solution and preparations were left to equilibrate for 30 min. Contractile activity of each segment was recorded with a Logitech Quickcam Pro camera positioned 7–8 cm above the preparation. Videos (2x20min) of each test condition were captured and saved in avi format using VirtualDub software (version 1.9.11). Recordings were used to construct spatiotemporal maps using in-house edge detection software (Gwynne et al., 2004). Spatiotemporal maps plot the diameter of the colon at all points during the recording allowing contractile motor patterns to be analysed with Matlab software (version 12). Colonic migrating motor complexes (CMMCs) were defined as propagating contractions directed from the proximal to the distal end of the colon which travelled more than 50% of the colon length (Spencer and Bywater, 2002; Roberts et al., 2007; Roberts et al., 2008). Contractions that propagated less than 50% of the colonic length were considered to be short contractions (SCs). Incomplete contractions occurring synchronously at different parts of the colon rather than propagating over the length of the colon were defined as fragmented contractions (FCs).

3.3.9 Gastrointestinal Transit

Gastrointestinal transit was studied by x-ray prior to first treatment (day 0) and at 3, 7 and 14 days and 7 days post-treatment of IRI treatment (n=5 mice/group). The contrast agent, 0.4 mL of suspended barium sulfate (X-OPAQUE-HD, 2.5g/mL), was administered via oral gavage. Prior to performing x-ray imaging, animals were trained/conditioned for oral gavage using a non-irritating substance such as 0.9% w/v saline or sterile water (volume 0.1-0.4 ml); this was repeated at least 3 times for each animal with at least 24 hours between each training session. Radiographs of the gastrointestinal tract were taken using a HiRay Plus Porta610HF x-ray apparatus (JOC Corp, Kanagawa, Japan; 50 kV, 0.3 mAs, exposure time 60 ms). Mice were immobilised in the prone position by placing them inside a transparent plastic restraint tube with partly open front side for breathing, this comfortably restrains animal movement for a maximum of 1-2 min which is essential for successful x-ray imaging. The training/conditioning with restraint was done by placing the restrainer into the mouse cages at least 24 hours prior to the x-ray procedure. X-rays were captured using Fujifilm cassettes (24×30 cm) immediately after administration of barium sulfate (0), every 5 minutes for the first hour, every 10 minutes for the second hour, then every 20 minutes through to 360 minutes (360). Between imaging mice were removed from restraining devices and placed into holding cage with free access to food and water. Mice were not fasted prior to x-ray imaging. Animals were closely monitored during and after all procedures. Images were developed via a Fujifilm FCR Capsula XLII and analysed using eFilm 4.0.2 software. Speed of gastrointestinal transit was calculated as time in minutes taken to reach each region of the gastrointestinal tract (stomach, small intestines, caecum, and large intestines). Organ emptying was calculated as the time taken for complete barium emptying from specific gastrointestinal regions (stomach, small intestines) (Cabezos et al., 2008;Cabezos et al., 2010;Girón et al., 2015).

3.3.10 Faecal Water Content and Colonic Faecal Content Analysis

Fresh faecal pellets were collected from both sham and IRI-treated mice (n=10 mice/group). Individual mice were placed in holding cages for a period of 15 minutes, the first 5 fresh pellets expelled were collected and weighed immediately to calculate average fresh wet weight. Pellets were then dehydrated for 72 hours at room temperature prior to measurement of the dry weight. Water content was calculated as the difference between the wet weight and dry weight. Pellet length was measured in arbitrary units in all x-ray images displaying discernible pellets in the distal colon using an Image J measurement tool. X-ray images were converted to TIFF format and set to 800x800 pixels.

3.3.11 Statistical Analysis

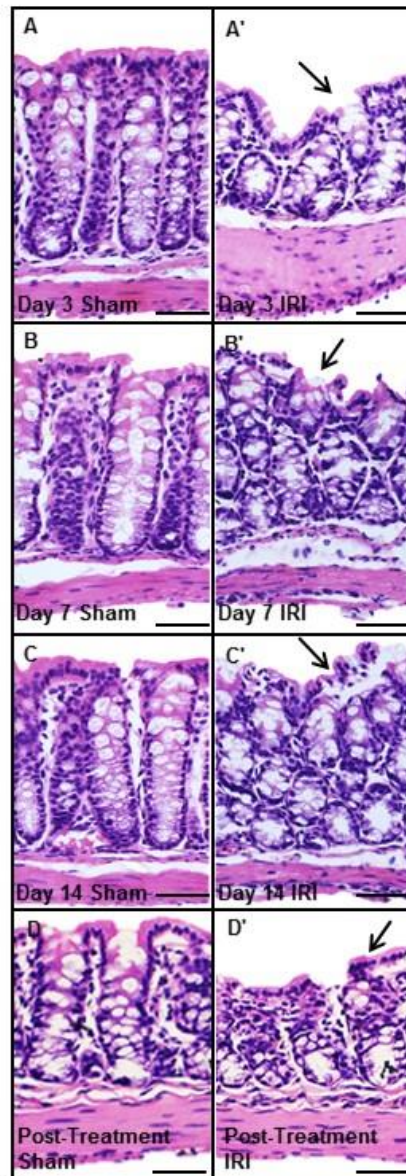
Data were assessed using two-way ANOVA, Welch's two-tailed *t* test and Student's two-tailed *t* test. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., CA, USA). Data are presented as mean \pm standard error of the mean (SEM). Value differences were considered statistically significant at $P < 0.05$.

3.4 Results

3.4.1 Morphological damage to the colon following irinotecan administration

Colonic architecture from the sham-treated animals at days 3, 7, 14 and post-treatment appeared healthy with a visible brush border as well as uniform crypts (**Figure 3.1A-D**). Severe mucosal ulceration in the epithelial layer was observed in the colon from the IRI-treated group at day 3 (**Figure 3.1A'**). Colon sections from the IRI-treated groups at days 7 (**Figure 3.1B'**) and 14 (**Figure 3.1C'**) displayed persistent ulceration of the epithelial brush border coupled with crypt hypoplasia and disorganisation, which were not observed in colonic preparations from sham-treated mice. Colon sections from post-treatment IRI mice displayed crypt hypoplasia and disorganisation, but the epithelial brush border was visible and appeared to be intact (**Figure 3.1D'**).

Figure 3.1 Gross morphological changes in the colon following repeated *in vivo* IRI administration. H&E staining in the colon from sham-treated and IRI-treated mice at 3 (**A, A'**), 7 (**B, B'**), 14 (**C, C'**) days, and 7 days' post-treatment (**D, D'**) (n=5 mice/group). Scale bar = 100µm.



3.4.2 Intestinal inflammation following irinotecan administration

To investigate if acute and chronic IRI treatment causes inflammation, immune cell infiltration in the colon was analysed. Immune cells in colonic cross sections were labelled with a pan leukocyte marker anti-CD45 antibody following 3, 7, 14 days and post IRI treatment (**Figure 3.2A'-D'**) compared to sham-treated mice (**Figure 3.2A-D**). Total numbers of CD45 positive cells were counted within a 2mm² area.

A significant increase in the number of CD45 positive cells was found in the colon following 3 (sham: 45±9; IRI: 109±3, $P<0.0001$), 7 (sham: 47±3; IRI: 92±5, $P<0.0001$) and 14 days (sham: 42±3; IRI: 98±2, $P<0.0001$) of IRI administration when compared to sham treatment (**Figure 3.3**). This increase persisted in post-treatment IRI mice (92±7, $P<0.0001$) compared to sham (44±1) group (**Figure 3.3**).

Figure 3.2 CD45+ leukocytes in the colon. Cross sections of the colon labelled with a pan leukocytes marker anti-CD45+ antibody (green) from sham-treated and IRI-treated mice at 3 (**A, A'**), 7 (**B, B'**), 14 (**C, C'**) days, and 7 days' post-treatment (**D, D'**). Scale bar = 100µm.

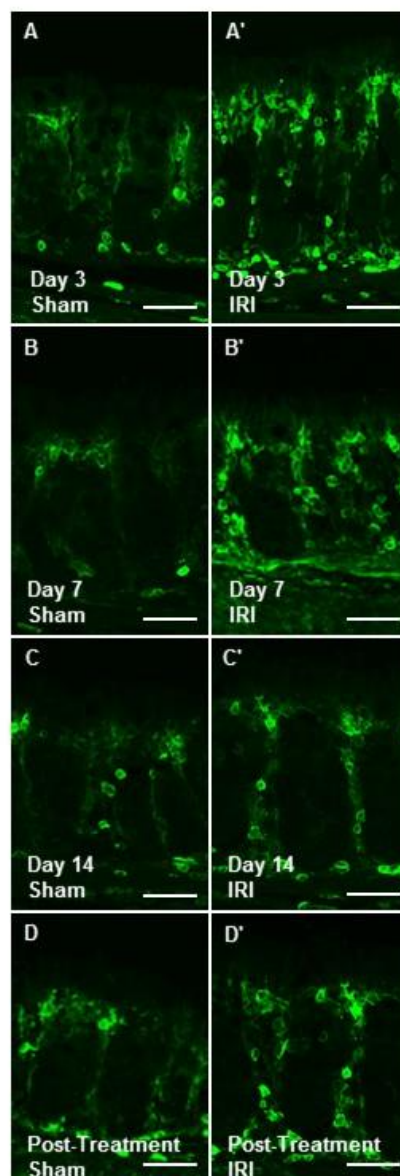
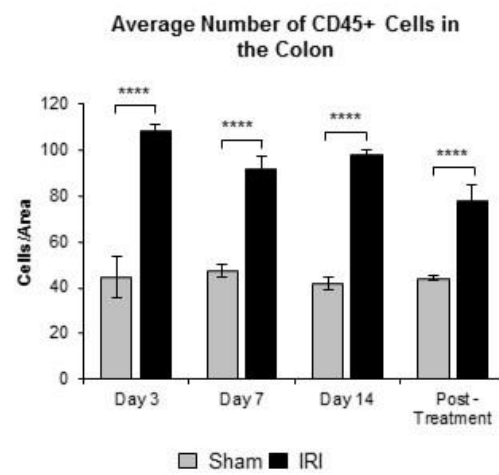


Figure 3.3 Quantification of CD45+ cells in the colon. Number of CD45+ cells was counted within 2mm² of the mucosa in the colon sections from sham and IRI-treated mice at 3, 7 and 14 days, and 7 days post-treatment. Grey columns: sham-treated, black columns: IRI-treated mice. Data represented as mean \pm S.E.M. **** P <0.0001, n=5 mice/group.



3.4.3 Neuronal loss and changes in cholinergic neurons and fibres following repeated *in vivo* administration of irinotecan

To investigate changes to the total number of myenteric neurons wholemount preparations were labelled with a pan neuronal marker anti-PGP9.5 antibody (**Figure 3.4**). Repeated *in vivo* administration of IRI induced myenteric neuronal loss when compared to the sham-treated groups at days 7 (sham: 1218 ± 17 ; IRI: 1092 ± 2 , $P < 0.05$) and 14 (sham: 1262 ± 34 ; IRI: 1072 ± 23 , $P < 0.05$) (**Figure 3.5A**). This neuronal loss persisted in mice post IRI treatment (sham: 1232 ± 32 ; IRI: 1038 ± 52 , $P < 0.01$). No significant difference in numbers of myenteric neurons was found at day 3 (sham 1222 ± 9 ; IRI 1171 ± 22) (**Figure 3.5A**).

To determine if IRI administration was associated with changes in a specific subpopulation of myenteric neurons, the average number and proportion of neurons immunoreactive (IR) for ChAT specific to cholinergic neurons was analysed in both sham and IRI-treated mice. Repeated *in vivo* administration of IRI induced a significant increase in the average number of ChAT-IR neurons when compared to sham-treated group at days 3 (sham: 293 ± 9 ; IRI: 372 ± 6 , $P < 0.0001$), 7 (sham: 325 ± 3 ; IRI: 390 ± 5 , $P < 0.001$), 14 (sham: 320 ± 15 ; IRI: 390 ± 3 , $P < 0.0001$) and post-treatment (sham: 306 ± 15 ; IRI: 401 ± 10 , $P < 0.0001$) (**Figure 3.5B**). The proportion of ChAT-IR neurons significantly increased at all time points (**Figure 3.5C**).

Cholinergic fibres were labelled within myenteric ganglia in wholemount preparations of the colon using anti-VACHT antibody at day 14 and post IRI treatment (**Figure 3.6A-B'**). Repeated *in vivo* administration of IRI induced a significant increase in the density of VACHT-IR fibres in the myenteric plexus at day 14 (sham: 4.0 ± 0.2 ; IRI: 10.3 ± 0.3 , $P < 0.0001$) and post-treatment compared to sham-treated group (sham: 4.2 ± 0.2 ; IRI: 12.6 ± 0.5 , $P < 0.0001$) (**Figure 3.6C**). The density of VACHT-IR fibres was higher in preparations from post treatment mice compared to day 14 IRI-treated mice ($P < 0.01$) (**Figure 3.6C**).

Figure 3.4 Wholemout preparations of myenteric plexus.

Myenteric neurons were labelled using a pan neuronal marker anti-PGP9.5 antibody (magenta) and cholinergic neurons were labelled using anti-ChAT antibody (green) in the wholemount preparations of the colon from sham-treated and IRI-treated mice at 3 (**A, A'**), 7 (**B, B'**), 14 (**C, C'**) days and 7 days' post-treatment (**D, D'**). Scale bar =50µm.

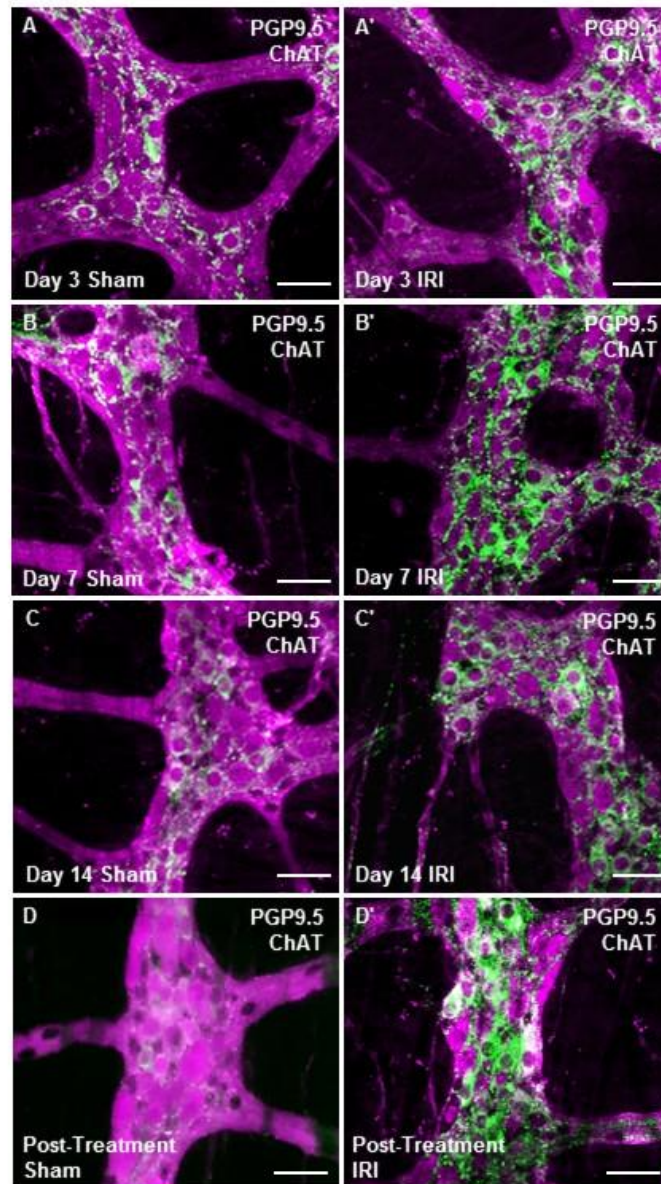


Figure 3.5 Effect of repeated *in vivo* IRI administration on the total number of neurons and average number and proportion of ChAT-IR myenteric neurons. (A) Average number of PGP9.5-IR neurons in the colon was counted per 2mm² at 3, 7, and 14 days, and 7 days' post-treatment in both sham and IRI-treated mice. (B) Average number of ChAT-IR neurons in the colon was counted per 2mm² at 3, 7 and 14 days and 7 days' post-treatment in both sham and IRI-treated mice. (C) Proportion of ChAT-IR neurons calculated to the total number of PGP9.5-IR myenteric neurons in the colon per 2mm² at 3, 7 and 14 days and 7 days' post-treatment in both sham and IRI-treated mice. Grey columns: sham-treated, black columns: IRI-treated mice. Data presented as mean \pm S.E.M. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001, n = 5 mice/group.

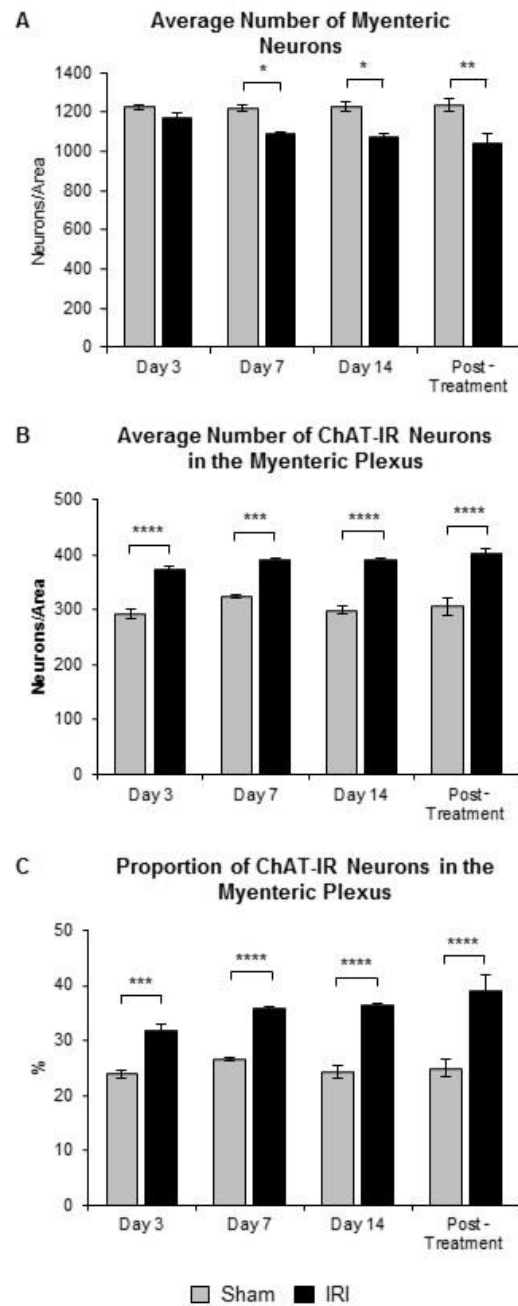
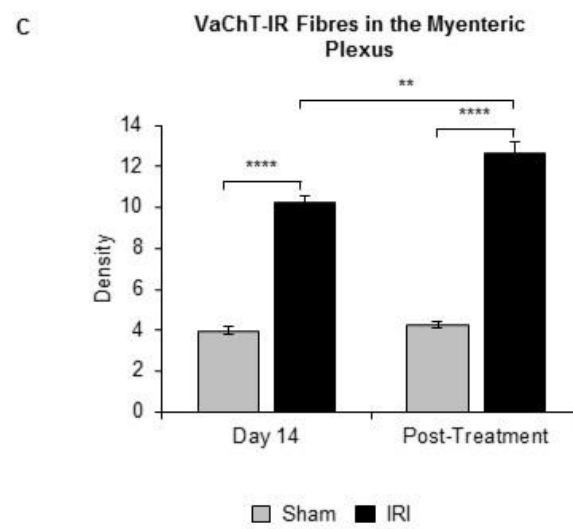
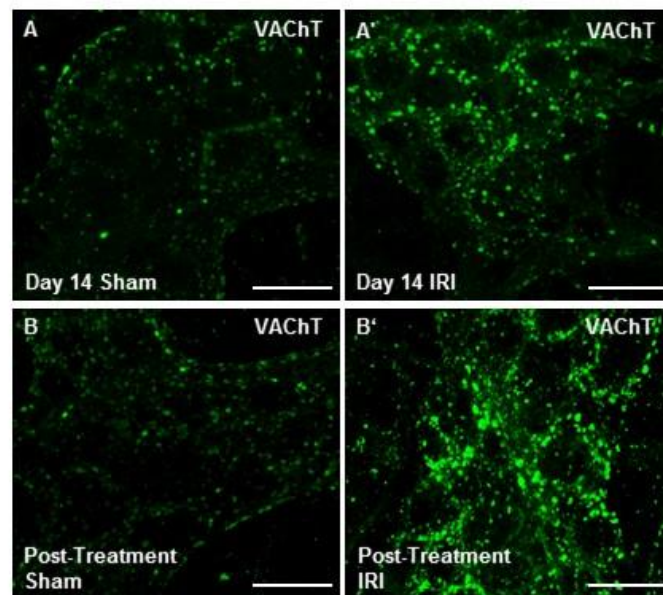


Figure 3.6 Effect of repeated *in vivo* IRI administration on the density of VACht-IR cholinergic fibres in the myenteric plexus.

Wholemout preparations of VACht-IR (green) fibres in the myenteric ganglia of the colon from sham and IRI-treated mice at 14 days (**A, A'**) and 7 days' post-treatment (**B, B'**). Scale bar =25µm. (**C**) Density of VACht-IR fibres in the myenteric plexus was analysed at 14 days and post-treatment in both sham and IRI-treated mice. Grey columns: sham-treated, black columns: IRI-treated mice. Data presented as mean ± S.E.M. ** $P<0.01$, **** $P<0.0001$, $n = 5$ per group/time point.



3.4.4 Changes in colonic motility following IRI administration

To investigate the effects of IRI on colonic motility, excised colons were studied in organ bath experiments at day 14 of IRI treatment and post-treatment. The total number of contractions (including all types of motor activity in the colon: CMMCs, short and fragmented contractions) (**Figure 3.7A**) was increased in the colons from day 14 IRI-treated ($P<0.01$) and post-treatment ($P<0.001$) animals compared to sham-treated mice (**Figure 3.7B, Table 4.1**). To determine if this increase was due to changes in a specific type of motor activity, the frequency and proportion were analysed for each type of motor contractions.

Colonic migrating motor complexes (CMMCs) were defined as sustained anally directed contractions propagating for more than 50% of the colon length (**Figure 3.7A**). A decrease in the frequency and proportion of CMMCs was observed in the colons from day 14 IRI-treated compared to sham-treated animals ($P<0.0001$ for both) (**Figure 3.7B, C, Table 3.1**). This decrease in frequency ($P<0.0001$) and proportion ($P<0.0001$) of CMMCs persisted in the colons from post-treatment mice compared to sham-treated animals (**Figure 3.7B, C, Table 3.1**).

Short contractions (SCs) were defined as contractions that propagated less than 50% of the colon length (**Figure 3.7A**). Following 14 days' treatment with IRI, the frequency of SCs in the colon increased when compared to sham-treated mice ($P<0.05$) (**Figure 3.7B, Table 3.1**). However, no significant difference in the proportion of SCs was found in day 14 IRI-treated compared to sham-treated mice (**Figure 3.7C**). Similarly, the frequency of SCs in the colon increased in post-treatment mice compared to sham-treated mice ($P<0.05$) (**Figure 3.7B, Table 3.1**), but without a change in the proportion of SCs in post-treatment mice when compared to sham-treated mice (**Figure 3.7C**).

Fragmented contractions (FCs) were defined as incomplete contractions that did not propagate but rather occurred simultaneously over the length of

the colon. Both the frequency and the proportion of FCs was significantly higher in day 14 IRI-treated mice when compared to sham-treated mice ($P<0.001$ for both) (**Figure 3.7B, C, Table 3.1**). Similarly, the frequency and proportion of FCs was significantly greater in the colon from post-treatment IRI mice than in sham-treated mice ($P<0.001$ for both) (**Figure 3.7B, C, Table 3.1**).

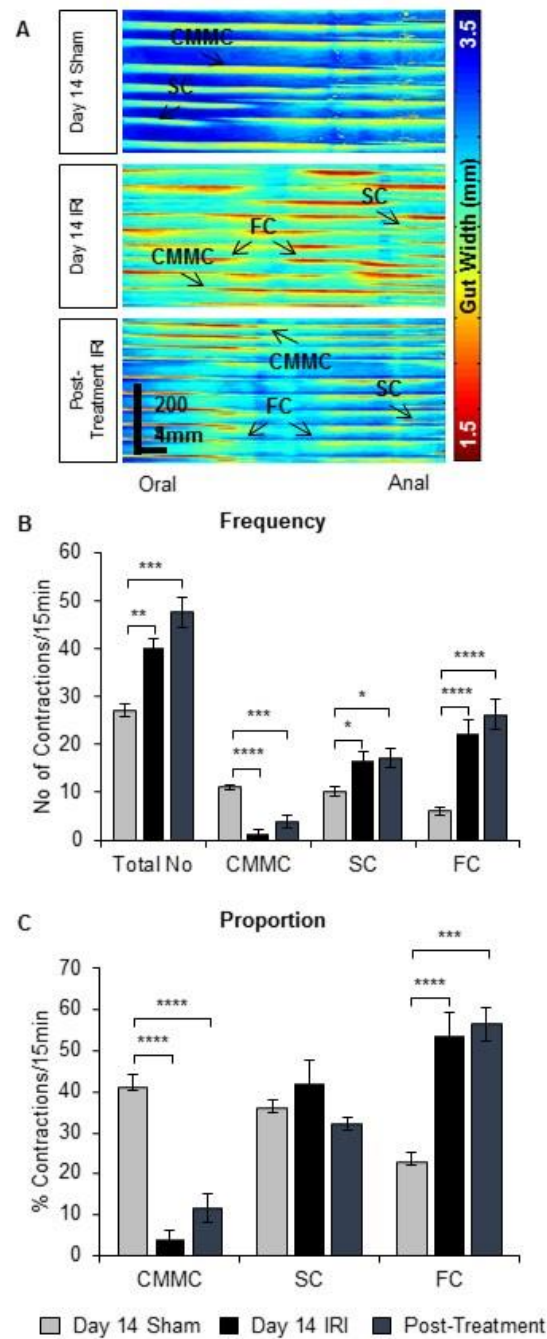
Table 3.1 Parameters of different types of colonic contractions following repeated *in vivo* irinotecan administration

| | Sham-treated | Day 14 IRI | Post-treatment IRI |
|---------------------------------|--------------|--------------|--------------------|
| Total Contractions (per/15 min) | 27.7±1.8 | 41.1±1.9** | 47.7±3.1*** |
| Frequency CMMCs (per/15 min) | 11.0±0.9 | 1.6±0.7**** | 3.8±1.4*** |
| Proportion CMMCs (%) | 40.6±2.5 | 4.6±2.1**** | 11.5±3.5**** |
| Frequency SCs (per/15 min) | 10.2±1.8 | 15.6±1.8* | 17.2±1.9* |
| Proportion SCs (%) | 35.8±3.6 | 38.2±3.9 | 32.1±1.6 |
| Frequency FCs (per/15 min) | 6.0±1.16 | 23.8±2.5**** | 26.7±3.1**** |
| Proportion FCs (%) | 23.7±2.3 | 57.3±4.4**** | 56.4±4*** |

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, significantly different to Day 14 sham, n=5 per group/time point.

Figure 3.7 Effects of *in vivo* IRI treatment on the colonic motility.

(A) Representative spatiotemporal maps generated from digital video recordings of colonic motility from sham and IRI-treated mice. Each contraction can be seen as a reduction in the gut width (red/yellow), while relaxation as an increase in the gut width (blue/green). Colonic migrating motor complexes (CMMCs) propagate >50% of the colon length, short contractions (SCs) propagate <50% of the colon length and fragmented contractions (FCs) are interrupted by period(s) of relaxation during contraction. (B) Frequency of contractions including all types of contractile activity and frequency of specific types of contractions in the colons from sham and IRI-treated mice. (C) The proportion of CMMCs, SCs and FCs to the total number of contractions. Grey columns: sham-treated, black columns: IRI-treated. Data presented as mean \pm S.E.M. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001, compared to day 14 sham, n = 5 mice/group.



3.4.5 Altered gastrointestinal transit following irinotecan administration

To determine the effects of IRI administration on gastrointestinal transit, a series of radiographic images were used to track the movement of barium sulfate through the gastrointestinal tract before the first injection (day 0), after 2 injections (day 3), 3 injections (day 7), 6 injections (day 14 and 7 days' post-treatment) (**Figure 3.8**). Speed of barium movement was calculated by tracing barium entry from one part of the gastrointestinal tract to the next. After 3 days of IRI administration, transit was significantly faster to the caecum and large intestines and pellet formation was significantly quicker (**Figure 3.9, Table 3.2**). No significant differences in transit time to the caecum or large intestines were found at days 7, 14 or post-treatment when compared to day 0 (**Figure 3.9, Table 3.2**).

Although tracing barium movement allowed for the analysis of real time transit speed, gastrointestinal organ filling and emptying does not happen simultaneously, therefore we further analysed the time taken for complete barium emptying from specific gastrointestinal regions. Significant delays in gastric emptying were found at all time points following IRI treatment and post-treatment (**Figure 3.10A, Table 3.2**). No significant differences in intestinal emptying were found following 3 or 7 days of IRI administration, however intestinal emptying was significantly delayed following 14 days of IRI treatment, as well as post-treatment, compared to day 0 (**Figure 3.10B, Table 3.2**).

Pellet formation time was calculated as the time taken (in minutes) for the first pellet to form. Pellet formation time was significantly quicker following 3 days of IRI-treatment when compared to all other time points (**Figure 3.10C, Table 3.2**).

Table 3.2 Speed of gastrointestinal transit and emptying following repeated *in vivo* irinotecan administration

| Parameters measured | | Day 0 | Day 3 | Day 7 | Day 14 | Post Treatment |
|---|----------------------------|-------|----------|----------|----------|-----------------|
| Speed of transit (time to reach each region, min) | Stomach | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| | Small Intestines | 5±0 | 5±0 | 5±0 | 5±0 | 5±0 |
| | Caecum | 70±3 | 45±4*** | 64±5 | 70±6 | 63±3 |
| | Large Intestines | 90±3 | 55±4**** | 92±6 | 88±3 | 83±3 |
| Time for complete barium emptying (min) | Gastric emptying | 17±1 | 36±3** | 37±2* | 40±3*** | 53±7**** |
| | Intestinal emptying | 88±2 | 80±4 | 100±7 | 104±5†† | 126±7** †††† |
| | Pellet Formation | 90±3 | 55±4**** | 92±6†††† | 88±3†††† | 83±3†† |

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ significantly different to Day 0. †† $P<0.01$, †††† $P<0.0001$ significantly different to Day 3, n=5 per group/time point.

Figure 3.8 X-ray images following repeated *in vivo* IRI administration. Representative x-ray images obtained from mice 0–210 min after intragastric barium sulfate (0.4mL, 2.5mg/mL) administration. X-ray imaging was performed at day 0 (prior to 1st injection) and following 3, 7 and 14 days of IRI administration and 7 days' post-treatment (n = 5 mice/group). Stomach (*), small intestines (#), caecum (Δ), pellet formation (†).

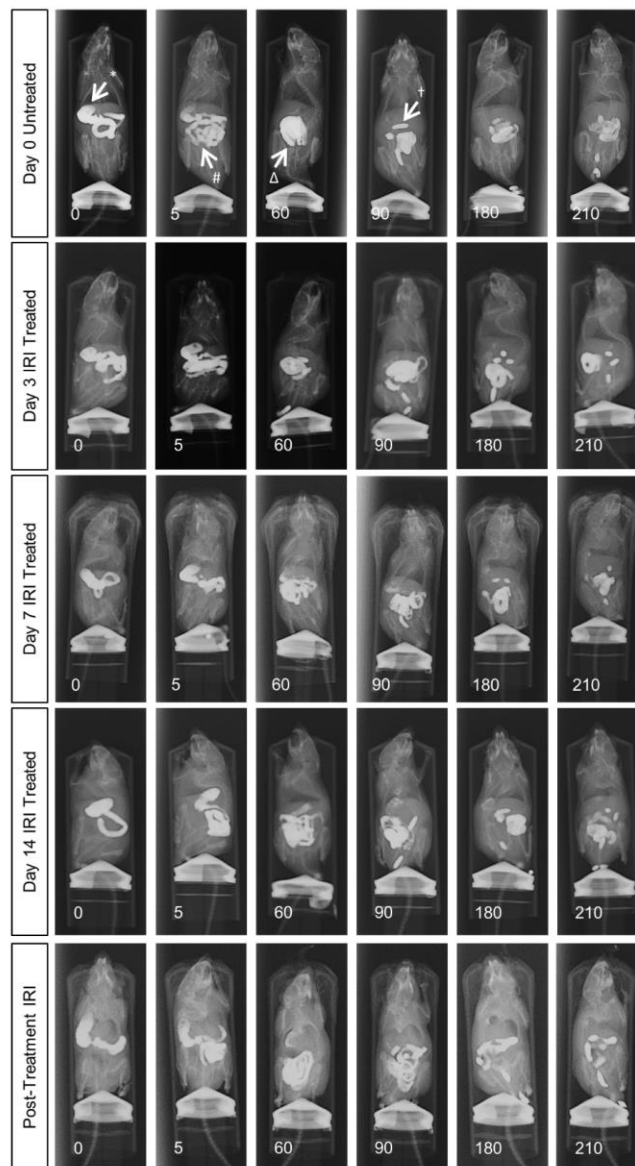
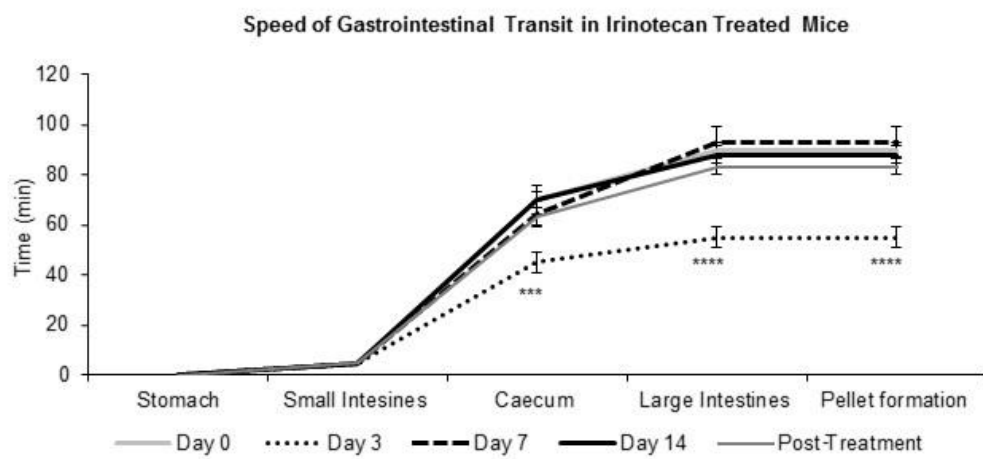
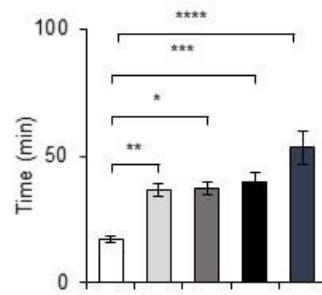


Figure 3.9. Gastrointestinal transit time following repeated *in vivo* IRI administration. Time (min) taken for barium sulfate to reach the stomach, small intestines, caecum and large intestines before (day 0) and at 3, 7 and 14 days and 7 days' post-treatment following IRI administration. Data presented as mean \pm S.E.M. *** $P < 0.001$, **** $P < 0.0001$ compared to day 0, n = 5 mice/group.

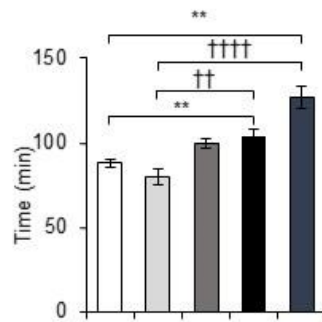


3.10 Gastric and intestinal emptying following repeated *in vivo* IRI administration. (A) Time (min) taken for complete emptying of barium from the stomach. (B) Time (min) taken for complete emptying of barium from the small intestines. (C) Time (min) taken to form first pellet before (day 0) and at 3, 7 and 14 days and 7 days post IRI administration. Data presented as mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to day 0. †† $P < 0.01$, †††† $P < 0.0001$ compared to day 3 IRI-treated group, n = 5 mice/group.

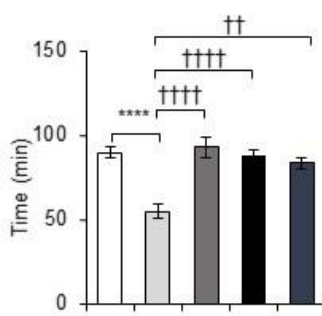
A Gastric Emptying



B Intestinal Emptying



C Pellet Formation



□ Day 0 □ Day 3 ■ Day 7 ■ Day 14 ■ Post-Treatment

3.4.6 Colonic faecal content

To define the clinical symptoms resulting from the altered patterns of colonic motor activity, pellet length in x-ray images and faecal water content in freshly collected faecal pellets were analysed. Pellet length was significantly increased following 7 and 14 days of IRI treatment ($P<0.01$ for both), as well as post-treatment ($P<0.05$), however no significant differences in pellet length were found following 3 days of IRI treatment when compared to day 0 (**Figure 3.11**).

Faecal water content was calculated as the difference between wet and dry pellet weight. The average wet weight of fresh pellets from IRI-treated mice was significantly greater than that of pellets from sham-treated mice at day 3 (sham: 54 ± 3.4 , IRI: 67 ± 2.8 mg, $P<0.01$), day 7 (sham: 57 ± 4.5 , IRI: 74 ± 2.0 mg, $P<0.01$), day 14 (sham: 51 ± 2.9 , IRI: 74 ± 2.9 mg, $P<0.0001$) and post-treatment (sham: 52 ± 1.9 , IRI: 95 ± 3.9 mg, $P<0.0001$) (**Figure 3.12A**). After dehydration, the average dry weight of pellets was significantly lighter from IRI-treated mice at day 3 when compared to sham-treated mice (sham: 26 ± 1.5 , IRI: 19 ± 2.7 mg, $P<0.05$) ($n=10$ mice/group) (**Figure 3.12B**). However, the average dry weight from IRI-treated mice was significantly greater than for pellets from sham-treated mice at day 7 (sham: 25 ± 2.3 , IRI: 34 ± 0.9 mg, $P<0.01$), day 14 (sham: 25 ± 1.4 , IRI: 39 ± 2.3 mg, $P<0.0001$) and post-treatment (sham: 25 ± 1.3 , IRI: 41 ± 2.3 mg, $P<0.0001$) ($n=10$ mice/group) (**Figure 3.12B**). The water content was significantly higher in pellets collected from IRI-treated mice than pellets collected from sham-treated mice at day 3 (sham: 52 ± 1.7 , IRI: $72\pm3.2\%$, $P<0.0001$), day 14 (sham: 51 ± 1.8 , IRI: $57\pm1.2\%$, $P<0.05$) and post-treatment (53 ± 1.3 , IRI: $57\pm1.6\%$, $P<0.05$) ($n=10$ mice/group) (**Figure 3.12C**). No significant difference in water content between sham-treated ($56\pm2.7\%$) and IRI-treated mice ($54\pm1.5\%$) was found at day 7.

Figure 3.11 Pellet length following repeated *in vivo* IRI administration. Representative x-ray images of faecal pellets before (day 0) (**A**), at 3 (**B**), 7 (**C**), 14 (**D**) days and 7 days' post IRI treatment (**E**). (**F**) Length (Arb. units) of faecal pellets before (day 0), at 3, 7, 14 days and 7 days' post IRI treatment. Data presented as mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$ compared to day 14 sham, n = 5 mice/group.

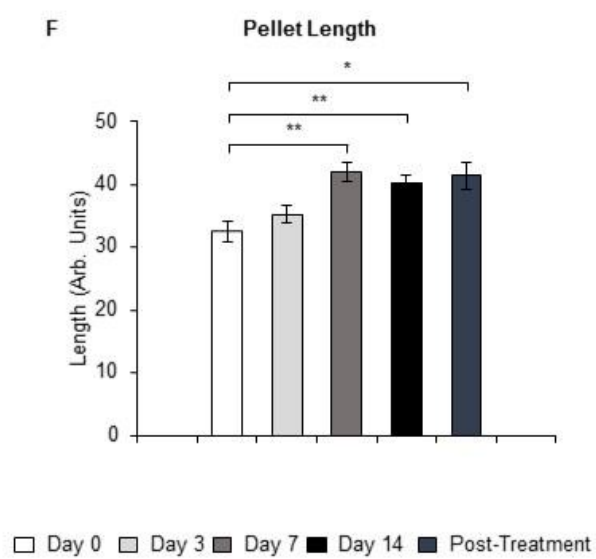
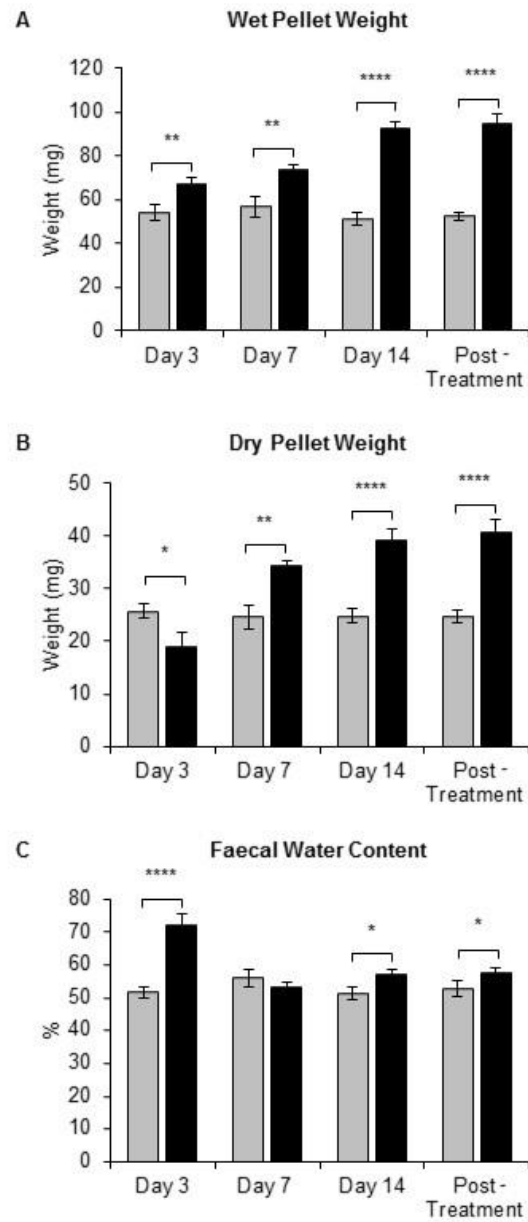


Figure 3.12 Faecal water content following repeated *in vivo* IRI administration. (A) Wet weight of faecal pellets measured immediately upon pellet expulsion. (B) Dry weight of faecal pellets measured after 72 hours of dehydration at room temperature. (C) Faecal water content calculated as the difference between the wet weight and dry weight. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ (n = 10 mice/group).



3.5 Discussion

This study is the first to investigate the effects of repeated *in vivo* administration of IRI on colon morphology, enteric neuropathy and gastrointestinal function in mice. The results show that repeated administration of IRI causes severe mucosal ulceration, crypt hypoplasia and disorganisation of the murine colon throughout the experimental period; however post-treatment colons showed signs of epithelial regeneration. A reduction in the average number of myenteric neurons was found at days 7 and 14 of IRI treatment, as well as post-treatment. The number and proportion of cholinergic ChAT-IR neurons were increased at all time points as well as the density of VACHT-IR cholinergic nerve fibres studied day 14 and post-treatment. These findings correlated with increased motor activity and changes in motility patterns studied at day 14 and post-treatment. Intestinal transit time and pellet formation were significantly faster at 3 days of IRI administration, but not at subsequent time points. Intestinal emptying was delayed at day 14 and post-treatment, whilst gastric emptying was delayed at all experimental time points. Faecal water content was significantly increased at days 3, 14 and post-treatment, indicative of diarrhoea.

Gastrointestinal mucositis is a frequent and debilitating complication resulting from the systemic effects of cytotoxic chemotherapy and the local effects of radiation (Avritscher et al., 2004). Inflammation, epithelial degradation and intestinal ulceration, manifesting as mucositis, is a well-established consequence of IRI administration (Duncan and Grant, 2003; Sonis et al., 2004; Stringer et al., 2009b; Stringer et al., 2009d). Although the incidence and severity of mucositis varies greatly according to patient characteristics and treatment regimens, the incidence of IRI-induced gastrointestinal mucotoxicity in the form of secretory diarrhoea has been reported to be as high as 87% (Rougier et al., 1998; Avritscher et al., 2004). Our results show severe mucosal ulceration, crypt hypoplasia and disorganisation in the colon following both short and long-term IRI treatment. This is in line with previous reports for the rat jejunum and colon

following IRI treatment, where villus blunting, epithelial atrophy and crypt ablation was reported (Logan et al., 2008). However, mucosal regeneration is evident in post-treatment mice in which colonic crypts are still disorganised, but the epithelial brush border is intact. Acute intestinal toxicity associated with anti-cancer treatment is believed to result from crypt cell death, which triggers mucosal inflammation and breakdown of the intestinal mucosal barrier, however controversy exists regarding whether this is a direct result of cytotoxicity or is mediated through a series of intermediate events (Sonis et al., 2004).

The basic pathophysiology of mucositis may be broken into 5 sequential phases i) initiation; ii) up-regulation and message generation; iii) signalling and amplification; iv) ulceration and inflammation; and v) healing (Sonis et al., 2004; Lee et al., 2014). In the healing phase, proliferation and differentiation of the gastrointestinal epithelium returns approximately two weeks post-chemotherapy (Sonis et al., 2004; Lee et al., 2014). Mucosal regeneration has been confirmed in human studies showing that early histological changes in the gastrointestinal tract following chemotherapeutic administration are resolved within days of treatment cessation, with no abnormal endoscopic findings in patients as early as 16 days post-chemotherapeutic treatment (Keefe et al., 2000). However, although histological damage has resolved, long-term diarrhoea following chemotherapy treatment persists up to 10 years post-treatment (Schneider et al., 2007; Denlinger and Barsevick, 2009; Numico et al., 2015). Similar results have been found in pelvic radiation therapy, during which intestinal permeability and mucosal injury peak mid-treatment then gradually improve, while nausea, diarrhoea and abdominal pain persist throughout the course of treatment (Carratù et al., 1998; Hovdenak et al., 2000). It has been suggested that crypt cell death in radiation syndrome is triggered indirectly by apoptotic endothelial lesions (Paris et al., 2001), highlighting that intestinal dysfunction at least in part, may be a consequence of an indirect cascade of events mediated by non-epithelial tissues (Sonis et al., 2004).

Early changes in gastrointestinal transit and symptoms of diarrhoea following IRI administration have been attributed specifically to mucosal damage and inflammation (Gibson et al., 2003;Stringer et al., 2007;Logan et al., 2008;Stringer et al., 2008;Stringer et al., 2009a;Stringer et al., 2009d). A significant increase in CD45+ leukocytes in colonic mucosa observed following 3 days of IRI administration persisted throughout the course of treatment as well as post-treatment in our study. Nuclear factor- κ B (NF- κ B) is activated by pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumour necrosis factor α (TNF α) (Lawrence, 2009). Significant increases in the level of NF- κ B and pro-inflammatory cytokines were found in the colon between 2 and 12 h following acute IRI administration (Logan et al., 2008). This increase in NF- κ B and pro-inflammatory cytokines coincided with initial signs of histological alteration of the tissues and occurrence of diarrhoea (Logan et al., 2008). Similarly, the production of pro-inflammatory cytokines such as IL-1 β , interferon (IFN)- γ and TNF- α is significantly up-regulated in the intestines of rats treated with IRI (Hu et al., 2006). Co-treatment with the anti-inflammatory agent St. John's wort significantly inhibited the expression of TNF- α mRNA in the intestine which was correlated with a reduction in diarrhoea and intestinal lesions following IRI-treatment in rats (Hu et al., 2006). These findings highlight the importance of inflammatory response in the manifestation of diarrhoea induced by IRI treatment.

Although the underlying mechanisms of delayed onset and long-term diarrhoea remain unclear, early mucosal damage and acute intestinal inflammation can lead to death and damage of enteric neurons resulting in long-term gastrointestinal dysfunction (Boyer et al., 2005;Linden et al., 2005;Nurgali et al., 2007;Nurgali et al., 2011). Our study is the first demonstrating that repeated administration of IRI results in neuronal loss of up to 16% from day 7 to post-treatment. Given its cell cycle specificity, IRI is believed to have relatively no toxic effect on differentiated post-mitotic cells such as neurons. However, the IRI analogue camptothecin administered at concentrations that inhibit Top-I has been found to induce apoptosis in cortical neurons which exhibited chromatin condensation, cytoplasmic shrinking, plasma membrane blebbing and DNA fragmentation

consistent with apoptotic cell death (Morris and Geller, 1996). Chemotherapy-induced neuronal loss in the myenteric plexus has been previously shown in mice and rats following administration of other chemotherapeutic agents, cisplatin and oxaliplatin, and this was associated with downstream effects to colonic motility and gastrointestinal transit (Vera et al., 2011; Wafai et al., 2013; Pini et al., 2016). These findings highlight that enteric neuropathy may be a critical component in the development of long-term chemotherapy-induced gastrointestinal dysfunction.

The enteric nervous system is involved in most physiological processes of the gastrointestinal tract and has a profound influence on various functions including motility (Hansen, 2003). The results of this study show alterations in colonic motility alongside loss of myenteric neurons in IRI-treated mice. The total number of contractions following IRI administration was significantly increased due to increases in both the number and proportion of short and fragmented contractions. Short and segmenting contractions play a central role in the formation of productive motor patterns in the healthy intestine (Gwynne et al., 2004). Short distance contractions result in segmentation of the colon which is essential for mixing and absorption of colonic contents (Huizinga and Chen, 2014). Alteration in frequency of short segmenting contractions has been found to contribute to gastrointestinal dysfunction (Fung et al., 2010). Effect of IRI treatment on colonic dysmotility may also be correlated with morphological and functional changes of interstitial cells of Cajal, this requires further investigation.

Cholinergic neurons are a vital signalling component in excitatory motor innervation of the colon (Furness, 2012). Our results have shown phenotypic changes in myenteric neurons as early as 3 days following IRI treatment, with increases of up to 17% in the numbers of detectable ChAT-IR neurons persisting throughout all experimental time-points and in post-treatment mice. ChAT is the catalytic enzyme in the synthesis of acetylcholine (Okuda and Haga, 2003). Acetylcholine is synthesised from choline and acetyl coenzyme A; reuptake of choline from the extracellular space is the rate limiting step in acetylcholine synthesis. Three different

transport systems participate in choline uptake; high-affinity choline transporter 1 (CHT1), choline transporter-like 1 (CTL1) and organic cation transporters (OCTs) such as OCT1, OCT2 and OCT3. CHT1 and OCT-1 immunoreactivity has been observed in the submucosal and myenteric plexi as well as the mucosal epithelium in the small and large intestines (Chen et al., 2001; Harrington et al., 2010). In human intestine, various OCTs have been shown to participate in the absorption of anti-cancer chemotherapeutics including IRI (Shnitsar et al., 2009; Koepsell, 2015). Increased expression of both OCT1 and OCT3 has been linked to IRI chemosensitivity in kidney carcinoma cells and lymphoma cells (Shnitsar et al., 2009; Gupta et al., 2012). The role of OCTs expressed on enteric neurons in IRI transport is yet to be studied; however binding of IRI to OCTs may affect the uptake of choline into neurons resulting in downstream effects on acetylcholine synthesis and the release of acetylcholine (Koepsell, 2013). Thus, it can be speculated that IRI might cause increased reuptake of choline leading to increased level of ChAT in enteric neurons observed in our study. Moreover, IRI increases cholinergic activity by binding to the active site of acetylcholinesterase resulting in functional inhibition of the enzyme and increased persistence of extracellular acetylcholine (Dodds and Rivory, 1999; Harel et al., 2005). To determine whether increase in ChAT expression in enteric neurons is due to increased synthesis or decreased release of acetylcholine, we have quantified the density of cholinergic fibres containing VACHT, a transporter responsible for transferring acetylcholine into vesicles. IRI-treated mice had significantly higher density of VACHT-IR fibres studied at day 14 and post-treatment. Taken together, these data suggest that IRI-induced increase in acetylcholine synthesis and release as well as reduced degradation leads to excessive amount of acetylcholine known as cholinergic syndrome affecting physiological functions of the gut. Acetylcholine is a major excitatory neurotransmitter responsible for the contractions of circular and smooth muscle (Furness, 2012) and mucosal secretion (Cooke, 2000). Acute diarrhoea experienced within the first 24 of IRI treatment has been attributed to cholinergic syndrome in CRC patients (Hecht, 1998). Delayed onset and long-term diarrhoea however are believed to be multifaceted,

resulting from a primarily secretory mechanism with an exudative component (Bleiberg and Cvitkovic, 1996; Saliba et al., 1998).

The results of this study have shown that faecal water content was significantly increased at days 3, 14 and post-treatment, indicative of diarrhoea. Pellet formation occurred significantly more rapidly at day 3 in IRI-treated mice, but was unchanged at days 7, 14 and post-treatment. Given the delays in gastric and intestinal emptying it is possible that increased colonic transit speed is masked, resulting in overall pellet formation time remaining unchanged. It has previously been shown that colonic propulsion speed correlates positively with pellet length (Costa et al., 2015). The pellet length was significantly increased in IRI-treated mice at days 7, 14 and post-treatment when compared to control. These findings suggest increased colonic propulsive activity and presence of diarrhoea in IRI-treated mice.

3.6 Conclusion

In conclusion, this study demonstrates that IRI treatment induces mucosal damage and inflammation which may contribute to neuronal loss and phenotypic changes in the myenteric plexus. Increased number of cholinergic neurons and fibres in the colon may underlie alterations in colonic motor activity and gastrointestinal transit following IRI treatment resulting in chronic gastrointestinal dysfunction. This study is the first to show that neurogenic changes in the gut may be a key player in the manifestation of delayed-onset and long-term chronic diarrhoea that persists after the cessation of IRI treatment.

CHAPTER FOUR: GASTROINTESTINAL DYSFUNCTION AND ENTERIC NEUROTOXICITY FOLLOWING TREATMENT WITH ANTICANCER CHEMOTHERAPEUTIC AGENT 5-FLUOROURACIL

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4.1 Summary

The use of the anti-cancer chemotherapeutic agent 5-fluorouracil (5-FU) is often limited by nausea, vomiting, constipation and diarrhoea; these side-effects persist long after treatment. The effects of 5-FU on enteric neurons have not been studied and may provide insight into the mechanisms underlying 5-FU-induced gastrointestinal dysfunction.

Balb/c mice received intraperitoneal injections of 5-FU (23 mg/kg^{-1}) 3 times/week for 14 days. Gastrointestinal transit was analysed *in vivo* prior to and following 3, 7 and 14 days of 5-FU treatment via serial x-ray imaging. Following 14 days of 5-FU administration, colons were collected for assessment of *ex vivo* colonic motility, gross morphological structure and immunohistochemical analysis of myenteric neurons. Faecal lipocalin-2 and CD45+ leukocytes in the colon were analysed as markers of intestinal inflammation.

Short-term administration of 5-FU (3 days) increased gastrointestinal transit, induced acute intestinal inflammation and reduced the proportion of nNOS-immunoreactive neurons. Long-term treatment (7, 14 days) resulted in delayed gastrointestinal transit, inhibition of colonic migrating motor complexes, increased short and fragmented contractions, myenteric neuronal loss and a reduction in the number of ChAT-immunoreactive neurons after the inflammation was resolved. Gross morphological damage to the colon was observed following both short and long-term 5-FU treatment.

Our results indicate that 5-FU induces accelerated gastrointestinal transit associated with acute intestinal inflammation at day 3 after the start of treatment, which may have led to persistent changes in the ENS observed after days 7 and 14 of treatment contributing to delayed gastrointestinal transit and colonic dysmotility.

Abbreviations

| | |
|----------------|---------------------------------|
| 5-FU | 5-fluorouracil |
| ChAT | choline acetyltransferase |
| CMMC | colonic migrating motor complex |
| CRC | colorectal cancer |
| DMSO | dimethyl sulfoxide |
| ENS | enteric nervous system |
| FC | fragmented contraction |
| H&E | haematoxylin and eosin |
| IR | immunoreactive |
| nNOS | neuronal nitric oxide synthase |
| PBS | phosphate buffered saline |
| SC | short contraction |

4.2 Introduction

Since the discovery of fluoropyrimidines in the 1950's, 5-fluorouracil (5-FU) has been the backbone of therapy for many solid tumours and is considered as the standard first line therapy for metastatic CRC (Goldberg, 2005). 5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen (Wohlhueter et al., 1980). Upon entering a cell, 5-FU is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate and fluorouridine triphosphate. These active metabolites exert their cytotoxic effects via misincorporation of fluoronucleotides into RNA as well as inhibition of nucleotide synthetic enzyme thymidylate synthase (Longley et al., 2003). Combination therapies including 5-FU are reported to improve response rates for advanced CRC by 40-50% (Douillard et al., 2000; Giacchetti et al., 2000), however severe gastrointestinal side-effects such as nausea, vomiting, constipation and diarrhoea remain significant hurdles in the clinical application of 5-FU. It has been suggested that 5-FU-induced gastrointestinal dysfunction results from inflammation, epithelial degradation and intestinal ulceration triggering intestinal mucositis (Duncan and Grant, 2003). However, recent evidence suggests that 5-FU induced gastrointestinal dysmotility outlasts intestinal mucositis (Soares et al., 2008). One system that has been overlooked in 5-FU-induced gastrointestinal dysfunction is the enteric nervous system (ENS), embedded within the wall of the gastrointestinal tract and controlling its functions (Furness, 2012). Neuronal loss and phenotypic changes within the ENS following chemotherapeutic administration of other agents, cisplatin and oxaliplatin, have been found to result in downstream effects on gastrointestinal muscle tone and transit (Vera et al., 2011; Wafai et al., 2013).

The aims of this study were to investigate the effects of short and long-term 5-FU treatment on: i) gastrointestinal transit time and gastric emptying; ii) *ex vivo* colonic motility functions; iv) histological architecture of the colonic mucosa; v) level of inflammation in the colon; v) the total number of

myenteric neurons and subpopulations of inhibitory and excitatory neurons controlling colonic motility.

4.3 Methods

4.3.1 Ethical Approval

All procedures were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

4.3.2 Animals

Male Balb/c mice aged 6-8 weeks (18–25g) supplied from the Animal Resources Centre (Perth, Australia) were used for the experiments. Mice had free access to food and water and were kept under a 12 hour light/dark cycle in a well-ventilated room at an approximate temperature of 22°C. Mice acclimatised for up to 7 days prior to the commencement of *in vivo* intraperitoneal injections. A total of 42 mice were used for this study.

4.3.3 *In vivo* 5-Fluorouracil Injections

Mice received intraperitoneal injections of 5-FU (23 mg/kg⁻¹) (Sigma-Aldrich, Australia), 3 times a week via a 26-gauge needle. 5-FU was dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Australia) to make 1M L⁻¹ stock solution refrigerated at -20°C. The stock was then defrosted and diluted with sterile water to make 0.1M L⁻¹ (10% DMSO) solutions for intraperitoneal injections. The dose of 5-FU was calculated to be equivalent to standard human dose per body surface area (Reagan-Shaw et al., 2008). The low doses of 5-FU (10-40 mg/kg⁻¹) have been shown to have anti-tumour efficacy in mouse models of cancer (Cao et al., 2014). Sham-treated mice received 10% DMSO in sterile water via intraperitoneal injection 3 times a week via a 26 gauge needle. The injected volumes were calculated

to the body weight; the maximum volume did not exceed 200 μ L per injection. Mice were euthanized via cervical dislocation at 3 (2 treatments), 7 (3 treatments) and 14 (6 treatments) days after the first injection and colon was collected for *in vitro* experiments.

4.3.4 Gastrointestinal Transit

Gastrointestinal transit was carried out as described in Chapter 3, section 3.3.9. Briefly, prior to performing x-ray imaging, animals were trained/conditioned for oral gavage using a non-irritating substance such as 0.9% w/v saline (volume 0.1-0.4 ml); this was repeated at least 3 times with each animal with at least 24 hours between each training. The training/conditioning with restraint were done by placing the restrainer into the mouse cages at least 24 hours prior to the X-Ray procedure. Gastrointestinal transit was studied by x-ray prior to first treatment (day 0) and following 3, 7 and 14 days of 5-FU treatment. The contrast agent, 0.4 mL of suspended barium sulfate (X-OPAQUE-HD, 2.5g/mL), was administered via oral gavage. Radiographs of the gastrointestinal tract were performed using a HiRay Plus Porta610HF x-ray apparatus (JOC Corp, Kanagawa, Japan; 50 kV, 0.3 mAs, exposure time 60 ms). Mice were immobilised in the prone position by placing them inside a transparent plastic restraint tube with partly open front side for breathing which comfortably restrains animal movement essential for maximum of 1-2 min for successful x-ray imaging. X-rays were captured using Fujifilm cassettes (24×30 cm) immediately after administration of barium sulfate (T0) every 5 minutes for the first hour, every 10 minutes for the second hour, then every 20 minutes through to 480 minutes (T480). Animals were closely monitored during and after all procedures. Images were developed via a Fujifilm FCR Capsula XLII and analysed using eFilm 4.0.2 software. Speed of gastrointestinal transit was calculated as time in minutes taken to reach each region of the gastrointestinal tract (stomach, small intestines, caecum, and large intestines). Organ emptying was calculated as the time taken for complete barium emptying from specific gastrointestinal regions (stomach, small intestines) (Cabezós et al., 2008;Cabezós et al., 2010;Girón et al., 2015).

4.3.5 Colonic Motility Experiments

The entire colon was removed from day 14 sham and 5-FU-treated mice and set up in organ-bath chambers to record motor patterns *in vitro* (Wafai et al., 2013), as described in Chapters 2 and 3, sections 2.3.12 and 3.3.8. Briefly, the colon was placed into warmed (35°C), oxygenated physiological saline until the fecal pellets were expelled. The empty colon was cannulated at both ends and arranged horizontally in an organ-bath chamber. The proximal end of the colon was connected to a reservoir containing oxygenated physiological saline to maintain intraluminal pressure. The distal end was attached to an outflow tube that provided a maximum of 2 cm H₂O back-pressure. Organ baths were continuously superfused with oxygenated physiological saline solution and preparations were left to equilibrate for 30 min. Contractile activity of each segment was recorded with a Logitech Quickcam Pro camera positioned 7–8 cm above the preparation. Videos (2x20min) of each test condition were captured and saved in *avi* format using VirtualDub software (version 1.9.11).

Colonic migrating motor complexes (CMMCs) were defined as propagating contractions directed from the proximal to the distal end of the colon which travelled more than 50% of the colon length (Spencer and Bywater, 2002; Roberts et al., 2007; Roberts et al., 2008). Contractions that propagated less than 50% colon length were considered to be short contractions (SCs). Incomplete non-propagating phasic contractions occurring concurrently at different parts of the colon rather than propagating over the length of the colon were defined as fragmented contractions (FCs). Recordings were used to construct spatiotemporal maps using in-house edge detection software (Gwynne et al., 2004). Spatiotemporal maps plot the diameter of the colon at all points during the recording allowing contractile motor patterns to be analysed with Matlab software (version 12).

4.3.6 Drugs Used

Hexamethonium Bromide (HEX) (Sigma-Aldrich, Australia) and tetrodotoxin (TTX) (Abcam, MA, USA) were prepared as stock solutions and diluted in physiological saline daily before addition to preparations.

4.3.7 Histology

To examine the morphological changes to the colon, standard Haematoxylin and Eosin (H&E) staining protocol was followed (Nurgali et al., 2011;McQuade et al., 2016a), as described in Chapters 2 and 3, sections 2.3.7 and 3.3.4. Briefly, the colon was harvested and placed in a 10% formalin solution overnight and then transferred into 70% ethanol the following day. Paraffin embedded colon sections were cut 5µm thick and de-waxed in a 60°C oven for 30 minutes. Ten sections per preparation were analysed. All images were analysed blindly.

4.3.8 Immunohistochemistry in Wholemout Preparations

Colon sections (2-3 cm) were placed in oxygenated phosphate-buffered saline (PBS) (pH 7.2) containing nicardipine (3 µM) (Sigma-Aldrich, Australia) for 20 mins to inhibit smooth muscle contractions. Samples were cut open along the mesenteric border, cleared of their contents, maximally stretched and dissected mucosa up to expose the myenteric plexus attached to the longitudinal muscle layer. Tissues were fixed with Zamboni's fixative (2% formaldehyde, 0.2% picric acid) overnight at 4°C. Preparations were cleared of fixative by washing 3 x 10 min with DMSO followed by 3 x 10 min washes with PBS. Fixed tissues were stored at 4°C in PBS for a maximum of 5 days.

Wholemout preparations were incubated with 10% normal donkey serum (Chemicon, USA) for 1 hour at room temperature. Tissues were then washed (2 x 5 mins) with PBS and incubated with primary antibodies against Protein Gene Product 9.5 (PGP9.5) (chicken, 1:500, Abcam, MA, USA), neuronal nitric oxide synthase (nNOS) (goat, 1:500, Abcam, MA, USA) and choline acetyl transferase (ChAT) (goat, 1:200, Abcam, MA, USA) overnight at 4°C. Tissues were then washed in PBS (3 x 10 min) before incubation

with species-specific secondary antibodies labelled with different fluorophores: donkey anti-chicken Alexa 594 (1:200, Jackson Immuno research Laboratories, PA, USA) and donkey anti-goat Alexa 488 (1:200, Jackson Immuno research Laboratories, PA, USA) for 2 hours at room temperature. Wholemound preparations were given 3 x 10 min final washes in PBS and then mounted on glass slides using fluorescent mounting medium (DAKO, Australia). Wholemound preparations were observed under a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan), 8 randomly chosen images from each preparation were captured with a 20 x objective and processed using NIS Elements software (Nikon, Japan). The number of PGP9.5, nNOS and ChAT immunoreactive neurons was quantified in the myenteric ganglia within a 2 mm² area of each preparation.

4.3.9 Immunohistochemistry in Cross Sections

Colon sections (1-2 cm) were placed in oxygenated PBS containing nifedipine (3 μ M) (Sigma-Aldrich, Australia) for 20 mins to inhibit smooth muscle contractions. Samples were cut open along the mesenteric border, cleared of their contents, and pinned mucosa up without stretching. Tissues were fixed with Zamboni's fixative overnight at 4°C. Preparations were cleared of fixative by washing 3 x 10 min with DMSO (Sigma-Aldrich, Australia) followed by 3 x 10 min washes with PBS. After washing, tissues were embedded in 100% OCT and frozen using liquid nitrogen (LN₂) and isopentane (2-methyl butane) and stored in -80°C freezer. Tissues were cut at 20 μ m section thickness using Leica CM1950 cryostat (Leica Biosystems, Germany), adhered to slides and allowed to rest for 30 minutes at room temperature before processing.

Cross section preparations were incubated with 10% normal donkey serum (Chemicon, USA) for 1 hour at room temperature. Tissues were then washed (2 x 5 mins) with PBS and incubated with primary antibodies against CD45 (Rat, 1:500, BioLegend, Australia), overnight at 4°C. Sections were then washed in PBS (3 x 10 min) before incubation with secondary antibodies labelled with fluorophore donkey anti-rat Alexa 488 (1:200, Jackson Immunoresearch Laboratories, PA, USA) for 2 hours at room temperature. The sections were given 3 x 10 min final washes in PBS and

then cover slipped using fluorescence mounting medium (DAKO, Australia). Sections were viewed under a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan), 8 randomly chosen images from each preparation were captured with a 20 x objective and processed using NIS Elements software (Nikon, Japan). The number of CD45+ immunoreactive cells was quantified within a 2 mm² area in every colonic section.

4.3.10 Imaging

Three dimensional (z-series) images of wholemount preparations were taken using a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan), as described in Chapters 2 and 3, sections 2.3.8 and 3.3.7. Eight randomly chosen three dimensional (z-series) images from each preparation were captured with a 20 x objective and processed using NIS Elements software (Nikon, Japan). Fluorophores were visualized using excitation filters for Alexa 594 Red (excitation wavelength 559nm), Alexa 488 (excitation wavelength 473nm), and Alexa 405 (excitation wavelength 405nm). Z-series images were taken at step size of 1.75 µm (1600 x 1200 pixels).

4.3.11 Quantification of faecal Lipocalin-2

Fresh faecal pellets were collected from mice after 3, 7 and 14 days of 5-FU administration and stored immediately at -80°C. Faecal samples were thawed and suspended in PBS with 0.1% Tween (100mg/mL) overnight prior to processing. The next day samples were centrifuged at 12, 000 rpm at 4°C for 10 minutes. Samples were then agitated and centrifuged for another 5 minutes at 12, 000 rpm at 4°C. Supernatant was collected and lipocalin-2 levels were quantified using DuoSET ELISA Mouse Lipocalin-2 kit (R&D Systems, Minneapolis, USA).

4.3.12 Statistical Analysis

Data were assessed using two-way ANOVA, Welch's two-tailed *t* test and Student's two-tailed *t* test. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., CA, USA). Data are presented as mean \pm standard error of the mean (SEM). Value differences were considered statistically significant at $P < 0.05$.

4.4 Results

4.4.1 Altered gastrointestinal transit following 5-FU administration

To determine the effects of 5-FU administration on gastrointestinal transit, series of radiographic images were used to track barium sulfate throughout the gastrointestinal tract before the first injection (day 0), after 2 injections (day 3), 3 injections (day 7) and 6 injections (day 14) (**Figure 4.1, Table 4.1**). The speed of barium movement was calculated by tracing barium entry from one part of the gastrointestinal tract to the next. After 3 days of 5-FU administration, movement of barium in both the caecum and colon was faster than before treatment, however, after 7 and 14 days of 5-FU administration barium movement was significantly delayed compared to day 0 in the caecum and colon (**Figure 4.2, Table 4.1**).

Although tracing barium movement allowed analysis of real time transit speed, gastrointestinal organ filling and emptying does not happen simultaneously, thus we further analysed the time taken for complete barium emptying from specific regions. No changes in gastric emptying time were observed at day 3 of 5-FU administration, but significant delays in gastric emptying were seen after 7 and 14 days of 5-FU administration (**Figure 4.3A, Table 4.1**). Intestinal emptying was faster after 3 days of 5-FU administration, but significantly delayed after both 7 and 14 days of 5-FU administration (**Figure 4.3B, Table 4.1**).

Pellet formation time was decreased after 3 days of 5-FU administration (**Figure 4.3C**), but significantly increased after 7 and 14 days of 5-FU administration (**Figure 4.3C**). Thus, increased intestinal transit was observed after short-term 5-FU treatment, while prolonged treatment induced delays in gastrointestinal transit. No significant difference in percentage body weight was found when comparing 5-FU-treated mice ($103\pm0.6\%$) to sham-treated mice ($105.9\pm1.9\%$).

Figure 4.1 X-ray images following repeated *in vivo* 5-FU administration. Representative x-ray images obtained from mice 0 – 210 min after intragastric barium sulfate (0.4mL, 2.5mg/mL) administration at day 0 (prior to 1st injection) and following 3, 7 and 14 days of 5-FU administration. Stomach (*), small intestines (#), caecum (Δ), pellet formation (†).

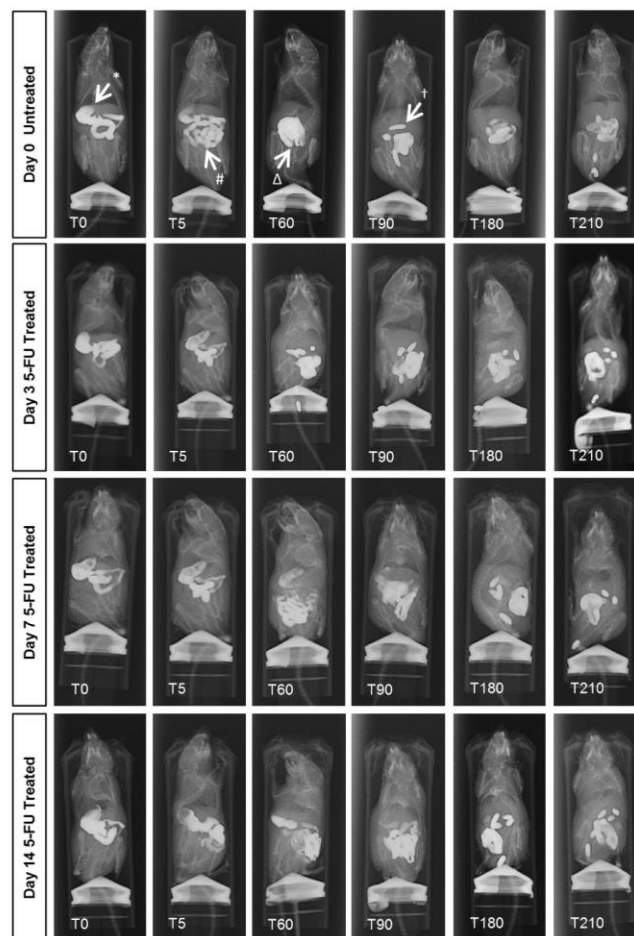


Figure 4.2 Gastrointestinal transit time following repeated *in vivo* 5-FU administration Time (min) taken for barium sulfate to reach the stomach, small intestines, caecum and large intestines before (day 0) and at 3, 7 and 14 days following 5-FU administration. Data represented as mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, $n = 6$ per group/time point.

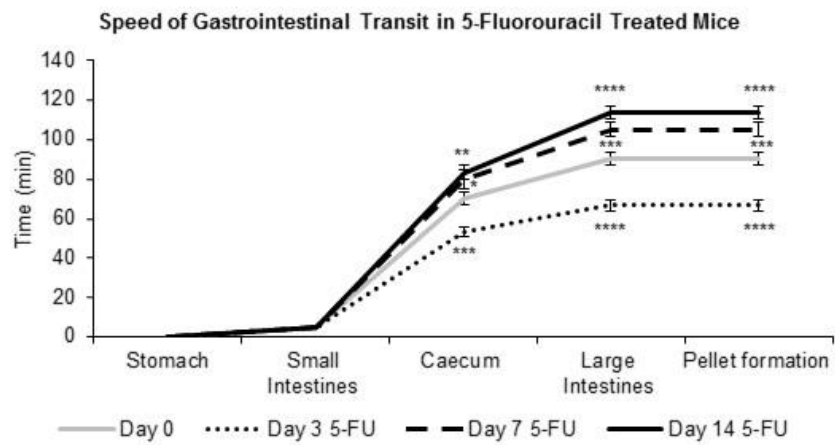
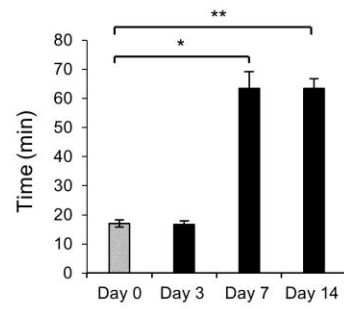
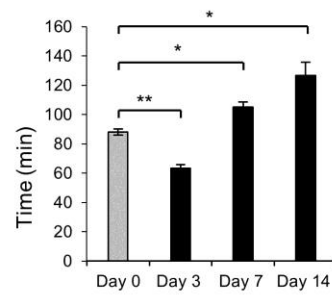


Figure 4.3 Gastric and intestinal emptying following repeated *in vivo* 5-FU administration. (A) Time (min) taken for complete emptying of barium from the stomach. (B) Time (min) taken for complete emptying of barium from the small intestines. (C) Time (min) taken to form first pellet before (day 0) and at 3, 7 and 14 days following 5-FU administration. Data represented as mean \pm S.E.M. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001, $n = 6$ per group/time point.

A Gastric Emptying



B Small Intestines Emptying



C Pellet Formation

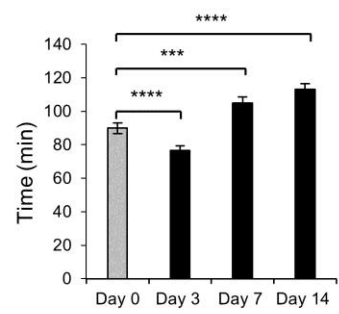


Table 4.1 Speed of transit and emptying following repeated *in vivo* 5-fluorouracil administration

| Parameters measured | | Day 0 | Day 3 | Day 7 | Day 14 |
|--|----------------------------|-------|----------|----------|-----------|
| Speed of transit (time to reach each region, min) | Stomach | 0±0 | 0±0 | 0±0 | 0±0 |
| | Small Intestines | 5±0 | 5±0 | 5±0 | 5±0 |
| | Caecum | 70±3 | 53±3*** | 80±5* | 83±4** |
| | Large Intestines | 90±3 | 66±3**** | 105±4*** | 113±4**** |
| Time for complete barium emptying (min) | Gastric emptying | 17±1 | 16±1 | 63±6* | 63±3** |
| | Intestinal emptying | 88±2 | 63±3** | 105±4* | 126±8* |
| | Pellet Formation | 90±3 | 66±3**** | 105±4*** | 113±4**** |

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, significantly different to Day 0. (n=5 per/group).

4.4.2 Changes in colonic motility following 5-FU treatment

To investigate effects of 5-FU treatment on colonic motility, excised colons were studied in organ bath experiments at day 14 of 5-FU treatment. The total number of contractions (including all types of motor patterns in the colon: CMMCs, short and fragmented contractions, **Figure 4.4A**) was increased in colons from 5-FU-treated animals compared to sham-treated mice (sham: 27.7 ± 1.4 , 5-FU: 36.1 ± 2.1 , $P < 0.01$, **Figure 4.4B**). To determine if this increase was due to changes in a specific type of motor activity, the frequency, proportion and propagation speed were analysed for each type of motor pattern.

Colonic migrating motor complexes (CMMCs) were defined as sustained anally directed contractions propagating more than 50% of the colon length. CMMCs are mediated mainly by myenteric neurons, although inputs from the mucosa may modulate their activity (Keating and Spencer, 2010). *In vivo* treatment with 5-FU was associated with a decrease in the frequency of CMMCs in comparison with sham-treated mice (sham: 11.2 ± 0.6 , 5-FU: 3.5 ± 1.0 , $P < 0.0001$, **Figure 4.4B**). Similarly, the proportion of CMMCs following 5-FU treatment was significantly decreased compared to sham-treated mice (sham: $41.1 \pm 3.0\%$, 5-FU: $12.4 \pm 3.8\%$, $P < 0.0001$, **Figure 4.4C**). No significant difference in the speed of CMMCs was found between sham-treated and 5-FU-treated mice (sham: $2.9 \pm 0.2 \text{ mm/s}$, 5-FU: $2.3 \pm 0.3 \text{ mm/s}$, **Figure 4.4D**).

Contractions that propagated less than 50% of the colon length were termed short contractions (SCs) (**Figure 4.4A**). Treatment with 5-FU resulted in both increased frequency (sham: 10.1 ± 1.1 , 5-FU: 20.5 ± 3.0 , $P < 0.01$, **Figure 4.4B**) and increased proportion (sham: $36.0 \pm 2.2\%$, 5-FU: $54.2 \pm 4.1\%$, $P < 0.01$, **Figure 4.4C**) of SCs in the colon. A significant increase in the speed of SCs was found following 5-FU administration when compared to sham-treated mice (sham: $0.7 \pm 0.0 \text{ mm/s}$, 5-FU: $1.9 \pm 0.3 \text{ mm/s}$, $P < 0.01$, **Figure 4.4D**). Incomplete contractions that did not propagate, but rather occurred concurrently over the length of the colon were defined as fragmented

contractions (FCs). Both the frequency (sham: 6.4 ± 0.8 , 5-FU: 11.8 ± 1.2 , $P < 0.01$, **Figure 4.4B**) and the proportion (sham: $22.9 \pm 2.3\%$, 5-FU: $32.5 \pm 1.8\%$, $P < 0.01$, **Figure 4.4C**) of FCs were significantly higher in 5-FU-treated compared to sham-treated mice. No significant difference in the speed of FCs was found between sham-treated and 5-FU-treated mice (sham: 1.5 ± 0.1 mm/s, 5-FU: 2.1 ± 0.3 mm/s, **Figure 4.4D**).

To investigate whether neurogenic components are involved in the recorded colonic motor patterns, a voltage-gated sodium channel blocker tetrodotoxin (TTX) and a nicotinic cholinergic antagonist hexamethonium (HEX) were added into the organ baths. TTX ($1 \mu\text{M}$, 20 min) abolished CMMCs, SCs and FCs in colons from both sham and 5-FU-treated mice (**Figure 4.5**), indicating that these motility patterns are neurogenic. However a persistent TTX-resistant tonic constriction in the distal part of the colon blocked by HEX ($100 \mu\text{M}$, 20 min) was observed in sham-treated mice. In 5-FU-treated mice, TTX-resistant tonic constrictions were prominent in both proximal and distal parts of the colon; the proximal tonic constriction was not blocked by application of HEX (**Figure 4.5**).

Figure 4.4 Total number, proportion and speed of different contractions following repeated *in vivo* 5-FU administration. (A) Representative spatiotemporal maps generated from digital video recordings of colonic motility from sham and 5-FU-treated mice. Each contraction can be seen as a reduction in the gut width (red), while relaxation as an increase in the gut width (blue). Colonic migrating motor complexes (CMMCs) propagate >50% of the colon length, short contractions (SCs) propagate <50% of the colon length and fragmented contractions (FCs) are interrupted by period(s) of relaxation during contraction. (B) Total number of contractions including all types of contractile activity in the colons from sham and 5-FU-treated mice. (C) The proportion of CMMCs, SCs and FCs to the total number of contractions. (D) The speed of CMMCs, SCs and FCs. Data represented as mean \pm S.E.M. ** $P < 0.01$, **** $P < 0.0001$, $n = 6$ per group/time point.

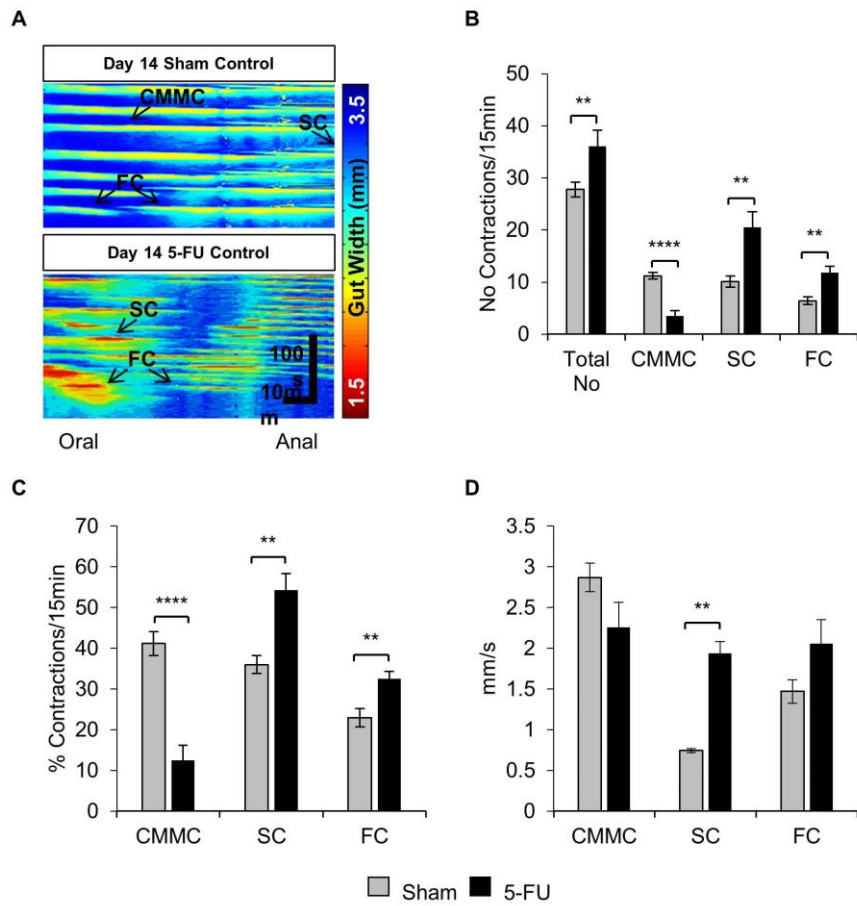
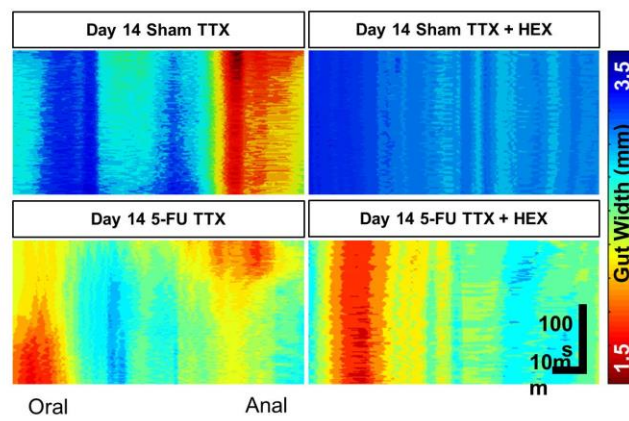


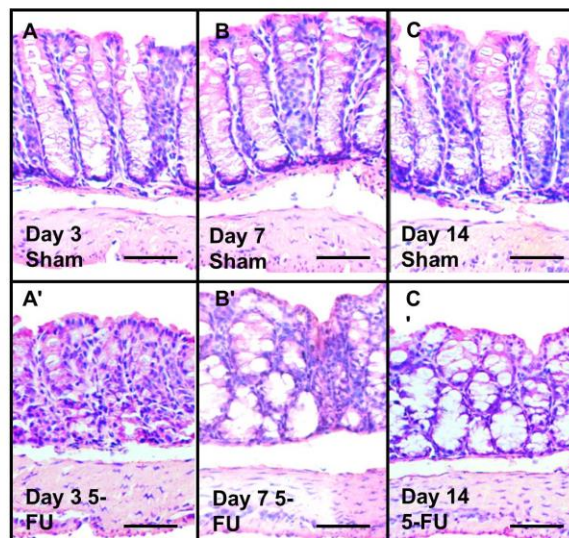
Figure 4.5 Spatiotemporal maps of colonic motility following repeated *in vivo* 5-FU administration. Spatiotemporal maps from day 14 sham and 5-FU-treated mice after addition of tetrodotoxin (TTX, 1 μ M, 20min) and hexamethonium (HEX, 100 μ M, 20 min).



4.4.3 Morphological damage to the colon following 5-FU administration

Colonic architecture from sham-treated animals at days 3, 7 and 14 appeared healthy with a visible brush border as well as uniform crypts (**Figure 4.6A, B, C**). Histological examination of the colon at day 3 from the 5-FU-treated group demonstrated no obvious changes to the epithelial brush border; however, there was a severe loss of colonic crypts and goblet cells and cellular infiltration within the lamina propria when compared to the sham-treated group (**Figure 4.6A, A'**). By days 7 and 14, there was thickening of the epithelial brush border indicative of regeneration of the colonic crypts in the 5-FU-treated groups, but they still appeared short, distended and disorganised with an increased number of goblet cells (**Figure 4.6B', C'**) when compared to colon from the sham-treated group (**Figure 4.6B, C**).

Figure 4.6 Gross morphological changes in the colon following repeated *in vivo* 5-FU administration. H&E staining in the colon from sham and 5-FU -treated mice at 3 (**A, A'**), 7 (**B, B'**) and 14 (**C, C'**) days. Scale bar = 100µm, n = 4 per group/time point.



4.4.4 Intestinal inflammation following 5-FU treatment

To investigate if 5-FU treatment causes inflammation, immune cell infiltration in the colon and the concentration of a neutrophil gelatinase-associated protein, lipocalin-2, a highly sensitive biomarker for intestinal inflammation (Bachman et al., 2009),(Chassaing et al., 2012) in faecal samples were analysed.

Immune cells in colonic cross sections were labelled with a pan leukocyte marker anti-CD45 antibody following 3 (**Figure 4.7A, A'**), 7 (**Figure 4.7B, B'**) and 14 (**Figure 4.7C, C'**) days of sham and 5-FU treatment. Total numbers of CD45 positive cells were counted within a 2mm² area. A significant increase in the number of CD45 positive cells was found in the colon following 3 days of 5-FU administration (99 ± 2 , $P < 0.0001$) when compared to sham (48 ± 1 , **Figure 4.8A**). No significant changes in the number of CD45 positive leukocytes were found after 7 (sham: 49 ± 2 , 5-FU: 53 ± 3) and 14 (sham: 51 ± 4 , 5-FU: 54 ± 1) days of treatment (**Figure 4.8A**). A significant increase in the concentration of lipocalin-2 was observed at day 3 after 5-FU administration (1592 ± 160 ng, $P < 0.05$) compared to sham-treated mice (883 ± 100 ng), but not at days 7 (sham: 946 ± 230 ng, 5-FU: 867 ± 210 ng) and 14 (sham: 1122 ± 160 ng, 5-FU: 881 ± 120 ng) (**Figure 4.8B**).

Figure 4.7 CD45+ cells in colonic cross sections following repeated *in vivo* 5-FU administration. Cross sections of the colon labelled with antibody against CD45+ leukocytes (green) following 3 (**A, A'**), 7 (**B, B'**) and 14 (**C, C'**) days of sham or 5-FU treatment, scale bar = 50µm.

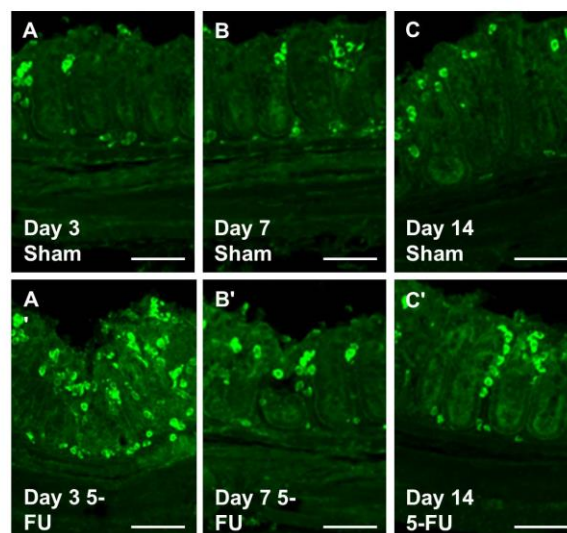
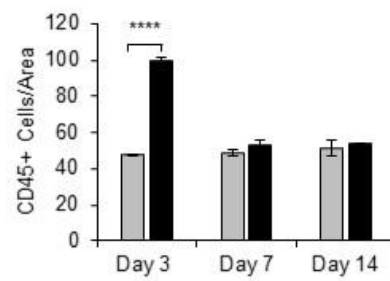


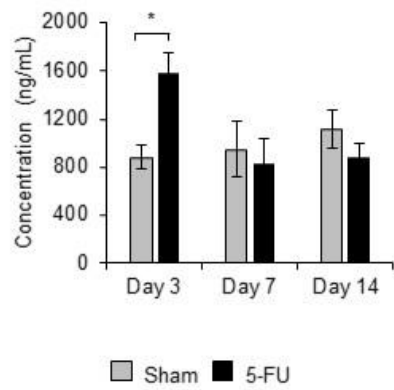
Figure 4.8 Inflammatory marker in the colonic cross sections and faecal pellets following repeated *in vivo* 5-FU administration (A)

Average number of CD45+ cells in the colon was counted per 2mm² at 3, 7 and 14 days in both sham and 5-FU treated mice. **(B)** Concentration of lipocalin-2 (ng/mL) in faecal pellets collected following 3, 7 and 14 days of 5-FU administration. Data represented as mean \pm S.E.M. * P <0.05, **** P <0.0001, n = 4 per group/time point.

A Average Number of CD45+ Cells in the Colon



B Level of Faecal Lipocalin-2



4.4.5 Reduction in the total number of myenteric neurons and changes in neuronal subpopulations following administration of 5-FU

To investigate any changes to the total number of myenteric neurons, wholemount preparations of the colon were labelled with anti-PGP9.5 antibody to count neurons within a 2mm² area. Repeated *in vivo* administration of 5-FU induced myenteric neuronal loss at days 7 (sham: 1225±6; 5-FU: 1150±5, $P<0.001$) and 14 (sham: 1229±3; 5-FU: 1091±5, $P<0.001$), but not at day 3 (sham: 1234±4; 5-FU: 1231±10, **Figure 4.9**).

To determine if 5-FU administration was associated with changes in subpopulations of myenteric neurons controlling intestinal muscle activity, inhibitory muscle motor and interneurons IR for nNOS (**Figure 4.10, 4.11**) and neurons IR for ChAT (**Figure 4.12, 4.13**) were analysed.

Fewer nNOS-IR neurons were observed at days 3 (sham: 357±7; 5-FU: 286±3, $P<0.0001$) and 14 (sham: 380±11; 5-FU: 310±3, $P<0.0001$) following 5-FU administration when compared to sham (**Figure 4.11A**). However, no reduction in the number of nNOS-IR neurons from the 5-FU-treated group was observed at day 7 (sham: 363±5; 5-FU: 341±4) (**Figure 4.11A**). The proportion of nNOS-IR neurons was reduced on day 3 (sham: 29.5±0.3%; 5-FU: 23.8±0.3%) when compared to sham ($P<0.01$, **Figure 4.11B**), but not on days 7 (sham: 29.3±0.6; 5-FU: 29.7±0.3) and 14 (sham: 30.9±0.9; 5-FU: 28.5±0.4, **Figure 4.11B**).

The total number of ChAT-IR neurons increased on day 3 (sham: 294±4; 5-FU: 319±4), but decreased on days 7 (sham: 322±2; 5-FU: 300±4) and 14 (sham: 320±3; 5-FU: 282±5) following 5-FU administration when compared to the sham-treated group (**Figure 4.13A**). However, no significant difference in the proportion of ChAT-IR neurons was observed at any time point after 5-FU administration began (**Figure 4.13B**).

Figure 4.9 Effect of repeated *in vivo* 5-FU administration on the total number of myenteric neurons. Average number of PGP9.5-IR neurons in the colon was counted per 2mm² at 3, 7 and 14 days in both sham and 5-FU-treated mice. Data represented as mean \pm S.E.M. *** $P < 0.001$, n = 6 per group/time point.

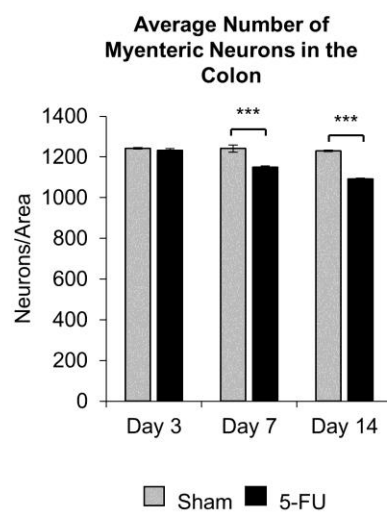


Figure 4.10 Wholemout preparations of nNOS-IR myenteric neurons in the colon following repeated *in vivo* 5-FU administration. Wholemout preparations of myenteric neurons in the colon following 3 (**A, A'**), 7 (**B, B'**) and 14 (**C, C'**) days of sham or 5-FU. Average number of PGP9.5-IR (magenta) and nNOS-IR (green) neurons in the colon was counted per 2mm² at 3, 7 and 14 days, scale bar =50µm.

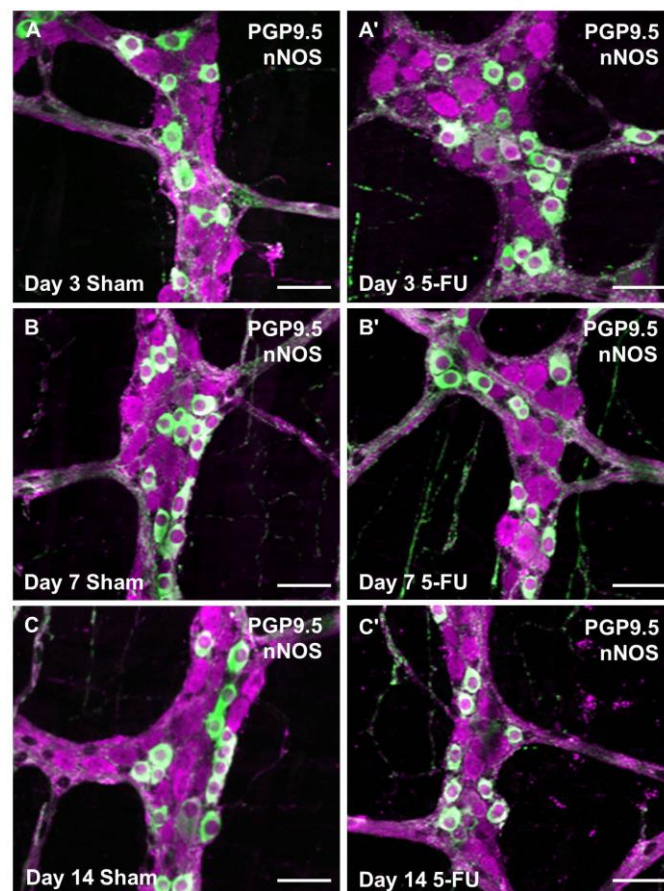


Figure 4.11 Effect of repeated *in vivo* 5-FU administration on average number and proportion of nNOS-IR myenteric neurons. (A) Average number of nNOS-IR neurons in the colon was counted per 2mm² at 3, 7 and 14 days in both sham and 5-FU treated mice. (B) Proportion of nNOS-IR neurons to the total number of PGP9.5-IR myenteric neurons in the colon was counted at 3, 7 and 14 days in both sham and 5-FU-treated mice. Grey column: sham-treated, black column: 5-FU-treated. Data represented as mean \pm S.E.M. ** $P < 0.01$, **** $P < 0.0001$, n = 6 per group/time point.

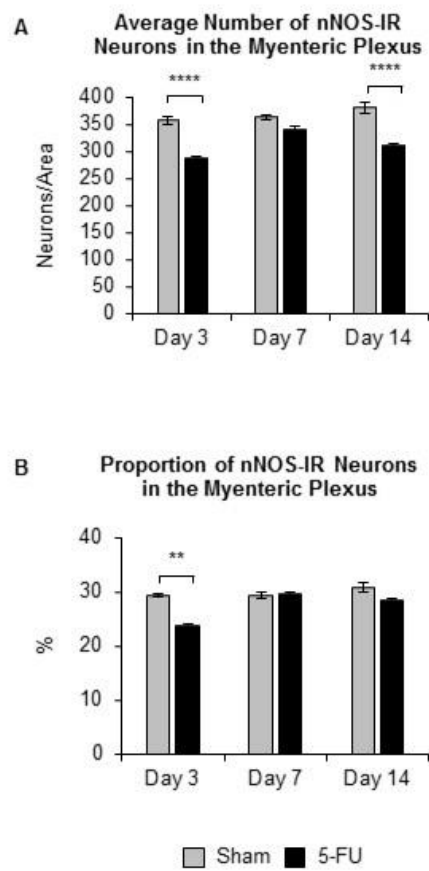


Figure 4.12 Wholemout preparations of ChAT-IR myenteric neurons in the colon following repeated *in vivo* 5-FU administration

Wholemout preparations of myenteric neurons in the colon following 3 (**A, A'**), 7 (**B, B'**) and 14 (**C, C'**) days of sham or 5-FU. Average number of PGP9.5-IR (magenta) and ChAT-IR (green) neurons in the colon was counted per 2mm² at 3, 7 and 14 days, scale bar =50µm.

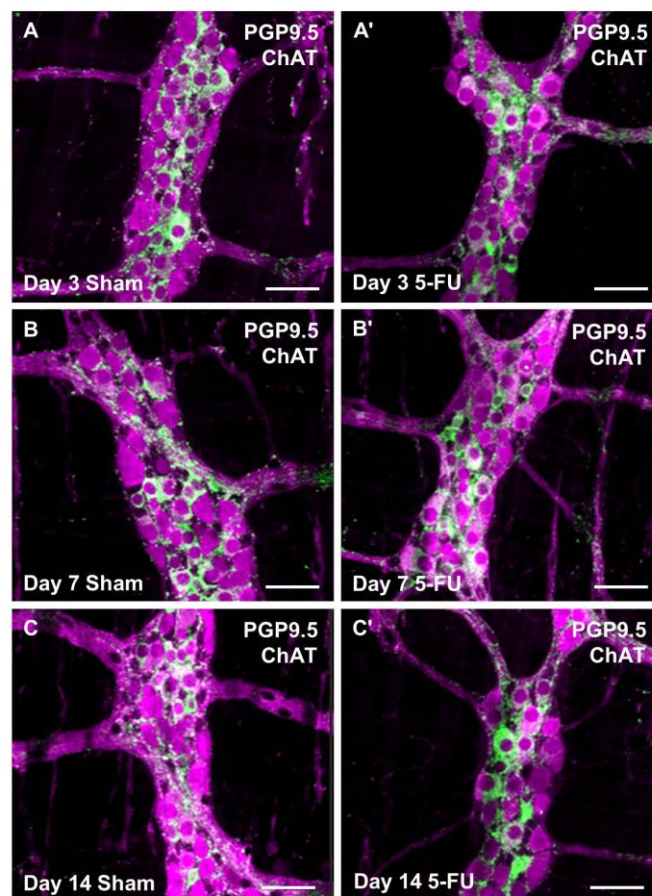
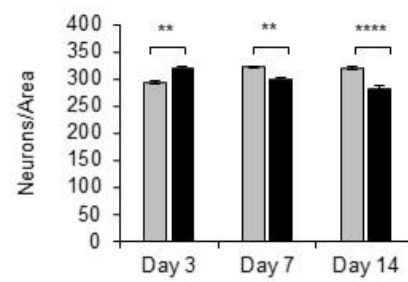
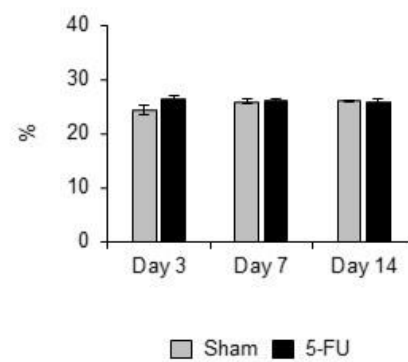


Figure 4.13 Effect of repeated *in vivo* 5-FU administration on the average number and proportion of ChAT-IR myenteric neurons. (A) Average number of ChAT-IR neurons in the colon was counted per 2mm² at 3, 7 and 14 days in both sham and 5-FU-treated mice. (B) Proportion of ChAT-IR neurons was counted to the total number of PGP9.5-IR myenteric neurons in the colon was counted per 2mm² at 3, 7 and 14 days in both sham and 5-FU treated mice. Grey column: sham-treated, black column: 5-FU-treated. Data represented as mean \pm S.E.M. ** $P < 0.01$, **** $P < 0.0001$, n = 6 per group/time point.

A Average Number of ChAT-IR Neurons in the Myenteric Plexus



B Proportion of ChAT-IR Neurons in the Myenteric Plexus



4.5 Discussion

This study is the first to examine 5-FU-induced enteric neuropathy and its effect on gastrointestinal function. Our results show that 5-FU administration causes a significant increase in gastrointestinal transit time at day 3, but delays in intestinal and gastric emptying at days 7 and 14. Significant reductions in the frequency and proportion of CMMCs, but increased frequency and proportions of short and fragmented contractions were prominent after 14 days of 5-FU treatment. All types of contractions were abolished by tetrodotoxin; a residual tonic constriction in the distal colon was blocked by hexamethonium in both sham and 5-FU-treated mice. A TTX-resistant tonic constriction of the proximal colon seen in 5-FU-treated mice was not blocked by hexamethonium. Severe crypt ablation and mucosal destruction occurred in the colon at day 3. Consistent crypt disorganisation and hypoplasia persisted throughout the experimental period; however mucosal barrier regeneration was evident after both 7 and 14 days. Acute inflammation, confirmed by increased levels of faecal lipocalin-2 and CD45 positive leukocytes in the colon, was seen on day 3 of 5-FU treatment but subsided by days 7 and 14. Loss of myenteric neurons was seen on days 7 and 14, accompanied by reduced numbers of ChAT-IR neurons at these time points.

Although 5-FU is routinely used in the treatment of CRC and is known to cause gastrointestinal side-effects, the neurological mechanisms underlying these side-effects have not been studied (Cao et al., 1994;Cao et al., 1996). Our study demonstrates that, while short-term 5-FU treatment accelerates overall gastrointestinal transit without a change in gastric emptying, more prolonged treatment results in significant delays to overall gastrointestinal transit, gastric emptying and intestinal emptying. Our findings are consistent with previous studies showing that short and long-term administration of the chemotherapeutic agent cisplatin differentially alters gastrointestinal transit (Cabezos et al., 2008;Cabezos et al., 2010). Our findings suggest that mechanisms underlying these differential changes in transit are different. Accelerated gastrointestinal transit observed after 3 days of treatment was

associated with acute intestinal inflammation and reduced proportions of nNOS-IR neurons. Intestinal inflammation was resolved after days 7 and 14 when delays to overall gastrointestinal transit, gastric and intestinal emptying, and colonic dysmotility were observed. The loss of myenteric neurons observed at these time points might contribute to the functional changes in the gastrointestinal tract. Gastric and intestinal transit has previously been investigated using radioactivity retention technique in rats where it was found that delayed gastric emptying and increased fundus and duodenum muscle contractions occurred 3 and 15 days after a single high dose of 5-FU (150mg/kg^{-1}) injection (Soares et al., 2008). However, our study is the first to use radiographic analysis to evaluate overall gastrointestinal transit alongside ENS damage following 5-FU administration in mice. Post-inflammatory delays to gastric and intestinal emptying have been reported in a plethora of gastrointestinal disorders (De Jonge et al., 2003; Van der Voort et al., 2003; Kindt et al., 2009). Gastric emptying of solids is significantly delayed in patients with post-infectious functional dyspepsia, irritable bowel syndrome, non-obstructive Crohn's disease and colitis (Grill et al., 1985; Annese et al., 1995; Van der Voort et al., 2003; De Schepper et al., 2007; Kindt et al., 2009).

Mucosal inflammation, epithelial degradation and intestinal ulceration, manifesting as mucositis, following 5-FU administration have been suggested as causes of gastrointestinal dysfunction (Duncan and Grant, 2003). Our results show that, while short-term 5-FU treatment was associated with destruction of the epithelial brush border and severe loss of colonic crypts and goblet cells, chronic treatment did not worsen epithelial damage. In fact regeneration of mucosa was evident during chronic 5-FU treatment, although crypts still appeared shorter, distended and disorganised. These results are consistent with previous findings in the rat duodenum following 3 days of 5-FU treatment, where loss of crypt architecture, shortening of villi and inflammatory infiltration in the lamina propria was reported (Soares et al., 2008). This was followed by the restoration of intestinal villi and crypts following 15 days of 5-FU treatment, but with sparse neutrophil infiltration in the muscular layer (Soares et al.,

2008). Such evidence indicates that duodenal mucosal inflammation resolves during 15 days of 5-FU treatment.

Both *in vitro* and *in vivo* treatment with 5-FU promote the attraction of inflammatory cells (Cottone et al., 2015). Intestinal inflammation is associated with gastrointestinal dysmotility both at the site of inflammation as well as at distant non-inflamed sites (Moreels et al., 2001; Akiho et al., 2005; O'Hara et al., 2007). Further to this a number of inflammatory mediators have been implicated in both short- and long-term smooth muscle contractility changes (Demedts et al., 2006). Accordingly, many gastrointestinal side-effects experienced during and after 5-FU administration have been attributed to inflammation-induced mucosal damage which we observed in the rat model of acute toxicity induced by a high dose (150 or 300 mg/kg⁻¹) of 5-FU (R Abalo, unpublished data) and in this study after a short-term treatment of mice with a low dose (23 mg/kg⁻¹) of 5-FU. However 5-FU-induced gastrointestinal dysmotility appears to outlast intestinal mucositis (Soares et al., 2008), indicating that a more complex and multifaceted pathophysiology is at play.

Our results show that chronic 5-FU administration results in significant neuronal loss in the myenteric plexus of the colon at days 7 and 14 when intestinal inflammation subsided. Our findings are consistent with previous studies investigating effects of platinum-based anti-cancer chemotherapeutics, which also demonstrated that long-term treatments induce loss of myenteric neurons (Vera et al., 2011; Wafai et al., 2013). However, 5-FU treatment induced less severe damage to myenteric neurons with 12% loss at day 14 compared to 25% of myenteric neuronal loss at day 14 of oxaliplatin treatment (Wafai et al., 2013). Although previous studies demonstrated that the loss of enteric neurons does not necessarily lead to changes in CMMCs (Ro et al., 2006; Barnes and Spencer, 2015) our results demonstrated that the loss of myenteric neurons correlated with colonic dysmotility in 5-FU-treated mice. The loss of myenteric neurons leads to long-term alterations in gastrointestinal functions as was shown in rats after cisplatin treatment (Vera et al., 2011) and mice after oxaliplatin

treatment (Wafai et al., 2013). 5-FU treatment inhibited CMMCs, but the total number of contractions of the isolated colon was significantly increased due to an increase in the number and proportion of short and fragmented contractions. Short and fragmented contractions play a vital role in the construction of productive motor patterns in the healthy intestine (Gwynne et al., 2004). Short distance contractions result in a segmenting motor pattern essential for mixing and absorption of colonic contents (Huizinga and Chen, 2014). Alteration in short segmenting contractions induces intestinal dysmotility (Fung et al., 2010).

Our results demonstrated that all types of contractions were neurogenic; residual tonic constriction in the distal colon was blocked by hexamethonium in both sham and 5-FU-treated mice. Tetrodotoxin-resistant tonic constriction in the proximal colon observed in 5-FU-treated mice was not blocked by hexamethonium. These results suggest that there is TTX-insensitive tonic excitatory drive to the smooth muscle that presumably involves at least one nicotinic synapse and hence must involve neurons with TTX-resistant action potentials like those described by Foong et al (2014) in the mouse submucosal plexus (Foong et al., 2014). This activity seems to be differentially sensitive to 5-FU. Thus, initial acute inflammation caused by 5-FU treatment may have led to persistent changes in the ENS and gut dysfunction. Similarly, in animal models of intestinal inflammation, long-term gut dysfunction persists long after resolution of acute inflammation due to neuronal loss, axonal damage and neuronal dysfunction in the myenteric plexus leading to changes in synaptic transmission between neurons and to the smooth muscles (Linden et al., 2003; Lomax et al., 2005; Krauter et al., 2007; Lomax et al., 2007; Nurgali et al., 2007; Nurgali et al., 2009; Nurgali et al., 2011).

We found that long-term treatment with 5-FU induced loss of both excitatory (ChAT-IR) and inhibitory (nNOS-IR) neurons in the myenteric plexus. However, only nNOS-IR neuron proportions were reduced at day 3 post 5-FU treatment, which is consistent with increased intestinal transit at this time point. Loss of nNOS-IR neurons was also observed in cisplatin and

oxaliplatin-treated animals (Vera et al., 2011;Wafai et al., 2013) however ChAT-IR neurons were not investigated in these studies. Thus, our results suggest that the loss of neurons after 5-FU treatment is not restricted to a specific neurochemically defined subpopulation. Differential effects of various chemotherapeutic compounds on enteric neurons might be attributed to the differences in their mechanisms of action with platinum-based compounds having direct neurotoxicity due to platinum adducts to DNA (Stojanovska et al., 2014) compared to inflammation-induced neuropathy associated with 5-FU treatment (Soares et al., 2008).

Damage to the intestinal smooth muscles, alterations in neuromuscular transmission, smooth muscle sensitivity to neurotransmitters and morphological and functional changes of interstitial cells of Cajal might be also involved in 5-FU-induced dysmotility and these require further investigation.

4.6 Conclusion

In conclusion, this study is the first to demonstrate that 5-FU administration induces accelerated gastrointestinal transit associated with acute intestinal inflammation at day 3 post treatment, which may have led to persistent changes in the ENS and gastrointestinal dysfunctions: delayed gastrointestinal transit, gastric and intestinal emptying, and colonic dysmotility. The loss of myenteric neurons observed at days 7 and 14 of treatment may contribute to the functional changes in the gastrointestinal tract. Further research investigating the effects of 5-FU on the electrophysiological properties of myenteric neurons is warranted.

CHAPTER FIVE: OXALIPLATIN-INDUCED ENTERIC NEURONAL LOSS AND GASTROINTESTINAL DYSFUNCTION IS ALLEVIATED BY CO-TREATMENT WITH BGP-15

The material presented in this chapter has been submitted for publication and has been reproduced here with minor alterations:

RM McQuade, V Stojanovska, JC Bornstein, AC Petersen, E Rybalka, K Nurgali (2017) Oxaliplatin-induced enteric neuronal loss and gastrointestinal dysfunction is prevented by co-treatment with BGP-15. Submitted to *British Journal of Pharmacology*.

5.1 Summary

Gastrointestinal side-effects of chemotherapy are under-recognised clinical hurdle and a cause of dose reduction, delays and cessation of treatment, presenting a constant challenge for efficient and tolerable anti-cancer treatment. Chemotherapy-induced nausea, vomiting, constipation and/or diarrhoea may persist up to 10 years post-treatment. We have previously found that oxaliplatin treatment results in intestinal dysfunction, oxidative stress and loss of enteric neurons. In this study we present evidence that BGP-15 alleviates oxaliplatin -induced enteric neuropathy and gastrointestinal dysfunction. Balb/c mice received oxaliplatin (3mg/kg/d) with and without BGP-15 (15mg/kg/d) tri-weekly intraperitoneally for 14 days. Gastrointestinal transit was analysed via *in vivo* x-ray imaging prior to and following 14 days of treatment. At day 14 colons were collected for assessment of *ex vivo* colonic motility, neuronal mitochondrial superoxide and cytochrome *c* levels as well as immunohistochemical analysis of myenteric neurons. Oxaliplatin-induced neuronal loss, increased proportion of neuronal nitric oxide synthase-immunoreactive neurons and increased levels of mitochondrial superoxide and cytochrome *c* in the myenteric plexus were attenuated by BGP-15 co-treatment. Significant delays in gastrointestinal transit, intestinal emptying and pellet formation, as well as impaired colonic motor activity, reduced faecal water content and lack of weight gain associated with oxaliplatin treatment were restored to sham levels in mice co-treated with BGP-15. Our results indicate for the first time that BGP-15 ameliorates oxidative stress, increases enteric neuronal survival and alleviates gastrointestinal dysfunction produced by oxaliplatin, which suggests that BGP-15 may relieve the gastrointestinal side-effects associated with chemotherapy.

Abbreviations

| | |
|---------------|--|
| ATP | adenosine triphosphate |
| CMMC | colonic migrating motor complex |
| CRC | colorectal cancer |
| DMSO | dimethyl sulfoxide |
| ENS | enteric nervous system |
| FC | fragmented contraction |
| FOLFOX | 5-fluorouracil, oxaliplatin and leucovorin |
| HSP | heat shock proteins |
| LMMP | longitudinal muscle-myenteric plexus |
| mETC | mitochondrial electron transport chain |
| mPTP | mitochondrial permeability transition pore |
| mtDNA | mitochondrial dna |
| nNOS | neuronal nitric oxide synthase |
| OXL | oxaliplatin |
| PAR | poly(ADP-ribose) |
| PARP | poly(ADP-ribose) polymerase |
| PBS | phosphate buffered saline |
| ROS | reactive oxygen species |
| SC | short contraction |

5.2 Introduction

To date, few studies have investigated the effects of platinum-based chemotherapeutics on enteric neurons in animal models (Vera et al., 2011;Wafai et al., 2013). These studies, alongside results presented in Chapter 2 reported significant reductions in the number of neurons alongside heightened proportion of neuronal nitric oxide synthase (nNOS) immunoreactive neurons and oxidative stress in the myenteric and submucosal plexus following *in vivo* treatment with cisplatin (Vera et al. 2011) and OXL (Wafai et al., 2013;McQuade et al., 2016b). These changes in the ENS are correlated with inhibition of gastrointestinal transit and colonic propulsion in cisplatin (Vera et al., 2011) and OXL-treated animals (Wafai et al., 2013;McQuade et al., 2016b) leading to symptoms of constipation or diarrhoea.

Identifying drugs that effectively prevent the toxic complications of chemotherapies is imperative to improve the efficacy of anti-cancer treatment and patient quality of life. BGP-15 ($C_{14}H_{22}N_4O_2 \cdot 2HCl$) is a pharmacological modulator of the cytoprotective response to stress, acting as a poly(ADP-ribose) polymerase (PARP)1 inhibitor (Sarszegi et al., 2012), heat shock protein (HSP) co-inducer (Ciocca et al., 1993;Costa et al., 1997) and mild anti-oxidant (Racz et al., 2002) in a variety of tissues; it is suggested to have neuroprotective properties in cisplatin- and taxol-induced peripheral sensory neuropathy in rats (Bardos et al., 2003). Multiple studies have shown that neutralisation of reactive oxygen species (ROS) and pharmacological inhibition or genetic inactivation of PARP is therapeutically effective in a wide range of cardiovascular, inflammatory, vascular, and neurodegenerative diseases, by protecting against cell death (Pacher et al., 2007). Overactivation of PARP1 and downstream products resulting in cellular energy (ATP) depletion, have been identified following OXL application in SGC-7901 human gastric cancer cells (Wu et al., 2015), whilst PARP1 inhibiting agents have been found to attenuate OXL-induced painful neuropathy (Ta et al., 2013). Similarly, increased expression of HSPs is associated with improved symptoms of peripheral neuropathy in diabetic

and chronic constriction injury of sciatic nerve in rats (Chen et al., 2012;Chen et al., 2013). Collectively these data suggest that BGP-15 is a potential therapeutic treatment for OXL-induced neurological side-effects.

In this study we tested if long-term *in vivo* BGP-15 co-treatment attenuates oxidative stress, enteric neuropathy and intestinal dysfunction induced by oxaliplatin treatment.

5.3 Material and Methods

5.3.1 Ethical Approval

All procedures were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

5.3.2 Animals

Male Balb/c mice aged 6-8 weeks (18 – 25 g) supplied from the Animal Resources Centre (Perth, Australia) were used for the experiments. Mice had free access to food and water and were kept under a 12-hour light/dark cycle in a well-ventilated room at an approximate temperature of 22°C. Mice acclimatised for a minimum of 5 days prior to the commencement of *in vivo* intraperitoneal injections. A total of 60 mice were used for this study.

5.3.3 Intraperitoneal Injections

Mice received intraperitoneal injections of OXL (Tocris Bioscience, UK) (3 mg/kg/dose) with or without BGP-15 (kindly donated by N-Gene R&D Inc., USA) (15mg/kg/dose) 3 times a week via a 26-gauge needle. OXL was dissolved in sterile water in order to make 10^{-2} M L⁻¹ stock solutions and refrigerated at -20°C. The stock was then defrosted and diluted with sterile water further to make 10^{-3} M L⁻¹ solutions for intraperitoneal injections. The

dose of OXL was calculated to be equivalent to standard human dose per body surface area (Reagan-Shaw et al., 2008; Renn et al., 2011). BGP-15 was dissolved in DMSO to make 0.1M L⁻¹ stock solution and refrigerated at -20°C. The stock was then defrosted and diluted with sterile water to make 0.01M L⁻¹ solutions for intraperitoneal injections. Sham-treated mice were divided into three groups: sham H₂O-treated mice received sterile water; sham DMSO-treated mice received 10% DMSO in sterile water; and sham DMSO+H₂O-treated mice received both pure sterile water as well as 10% DMSO in sterile water via intraperitoneal injection 3 times a week. The maximum injection volume did not exceed 200µL per injection for all groups. Combination treatments (OXL+BGP-15, H₂O+DMSO) were delivered no more than 5 minutes apart. *In vivo* studies of gastrointestinal transit were performed as described below. Body weight was measured daily. Mice were euthanized via cervical dislocation 14 days after the first injection. Colons were collected for *ex vivo* experiments.

5.3.4 Assessment of Mitochondrial Superoxide Production

MitoSOX™ Red M36008 (Invitrogen, Australia), was used to visualise mitochondrially-derived superoxide in wholemount preparations of submucosal and myenteric ganglia of the colon, as described in Chapter 2, section 2.3.4. Freshly excised distal colon preparations were dissected to expose submucosal and myenteric ganglia. Preparations were incubated in oxygenated physiological saline with MitoSOX™ Red M36008 (5 µM) in a gently shaking incubator Unimax 1010 (Heidolph Instruments, Germany) at a constant temperature of 37°C for 40 min. Tissues were washed (2 x 30 min) with oxygenated physiological saline (composition in mM: NaCl 118, KCl 4.6, CaCl₂ 3.5, MgSO₄ 1.2, NaH₂PO₄ 1, NaHCO₃ 25, d-Glucose 11; bubbled with 95%O₂ and 5% CO₂) and fixed in 4% paraformaldehyde overnight at 4°C. The following day tissues were washed (2 x 30 min) with physiological saline and mounted on glass slides with DAKO fluorescent mounting medium for imaging under a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan). All images were captured using identical acquisition exposure-time conditions, calibrated to standardised minimum

baseline fluorescence and converted to binary. Changes in fluorescence from baseline were measured in arbitrary units (arb. units) using Image J software (NIH, MD, USA). The corrected total fluorescence was calculated in 32 5x5µm² boxes within myenteric ganglia from each preparation to exclude fluorescence outside the ganglia as previously described (Burgess et al., 2010;McQuade et al., 2016b).

5.3.5 Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential changes in cells may be detected with the use of cationic, lipophilic JC-10 dye, as described in Chapter 2, section 2.3.5. In normal cells, JC-10 concentrates in the mitochondrial matrix where it forms red fluorescent aggregates (JC aggregates). In contrast, apoptotic cells stain in green fluorescent colour (JC monomeric form) due to the JC-10-labelled release of cytochrome c diffusing out of the mitochondria as a result of mitochondrial depolarisation and increased permeability. JC-10 fluorescent mitochondrial membrane potential microplate assay kit (Abcam, MA, USA) was used to detect mitochondrial membrane potential changes and the release of cytochrome c from damaged mitochondria in the myenteric ganglia of the distal colon. Freshly excised distal colon preparations from sham and OXL-treated mice were bathed in oxygenated physiological saline and dissected to expose the myenteric ganglia. Immediately following dissection, preparations were incubated for 20 min with 500µL of JC-10 dye solution (buffer A) with gentle agitation (Unimax 1010, Heidolph Instruments, Germany) at a constant temperature of 37°C. After 20 min, 500µL of buffer B solution was added to tissue preparations and allowed to incubate for a further 20 min in the same conditions as we have previously described (McQuade et al., 2016b). Immediately following the final incubation, tissues were mounted on glass slides with DAKO fluorescent mounting medium for imaging under a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan). All images were captured at identical acquisition exposure-time conditions, calibrated to standardised minimum baseline fluorescence and converted to binary. Changes in fluorescence from baseline were measured in arbitrary units (arb. units) using Image J

software (NIH, MD, USA). The corrected total fluorescence was calculated in 32 5x5µm² boxes within myenteric ganglia from each preparation to exclude fluorescence outside the ganglia as previously described

5.3.6 Immunohistochemistry

Distal colon segments (length 2-3 cm) were placed in oxygenated phosphate-buffered saline (PBS) (pH 7.2) containing nicardipine (3 µM) (Sigma-Aldrich, Australia) for 20 mins to inhibit smooth muscle contractions. Samples were dissected along the mesenteric border, cleared of their contents, maximally stretched and the mucosa was removed to expose the myenteric plexus attached to the longitudinal muscle layer. Tissues were fixed with Zamboni's fixative (2% formaldehyde, 0.2% picric acid) overnight at 4°C. Preparations were cleared of fixative by washing 3 x 10 min with DMSO followed by 3 x 10 min washes with PBS. Fixed tissues were stored at 4°C in PBS for a maximum of 5 days.

Wholemout preparations were incubated with 10% normal donkey serum (Chemicon, USA) for 1 hour at room temperature. Tissues were then washed (2 x 5 mins) with PBS and incubated with primary antibodies against β Tubulin III (βTubIII) (chicken, 1:1000, Abcam, MA, USA) and neuronal nitric oxide synthase (nNOS) (goat, 1:500, Abcam, MA, USA) overnight at 4°C. Tissues were then washed in PBS (3 x 10 min) before incubation with species-specific secondary antibodies labelled with different fluorophores: donkey anti-chicken Alexa 594 (1:200, Jackson Immuno research Laboratories, PA, USA) and donkey anti-goat Alexa 488 (1:200, Jackson Immuno research Laboratories, PA, USA) for 2 hours at room temperature. The preparations were given 3 x 10 min final washes in PBS and then mounted on glass slides using fluorescent mounting medium (DAKO, Australia). From each preparation 8 randomly chosen images (total area 2 mm²) were captured with a 20x objective and processed using NIS Elements software (Nikon, Japan). The number of βTubIII and nNOS immunoreactive neurons was quantified in the myenteric ganglia of each preparation. Quantitative analyses were conducted blindly.

5.3.7 Imaging

Three dimensional (z-series) images of wholemount preparations were taken using a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan), as described in Chapters 2, 3 and 4, sections 2.3.8, 3.3.7 and 4.3.10. Eight randomly chosen three dimensional (z-series) images from each preparation were captured with a 20 x objective and processed using NIS Elements software (Nikon, Japan). Fluorophores were visualized using excitation filters for Alexa 594 Red (excitation wavelength 559nm), Alexa 488 (excitation wavelength 473nm), and Alexa 405 (excitation wavelength 405nm). Z-series images were taken at step size of 1.75 μm (1600 x 1200 pixels).

5.3.8 Gastrointestinal Transit

Gastrointestinal transit was studied by x-ray as described in Chapters 3 and 4, sections 3.3.4 and 4.3.4, prior to the first treatment (day 0) and after 14 days of treatment. The contrast agent, 0.4 mL of suspended barium sulfate (X-OPAQUE-HD, 2.5g/mL), was administered via oral gavage. Prior to x-ray imaging, animals were trained/conditioned for oral gavage using 0.9% w/v saline (volume 0.1-0.4 ml); this was repeated at least 3 times for each animal with at least 24 hours of rest period between each training. Radiographs of the gastrointestinal tract were performed using a HiRay Plus Porta610HF x-ray apparatus (JOC Corp, Kanagawa, Japan; 50 kV, 0.3 Milliampere second (mAs), exposure time 60 ms). Mice were immobilised in the prone position inside a transparent plastic restraint tube with partly open front side for breathing; the tube comfortably restrains animal movement for the maximum of 1-2 min essential for successful x-ray imaging. The training/conditioning with restraint were done by placing the restrainer into the mouse cages at least 24 hours prior to the x-ray procedure. X-rays were captured using Fujifilm cassettes (24x30 cm) immediately after administration of barium sulfate (T0) every 5 minutes for the first hour, every 10 minutes for the second hour, then every 20 minutes through to 360 minutes (T360). Animals were closely monitored during and

after all procedures. Images were developed via a Fujifilm FCR Capsula XLII and analysed using eFilm 4.0.2 software. The rate of gastrointestinal transit was inferred from the time (in minutes) taken for barium sulfate to reach each region of the gastrointestinal tract (stomach, small intestines, caecum, and large intestines), longer transit times indicated lower rate of transit. Organ emptying was calculated as the time taken for complete barium emptying from specific gastrointestinal regions (stomach, small intestines) (Cabezos et al., 2008; Cabezos et al., 2010; Girón et al., 2015).

5.3.9 Colonic Motility Experiments

The entire colon was removed from day 14 sham and 5-FU-treated mice and set up in organ-bath chambers to record motor patterns *in vitro* (Wafai et al., 2013), as described in Chapters 2, 3 and 4, sections 2.3.12, 3.3.8 and 4.3.5. Briefly, the colon was placed into warmed (35°C), oxygenated physiological saline until the faecal pellets were expelled. The empty colon was cannulated at both ends and arranged horizontally in organ-bath chambers. The proximal end of the colon was connected to a reservoir containing oxygenated physiological saline to maintain intraluminal pressure. The distal end was attached to an outflow tube that provided a maximum of 2cm H₂O back-pressure. Organ baths were continuously superfused with oxygenated physiological saline solution and preparations were left to equilibrate for 30 min. Contractile activity of each segment was recorded with a Logitech Quickcam Pro camera positioned 7–8 cm above the preparation. Videos (2x20 min) of each test condition were captured and saved in *avi* format using VirtualDub software (version 1.9.11).

Colonic migrating motor complexes (CMMCs) were defined as propagating contractions directed from the proximal to the distal end of the colon which travelled more than 50% of the colon length (Roberts et al., 2007; Roberts et al., 2008). Contractions that propagated less than 50% of the colon length were considered to be short contractions (SCs). Another form of incomplete contraction was identified as fragmented contractions (FCs) occurring simultaneously at different parts of the colon rather than propagating over

the length of the colon (McQuade et al., 2016a). Recordings were used to construct spatiotemporal maps using in-house edge detection software (Gwynne et al., 2004). Spatiotemporal maps plot the diameter of the colon at all points during the recording allowing contractile motor patterns to be analysed with Matlab software (version 12).

5.3.10 Faecal Water Content and Colonic Faecal Content

Faecal water content and colonic faecal content were analysed as described in Chapter 3, section 3.3.10. Briefly, the wet weight of faecal pellets was measured following 14 days *in vivo* OXL administration. Individual mice were placed in holding cages for a period of 15 minutes on 3 separate occasions throughout the day; the first 5 pellets expelled from every mouse at each holding time were collected and weighed immediately to determine wet pellet weight. Pellets were then dehydrated for 72 hours at room temperature prior to measurement of the dry weight. Water content was calculated as the difference between the wet weight and dry weight.

5.3.11 Statistical Analysis

Data were assessed using two-way ANOVA, Welch's two-tailed *t* test and Student's two-tailed *t* test. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., CA, USA). Data are presented as mean \pm standard error of the mean (SEM). Value differences were considered statistically significant at $P < 0.05$.

5.4 Results

Three vehicle control groups were used in this study: 1) sterile water (H₂O) as the vehicle for the OXL-treated group, 2) 10% DMSO as the vehicle for the BGP-15-treated group and 3) DMSO +H₂O as the vehicle for the OXL+BGP-15-treated group. No differences were found between these vehicle-treated groups in any measured parameters.

5.4.1 Co-administered BGP-15 prevents OXL-induced elevation of mitochondrial superoxide and changes to mitochondrial membrane potential

To evaluate production of reactive oxygen species (ROS), wholemount preparations of the myenteric plexus were probed with a fluorescent mitochondrial superoxide marker MitoSOX™ Red M36008 (**Figure 5.1**). MitoSOX fluorescence was increased in the myenteric plexus of OXL-treated mice (38±3 arbitrary units) (**Figures 5.1, 5.2**) compared to sham H₂O-treated animals (28±3 arbitrary units, $P<0.05$) and BGP-15-treated mice (20±1 arbitrary units, $P<0.001$) ($n=5$ mice/group, **Figure 5.2**). Treatment with BGP-15 alone had no effect on MitoSOX fluorescence when compared to sham DMSO-treated mice ($n=5$ mice/group, **Figure 5.2**). When given in combination with OXL, however, BGP-15 significantly reduced MitoSOX fluorescence (21±2 arbitrary units, $P<0.01$) compared to OXL-treated mice ($n=5$ mice/group, **Figure 5.2**) and there was no significant difference between this value and those from sham H₂O, sham DMSO or sham H₂O+DMSO-treated mice ($n=5$ mice/group, **Figure 5.2**).

Depletion of the inner mitochondrial membrane potential ($\Delta\Psi$) is a precursor to cytochrome c diffusion from the mitochondria and subsequently apoptosis, and can be measured via fluorescence (green) of monomeric JC-10 (**Figure 5.3**). A significant increase in JC-10 fluorescence was found in the myenteric plexus of OXL-treated mice (51±3 arbitrary units) (**Figures 5.3, 5.4**) compared to sham H₂O-treated animals (14±2 arbitrary units, $P<0.0001$) and BGP-15-treated mice (11±1 arbitrary units, $P<0.0001$) ($n=5$ mice/group, **Figure 5.4**). Treatment with BGP-15 alone had no effect on JC-

10 fluorescence when compared to sham DMSO-treated mice (n=5 mice/group, **Figure 5.4**). When given together with OXL, BGP-15 significantly reduced JC-10 fluorescence (21 ± 2 arbitrary units, $P < 0.0001$) compared to OXL-treated mice (n=5 mice/group, **Figure 5.4**), but the level of JC-10 fluorescence was higher in OXL+BGP-15-treated than in sham H₂O+DMSO-treated mice (11 ± 1 arbitrary units, $P < 0.01$) (n=5 mice/group, **Figure 5.4**). No significant difference in JC-10 fluorescence was found between sham H₂O, sham DMSO and sham H₂O+DMSO-treated mice (n=5 mice/group, **Figure 5.4**).

Figure 5.1 Mitochondrial superoxide in the myenteric plexus following repeated *in vivo* OXL+/- BGP-15 administration. Fluorescent wholemount preparations of myenteric neurons labelled with MitoSOX™ Red M36008 in the colons from H₂O (**A**), DMSO (**B**), DMSO+H₂O (**C**), OXL (**D**), BGP-15 (**E**) and OXL+BGP-15-treated (**F**) mice, scale bar = 50µm.

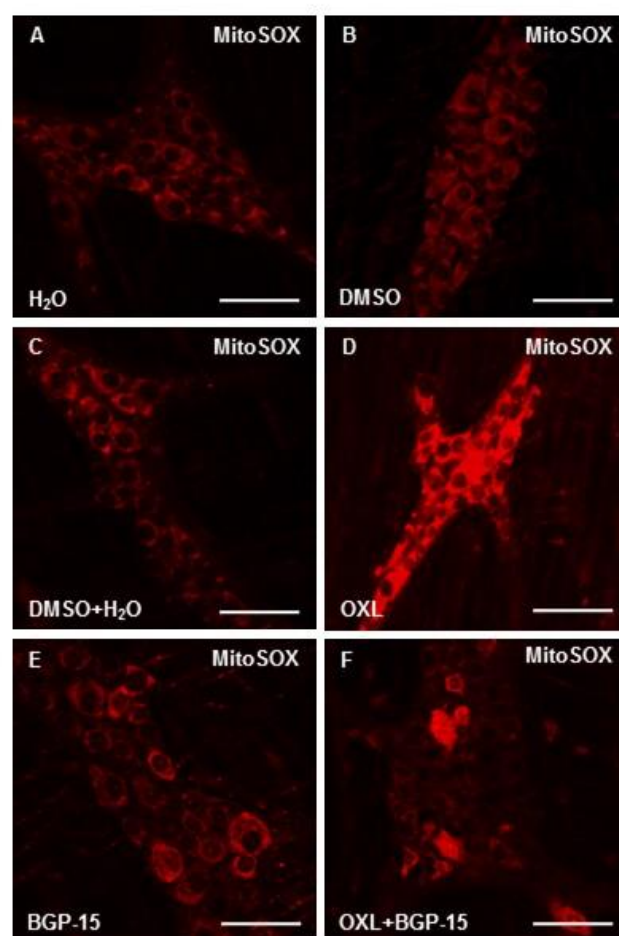


Figure 5.2 Mitochondrial superoxide fluorescence in the myenteric plexus following repeated *in vivo* OXL+/- BGP-15 administration.

Quantification of the levels of MitoSOXTM Red M36008 production visualised by fluorescent probe in myenteric ganglia in colonic preparations from H₂O, DMSO, DMSO+H₂O, OXL, BGP-15 and OXL+BGP-15-treated mice. Data represented as mean \pm S.E.M.

* $P < 0.05$ significantly different to sham, $^{**}P < 0.01$, $^{***}P < 0.001$ significantly different to OXL, n = 5 mice/group

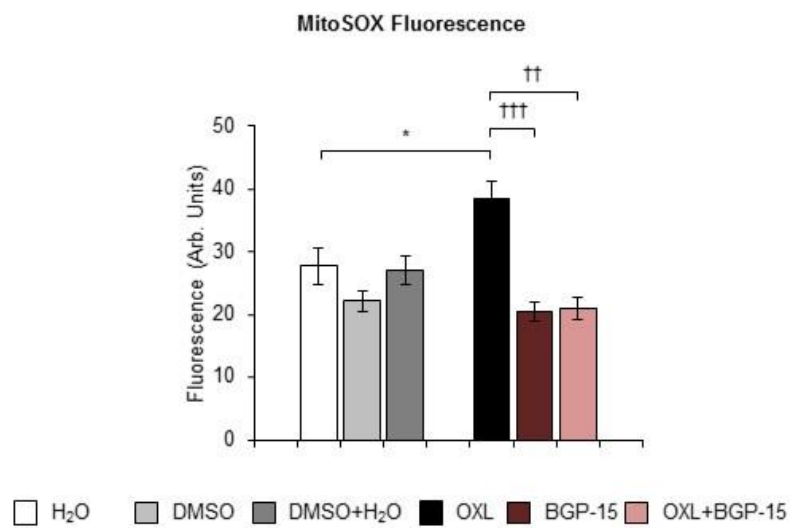


Figure 5.3 Mitochondrial membrane potential in the myenteric plexus following repeated *in vivo* OXL+/- BGP-15 administration.

Fluorescent wholemount preparations of myenteric neurons labelled with monomeric JC-10 in the colons from H₂O (**A**), DMSO (**B**), DMSO+H₂O (**C**), OXL (**D**), BGP-15 (**E**) and OXL+BGP-15-treated (**F**) mice, scale bar = 50µm.

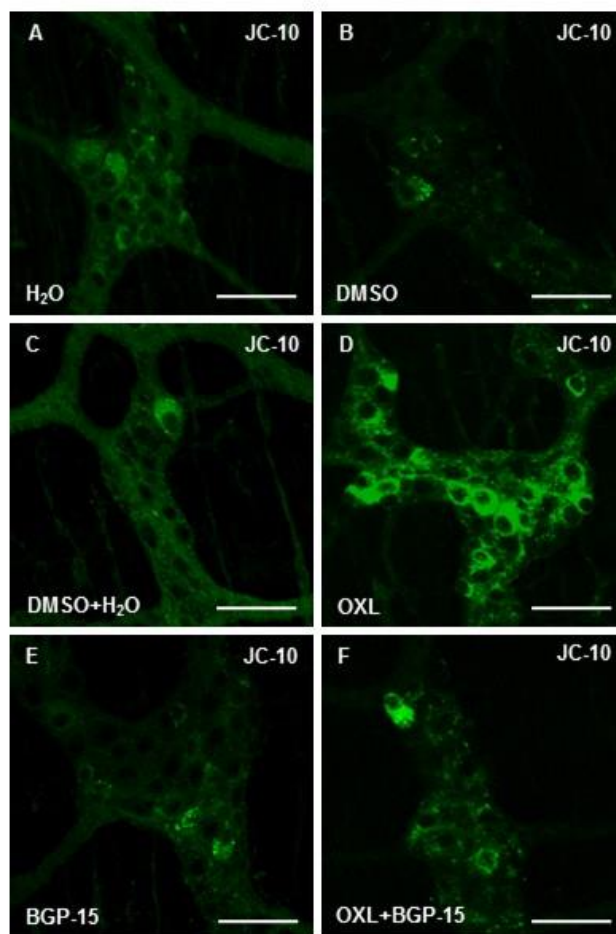
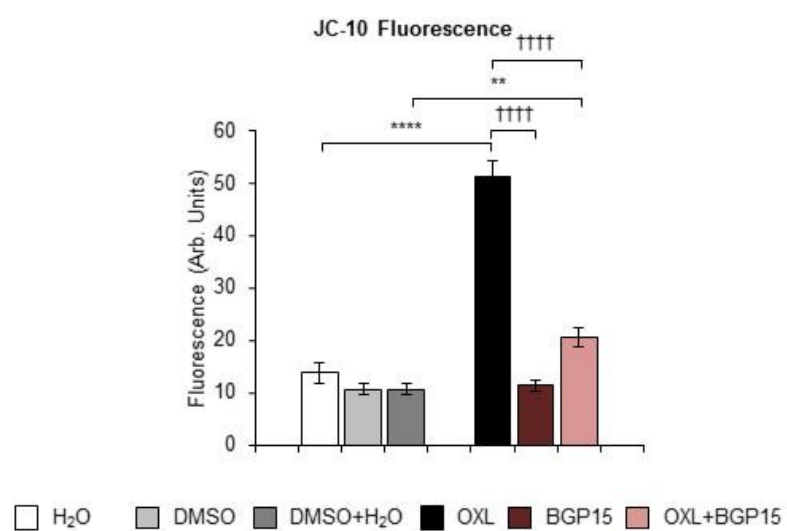


Figure 5.4 Monomeric JC-10 fluorescence in the myenteric plexus following repeated *in vivo* OXL+/- BGP-15 administration.

Quantification of the levels of monomeric JC-10 production visualised by fluorescent probe in myenteric ganglia in colonic preparations from H₂O, DMSO, DMSO+H₂O, OXL, BGP-15 and OXL+BGP-15-treated mice. Data represented as mean \pm S.E.M. ** P <0.01, **** P <0.0001 significantly different to sham, ††† P <0.0001 significantly different to OXL, n = 5 mice/group.



5.4.2 BGP-15 prevents OXL-induced enteric neuronal loss and reduces the proportion of nNOS-IR neurons

To investigate changes in the total number of myenteric neurons, wholemount preparations of colon collected at day 14 were labelled with pan neuronal marker anti- β Tubulin III for neuronal cell counting within a 2mm^2 area (**Figure 5.5**). Repeated *in vivo* administration of OXL induced significant myenteric neuronal loss (721 ± 8 neurons/area) when compared to sham H₂O-treated mice (1262 ± 34 , $P<0.0001$, $n=5$ mice/group) (**Figure 5.6**). BGP-15 treatment alone had no effect on the number of myenteric neurons (1214 ± 41) when compared to sham DMSO-treated mice (1229 ± 2), however when given in combination with OXL, BGP-15 protected against neuronal loss (1109 ± 78), with the number of neurons in the OXL+BGP-15-treated group significantly higher than in the OXL-treated group ($P<0.01$, $n=5$ mice/group). The number of neurons in OXL+BGP-15-treated mice was comparable to sham and did not differ significantly from any of the sham H₂O, DMSO or DMSO+H₂O (1142 ± 44) treated groups (**Figure 5.6**).

To determine if neuronal loss was associated with changes in a particular subpopulation of neurons, the effects of BGP-15 co-treatment on the nNOS-IR inhibitory motor and interneurons in the myenteric plexus in the colon were analysed (**Figure 5.7**). The proportion of nNOS-IR neurons in the myenteric plexus of the colon from OXL-treated mice ($48\pm 1.0\%$) was substantially increased when compared to sham H₂O-treated mice ($40\pm 0.2\%$, $P<0.01$) (**Figure 5.8**). The proportion of nNOS-IR neurons in BGP-15 treated mice ($41\pm 1.5\%$) was significantly lower than in OXL-treated mice ($48\pm 1.0\%$, $P<0.01$) but significantly higher than DMSO-treated mice ($31\pm 0.9\%$, $P<0.05$), and was comparable to sham H₂O and sham DMSO+H₂O-treated mice (**Figure 5.8**). When administered together with OXL, BGP-15 significantly reduced the proportion of nNOS-IR neurons ($23\pm 3\%$) when compared to sham DMSO+H₂O-treated mice ($36\pm 1.8\%$, $P<0.01$), OXL-treated mice ($48\pm 1.0\%$, $P<0.0001$) and BGP-15-treated mice ($41\pm 1.5\%$, $P<0.01$) (**Figure 5.8**).

Figure 5.5 Wholemount preparations of β Tub III-IR myenteric neurons following repeated *in vivo* OXL+/- BGP-15 administration.

β Tub III-IR neurons (red) with visible cell bodies (arrows) in the myenteric plexus of the distal colon in H₂O (**A**), DMSO (**B**), DMSO+H₂O (**C**), OXL (**D**), BGP-15 (**E**) and OXL+BGP-15-treated (**F**) mice, scale bar = 50 μ m.

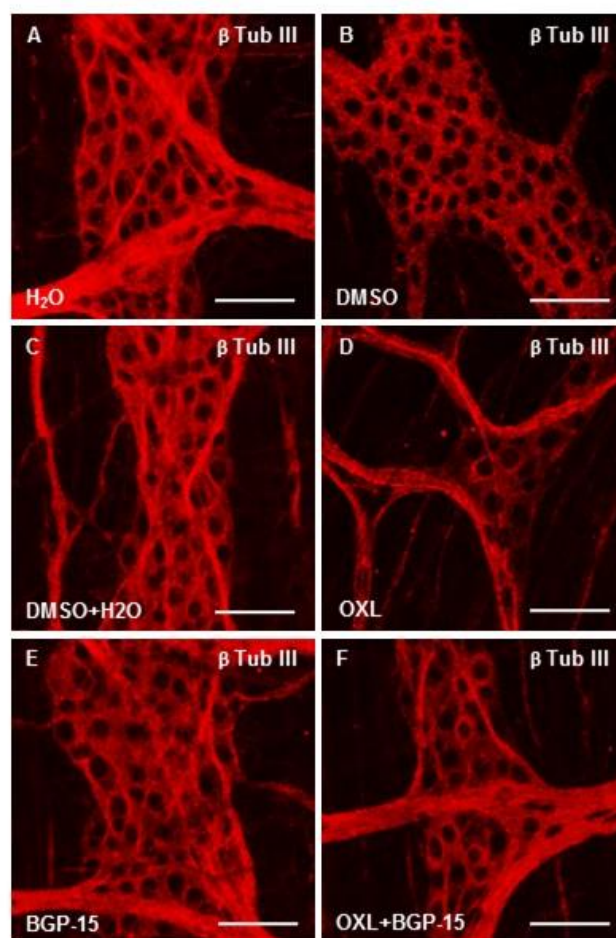


Figure 5.6 Effect of repeated *in vivo* OXL+/- BGP-15 administration number of myenteric neurons. Total number of β Tub III-IR neurons in the colon was counted per 2mm² at 14 days in H₂O, DMSO, DMSO+H₂O, OXL, BGP-15 and OXL+BGP-15-treated mice. Data represented as mean \pm S.E.M. **** P <0.0001 significantly different to sham, †† P <0.01, ††† P <0.001 significantly different to OXL, n = 5 mice/group.

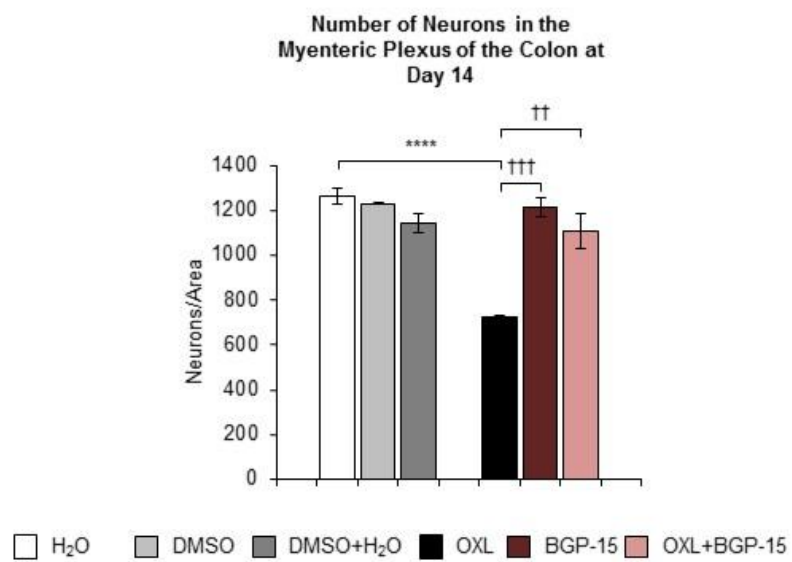


Figure 5.7 Wholemout preparations of nNOS-IR myenteric neurons following repeated *in vivo* OXL+/- BGP-15 administration. nNOS-IR neurons (green) (arrows) in the myenteric plexus of the distal colon in H₂O (**A**), DMSO (**B**), DMSO+H₂O (**C**), OXL (**D**), BGP-15 (**E**) and OXL+BGP-15-treated (**F**) mice, scale bar = 50µm.

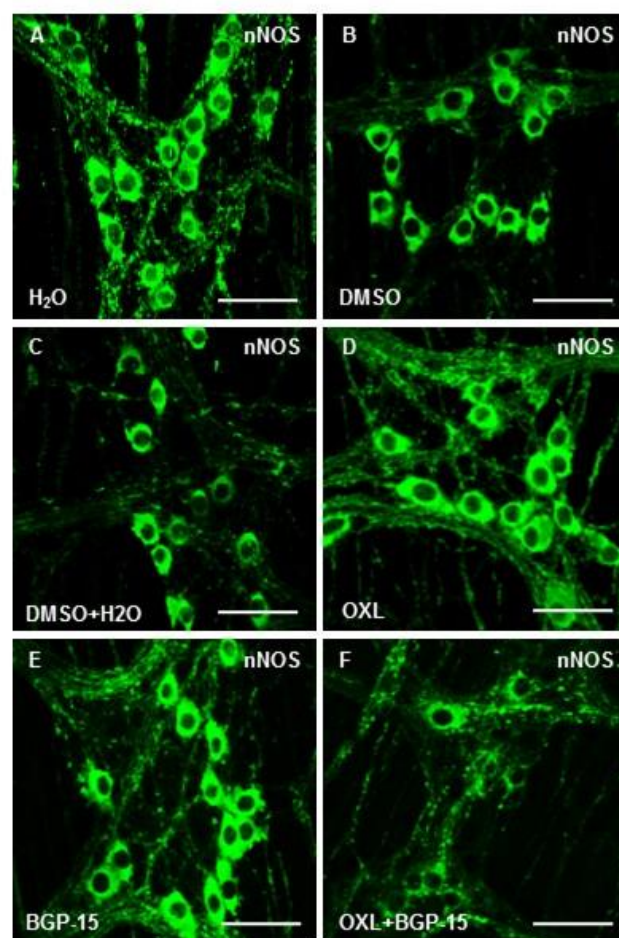
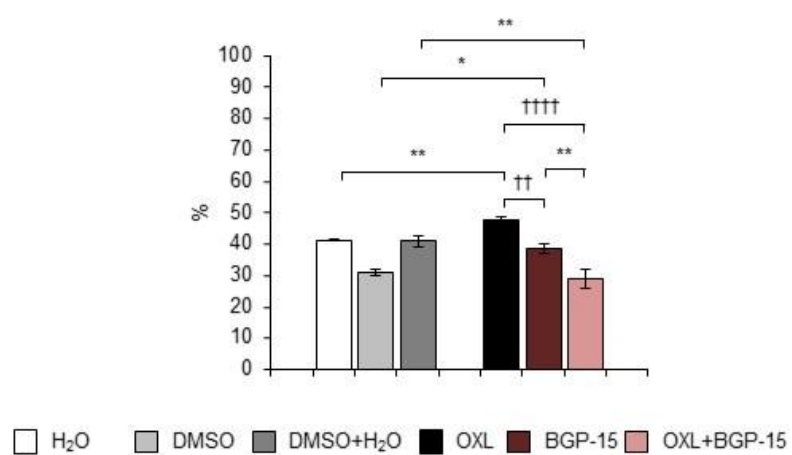


Figure 5.8 Effect of repeated *in vivo* OXL+/- BGP-15 administration on average number and proportion of nNOS-IR myenteric neurons.

Proportion of nNOS-IR neurons to the total number of β Tub III -IR myenteric neurons in the colon was counted at 14 days in H₂O, DMSO, DMSO+H₂O, OXL, bGP-15 and OXL+BGP-15-treated mice. Data represented as mean \pm S.E.M. * P <0.05, ** P <0.01 significantly different to sham, †† P <0.01, ††† P <0.0001 significantly different to OXL, n = 5 mice/group.

Proportion of nNOS-IR Neurons in the
Myenteric Plexus of the Colon at
Day 14



5.4.3 Co-administration of BGP-15 with OXL alleviates OXL-induced colonic dysmotility

To investigate the effects of OXL treatment on colonic motility, excised colons were studied in organ bath experiments at day 14 of OXL treatment with and without BGP-15 (n=5 mice/group) (**Figure 5.9**). Analysis of spatiotemporal maps from OXL-treated mice showed a significant decrease in the total number of contractions compared to sham H₂O-treated mice (**Figure 5.10A**) (**Table 5.1**). BGP-15 treatment alone did not affect the total number of contractions compared to sham DMSO-treated mice (**Figure 5.10A, Table 5.1**). When given in combination with OXL, BGP-15 alleviated OXL-induced inhibition of contraction, but the total number of contractions remained below the level of sham DMSO+H₂O -treated mice (**Figure 5.10A, Table 5.1**).

Treatment with OXL significantly decreased the proportion and frequency of CMMCs when compared to sham H₂O-treated mice (**Figures 5.10C, 11B, Table 5.1**). BGP-15 treatment alone did not affect either the proportion or the frequency of CMMCs when compared to sham DMSO-treated mice (**Figures 5.10C, 11B, Table 5.1**). When given together with OXL, BGP-15 increased the proportion and frequency of CMMCs, but these were still decreased when compared to H₂O+DMSO (**Figures 5.10C, 5.11B, Table 5.1**).

Treatment with OXL did not significantly alter the proportion or frequency of SCs but increased the proportions of FCs when compared to sham H₂O-treated mice (**Figures 5.10C, 5.11B, Table 5.1**). BGP-15 treatment alone also had no effect on the proportion or frequency of either SCs or FCs when compared to either sham DMSO-treated mice or OXL-treated mice (**Figures 5.10C, 5.11B, Table 5.1**). When given in combination with OXL, BGP-15 treatment increased the frequency of SCs when compared to OXL-treated mice (**Figure 5.10B, Table 5.1**) and increased the proportion of SCs when compared to H₂O+DMSO-treated mice (**Figures 5.10C, 5.11B, Table 5.1**). OXL+BGP-15 treatment reduced the proportion of FCs when compared to

OXL-treated mice, however this proportion did not differ from H₂O+DMSO-treated mice (**Figures 5.10C, 5.11B, Table 5.1**).

Figure 5.9 Colonic motility following repeated *in vivo* OXL+/- BGP-15 administration. Representative spatiotemporal maps generated from digital video recordings of colonic motility following 14 days of H₂O (A), DMSO (B), DMSO+H₂O (C), BGP-15 (D), OXL (E) and OXL+BGP-15 (F) administration. Each contraction can be seen as a reduction in the gut width (red), while relaxation as an increase in the gut width (blue). Colonic migrating motor complexes (CMMCs) propagate >50% of the colon length, short contractions (SCs) propagate <50% of the colon length and fragmented contractions (FCs) are interrupted by period(s) of relaxation during contraction.

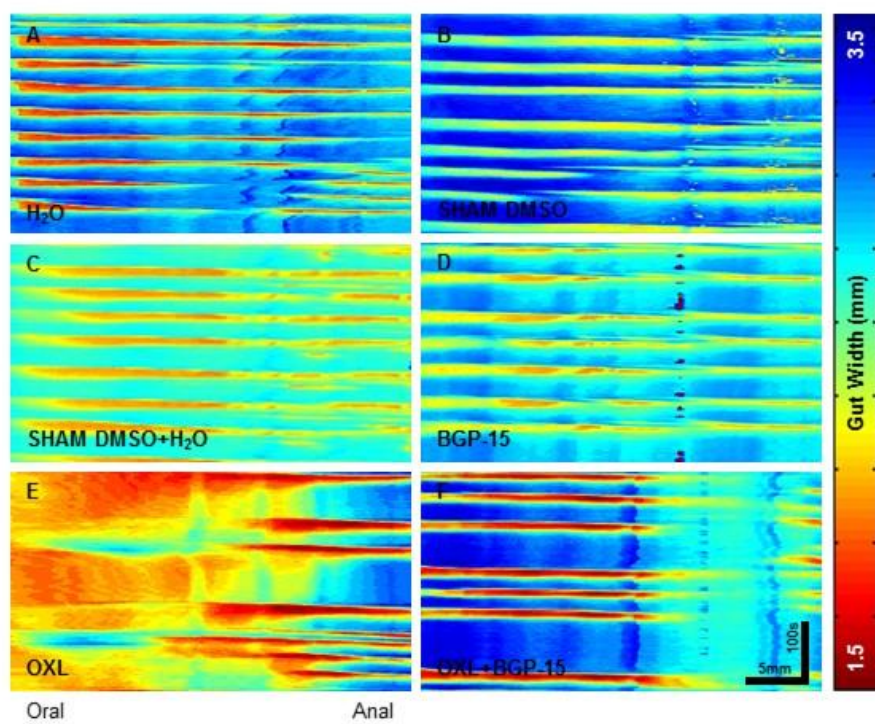


Figure 5.10 Total number and proportion of different contractions following repeated *in vivo* OXL+/- BGP-15 administration. Total number of contractions including all types of contractile activity in the colons from H₂O, DMSO, DMSO+H₂O, BGP-15, OXL, and OXL+BGP-15-treated mice (**A**). The proportion of CMMCs to the total number of contractions (**B**). The proportion of SCs to the total number of contractions (**C**). The proportion of FCs to the total number of contractions (**D**). Data represented as mean \pm S.E.M. * P <0.05, *** P <0.001 significantly different to sham, † P <0.05, †† P <0.01 significantly different to OXL, n = 5 per group/time point.

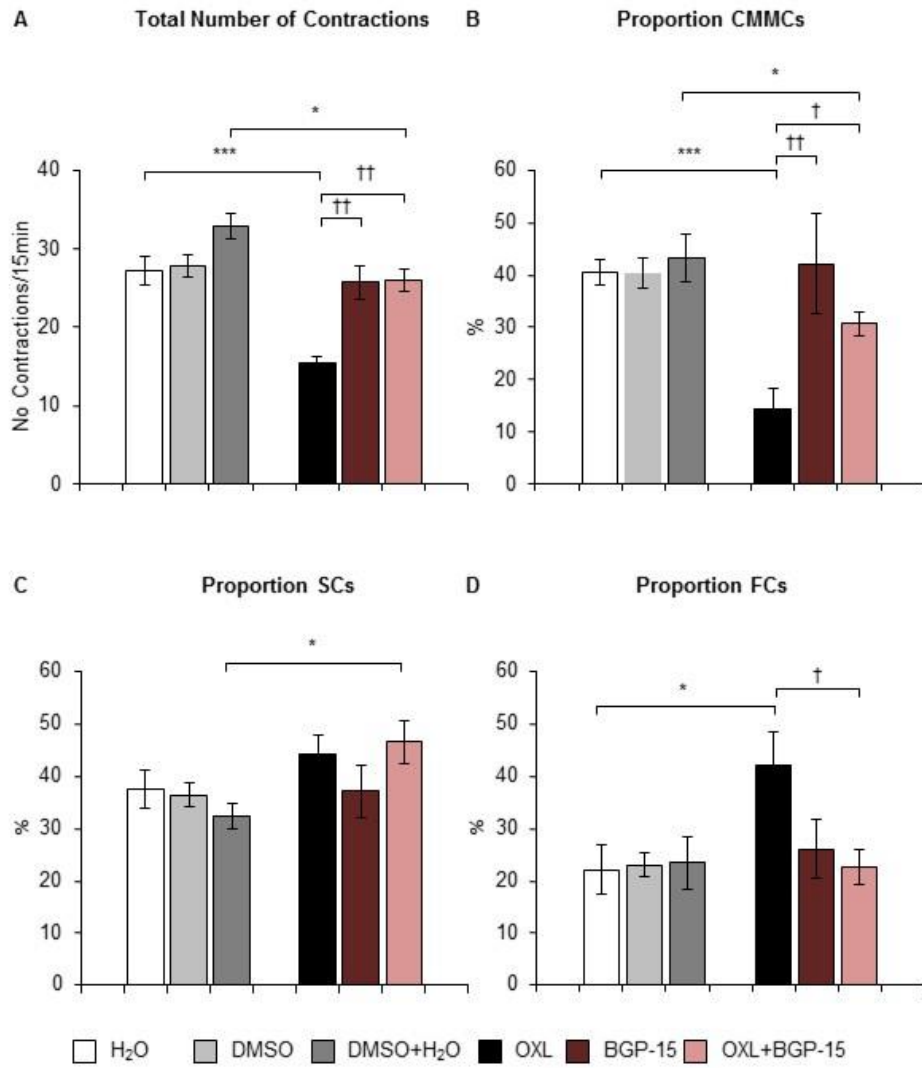


Figure 5.11 Frequency of different contractions following repeated *in vivo* OXL+/- BGP-15 administration. Number of CMMCs per 15 minutes in the colons from H₂O, DMSO, DMSO+H₂O, OXL, BGP-15 and OXL+BGP-15-treated mice (**A**). Number of SCs per 15 minutes in the colons from H₂O, DMSO, DMSO+H₂O, OXL, BGP-15 and OXL+BGP-15-treated mice (**B**). Number of FCs per 15 minutes in the colons from H₂O, DMSO, DMSO+H₂O, OXL, BGP-15 and OXL+BGP-15-treated mice (**C**). Data represented as mean \pm S.E.M. ** P <0.01 significantly different to sham, †† P <0.01, ††† P <0.001 significantly different to OXL, n = 5 mice/group.

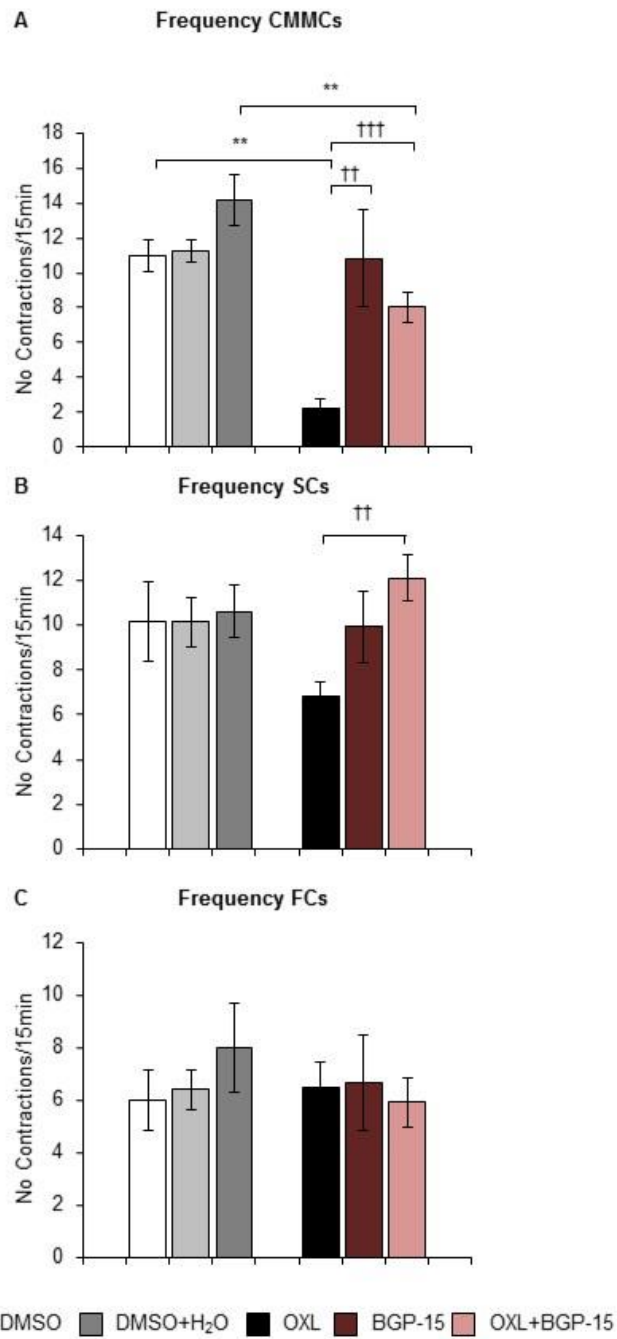


Table 5.1 Colonic motility following 14 days of treatment

| Parameters measured | | H ₂ O | DMSO | DMSO+H ₂ O | OXL | BGP-15 | OXL+BGP-15 |
|---|----------------------------------|------------------|--------|-----------------------|---------------|--------------|--------------------|
| Frequency (Number of Contractions/15 min) | Total Number Contractions | 28±1.8 | 28±1.4 | 33±1.6 | 15±0.8 *** | 26±2.2 †† | 26±1.0 * †† |
| | CMMCs | 11±0.9 | 11±0.6 | 14±1.5 | 2±0.6 ** | 11±2.8 †† | 8±0.9 ** ††† |
| | SCs | 10±1.8 | 10±1.1 | 11±1.1 | 7±0.7 | 9±1.5 | 12±1.0 †† |
| | FCs | 6±1.2 | 6±0.8 | 8±1.7 | 7±1.0 | 7±1.8 | 6±0.9 |
| Proportion (%) | CMMCs | 40±2.5 | 40±3.0 | 43±4.5 | 14±4.0 *** | 42±9.7 †† | 31±2.3 * † |
| | SCs | 37±3.7 | 36±2.2 | 32±2.5 | 44±3.7 | 37±5.1 | 47±4.0 * |
| | FCs | 22±4.8 | 23±2.3 | 23±5.1 | 42±6.1 * | 26±5.6 | 23±3.3 † |

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, significantly different to sham-treated group. † $P<0.05$, †† $P<0.01$, ††† $P<0.001$, significantly different to OXL-treated group (n=5 mice/group).

5.4.4 BGP-15 co-treatment largely restores gastrointestinal transit

To determine the effects of OXL treatment with and without BGP-15 on gastrointestinal transit, a series of radiographic images were used to track barium sulfate throughout the gastrointestinal tract before treatment (day 0/control), and after 6 injections (day 14) (n=5 mice/group) (**Figure 5.12**). The speed of barium movement was calculated by tracing barium entry from one part of the gastrointestinal tract to the next.

Following 14 days of OXL administration, movement of barium through the caecum and colon, gastric emptying, intestinal emptying and pellet formation were all significantly delayed when compared to control mice (**Figure 5.13A-D, Table 5.2**). Treatment with BGP-15 alone increased the speed of barium movement through the caecum and colon, but had no effect on intestinal emptying, gastric emptying or pellet formation when compared to sham DMSO-treated mice (**Figure 5.13A-D, Table 5.2**). When given in combination with OXL, BGP-15 treatment alleviated the delay in barium movement through the caecum and colon and significantly increased the speed of intestinal emptying and pellet formation when compared to OXL-treated mice, but had no effect on gastric emptying (**Figure 5.13A-D, Table 5.2**). No significant differences in the speed of barium movement through the caecum and colon, gastric emptying, intestinal emptying or pellet formation were found when comparing control, sham DMSO and H₂O+DMSO-treated mice (**Figure 5.13A-D, Table 5.2**)

Table 5.2 Speed of transit and emptying following 14 days of treatment

| Parameters measured | | Day 0 | DMSO | DMSO+H ₂ O | OXL | BGP-15 | OXL+BGP-15 |
|---|----------------------------|-------|------|-----------------------|----------------|--------------|----------------------|
| Speed of transit (time to reach each region, min) | Small Intestines | 5±0 | 5±0 | 5±0 | 5±0 | 5±0 | 5±0 |
| | Caecum | 70±3 | 77±5 | 76±4 | 170±3 **** | 60±5 † | 73±3 †††† |
| | Large Intestines | 90±3 | 90±5 | 88±4 | 205±5 **** | 70±5 †††† | 97±10 †††† |
| Time for complete barium emptying (min) | Gastric emptying | 17±1 | 33±3 | 27±4 | 65±9 *** | 53±8 | 55±11 |
| | Intestinal emptying | 88±2 | 83±3 | 86±2 | 155±11 **** | 90±5 †††† | 123±4 *** †††† |
| | Pellet Formation | 90±3 | 90±5 | 88±4 | 205±5 **** | 70±5 †††† | 97±10 †††† |

*** $P<0.001$, **** $P<0.0001$, significantly different to control/sham-treated group. † $P<0.05$, †††† $P<0.0001$, significantly different to OXL-treated (n=5 mice/group).

Figure 5.12 X-ray images following 14 days repeated *in vivo* OXL +/- BGP-15 administration. Representative x-ray images obtained from mice 5 – 210 min after intragastric barium sulfate (0.4mL, 2.5mg/mL) administration in control mice (prior to 1st injection) and following 14 days of DMSO, DMSO+H₂O, OXL, BGP-15 and OXL+BGP-15 administration. Stomach (*), small intestines (#), caecum (Δ), pellet formation (†).

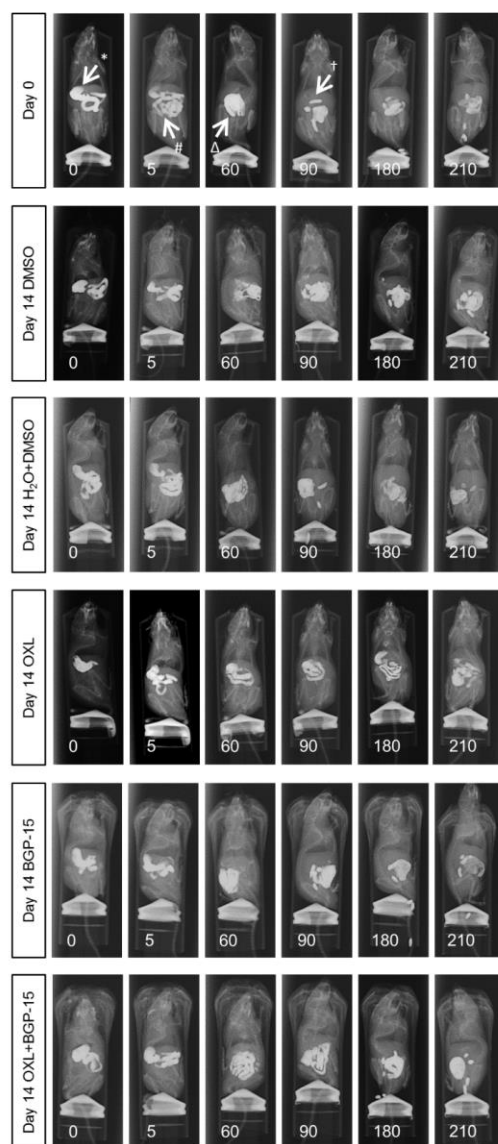


Figure 5.13 Gastrointestinal transit following 14 days repeated *in vivo* OXL+/- BGP-15 administration. Time (min) taken for barium sulfate to reach the stomach, small intestines, caecum and large intestines in control mice (prior to 1st injection) and at 14 days following DMSO, DMSO+H₂O, OXL, BGP-15 and OXL+BGP-15 administration. Data represented as mean \pm S.E.M. **** $P < 0.0001$ significantly different to sham, $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.0001$ significantly different to OXL, n = 5 mice/group.

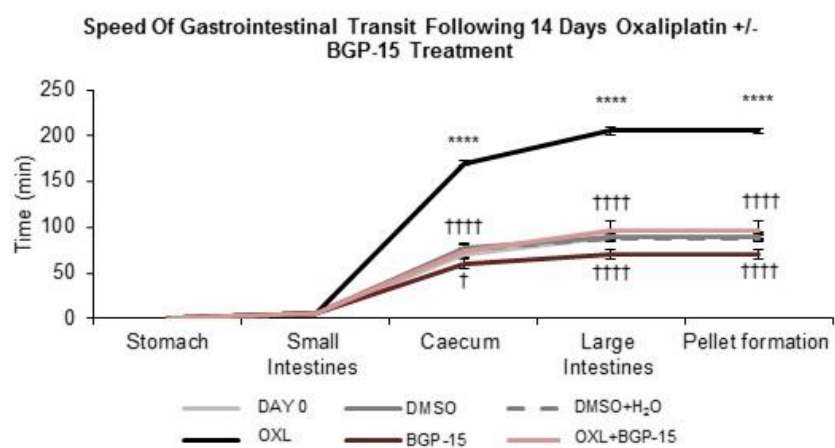
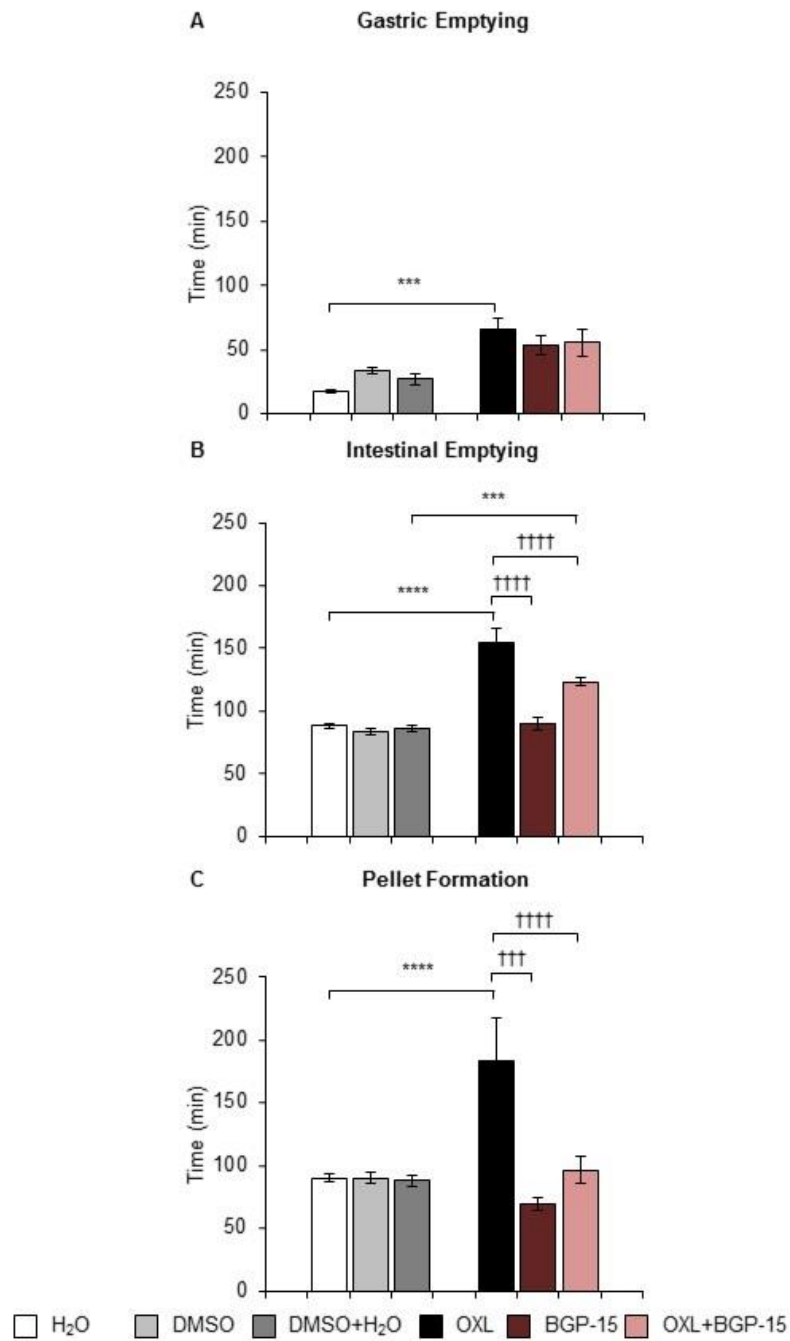


Figure 5.14 Gastric and intestinal emptying following 14 days repeated *in vivo* OXL+/- BGP-15 administration. Time (min) taken for complete emptying of barium from the stomach. Time (min) taken for complete emptying of barium from the small intestines. Time (min) taken to form first pellet in control mice (prior to 1st injection) and at 14 days following DMSO, DMSO+H₂O, OXL, BGP-15 and OXL+BGP-15 administration. Data represented as mean \pm S.E.M. *** P <0.001, **** P <0.0001 significantly different to sham, ††† P <0.0001 significantly different to OXL, n = 5 mice/group.



5.4.5 BGP-15 co-treatment prevents OXL effects on faecal content and animal body weight

To define the clinically-associated symptoms of gastrointestinal dysfunction resulting from OXL+/-BGP-15 administration, fresh faecal pellets were collected from all mice after 14 days of treatment (n=10 mice/group) and dehydrated for 72 hours'. Faecal water content was calculated as the difference between wet and dry pellet weight. Following 14 days of OXL treatment wet pellet weight was significantly reduced compared to sham H₂O-treated pellets ($P<0.001$, **Figure 5.15A**). BGP-15 treatment alone had no effect on wet pellet weight when compared to sham DMSO-treated mice (**Figure 5.15A**). When given together with OXL, BGP-15 significantly increased wet pellet weight compared to both sham DMSO+H₂O ($P<0.05$, **Figure 5.15A**), and OXL-treated mice ($P<0.0001$, **Figure 5.15A**). No significant difference in wet pellet weight was found when comparing sham H₂O, sham DMSO and sham H₂O+DMSO-treated mice. Likewise, no significant difference in dry pellet weight was found when comparing sham H₂O and OXL-treated mice at day 14 (**Figure 5.15B**). BGP-15 treatment alone had no effect on dry pellet weight when compared to sham DMSO-treated mice (**Figure 5.15B**). When given in combination with OXL, BGP-15 had no effect on dry pellet weight when compared to either OXL-treated or sham DMSO+H₂O-treated mice. No significant difference in dry pellet weight was found when comparing sham H₂O, sham DMSO and sham H₂O+DMSO-treated mice.

The water content of faecal pellets was significantly reduced in OXL-treated mice at day 14 ($P<0.0001$, **Figure 5.15C**) when compared to sham H₂O-treated mice. Treatment with BGP-15 alone had no effect on faecal water content when compared to sham DMSO-treated mice (**Figure 5.15C**). When given in combination with OXL, BGP-15 restored faecal water content to sham levels at day 14 ($P<0.0001$, **Figure 5.15C**) when compared to OXL-treated mice. No significant difference in faecal water content was found when comparing sham H₂O, sham DMSO and sham H₂O+DMSO-treated mice.

To establish whether chronic OXL administration was associated with weight loss or gain, the body weight of each mouse was monitored prior to and daily throughout the course of all treatments (n=10 mice/group) (**Figure 5.16**). OXL-treated mice did not gain body weight throughout the 2-week treatment regimen, and as a consequence, had a significantly reduced body weight from day 5 to the conclusion of the treatment regimen in comparison to sham H₂O-treated animals ($P<0.0001$, **Figure 5.16**). BGP-15 treatment alone had no significant effects on body weight when compared to sham DMSO-treated mice, however when compared to OXL-treated mice that gained no weight, BGP-15-treated mice gained significantly more weight from day 4 to the conclusion of the treatment regimen ($P<0.0001$, **Figure 5.16**). The addition of BGP-15 to OXL treatment protected mice from OXL-induced body weight suppression from day 10 to conclusion of the treatment regimen ($P<0.0001$, **Figure 5.16**) to sham levels.

Figure 5.15 Water content following OXL+/-BGP-15 administration.

(A) Wet faecal weight, (B) dry faecal weight and (C) faecal water content following 14 days' treatment with OXL+/-BGP-15. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ significantly different to sham, †† $P < 0.001$, ††† $P < 0.0001$ significantly different to OXL, n = 10 mice/group.

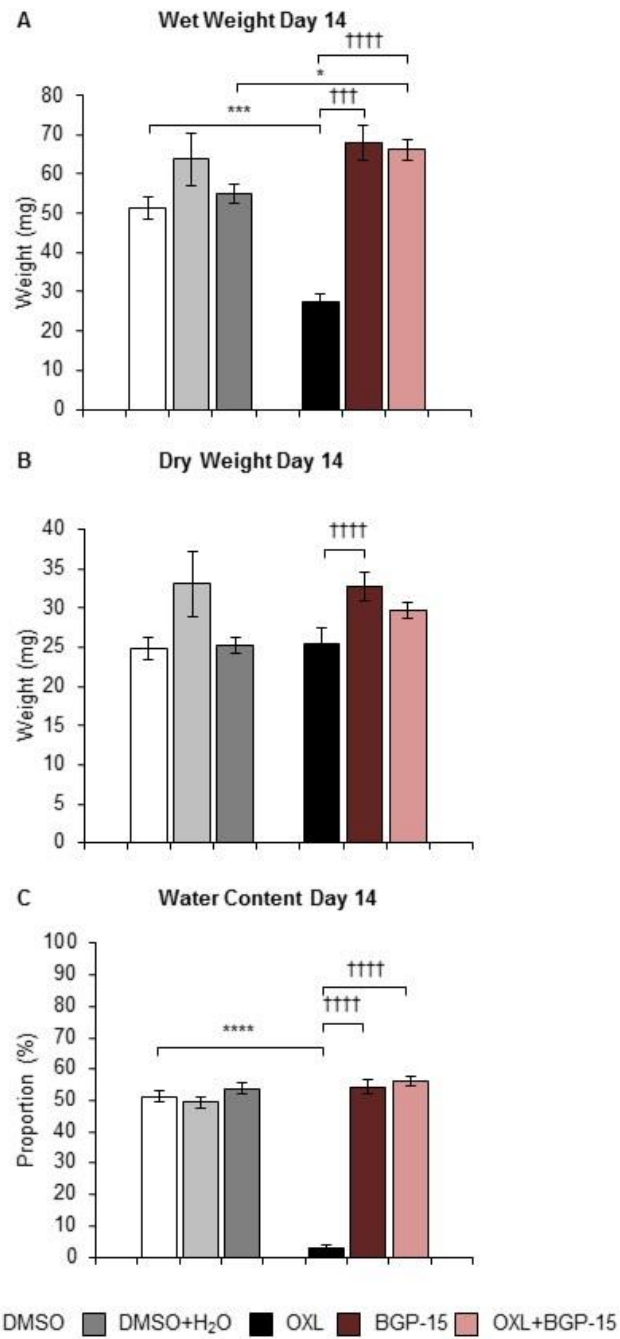
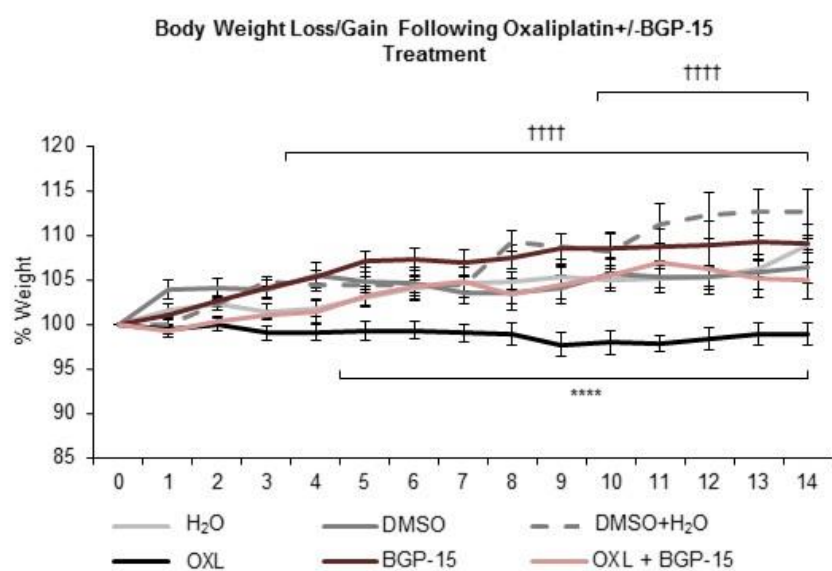


Figure 5.16 Percentage of weight loss/gain following repeated *in vivo* OXL+/- BGP-15 administration over 14 days. Data represented as mean \pm S.E.M. **** $P < 0.0001$ significantly different to sham, ††† $P < 0.0001$ significantly different to OXL, n = 10 mice/group.



5.5 Discussion

Previously we have shown that OXL treatment induces oxidative stress and loss of enteric neurons leading to gastrointestinal dysfunction, and suggested that reduction of oxidative stress in enteric neurons may offer a promising avenue for alleviation of chemotherapy-induced gastrointestinal side-effects (McQuade et al., 2016b). In the present study, the effect of BGP-15 co-administration with OXL chemotherapy on enteric neurons and gastrointestinal functions in mice was examined. Our data demonstrated that BGP-15 reduces OXL-induced mitochondrial superoxide production and protects against mitochondrial depolarization in colonic myenteric neurons. Subsequently, BGP-15 protected against myenteric neuronal loss which correlated with restored intestinal motility and transit, faecal water consistency and body weight over the treatment period. No significant differences were found in any parameters measured when comparing sham H₂O, sham DMSO and sham H₂O+DMSO-treated groups.

Mitochondrial dysfunction has emerged as a key player in the pathogenesis of platinum chemotherapy-induced neuronal death and dysfunction (Podratz et al., 2011;Zheng et al., 2011;Areti et al., 2014). Several studies of animal models suggest that mitochondrial dysfunction and axonal mitotoxicity contribute to neuropathic symptoms produced by various chemotherapeutic agents such as taxanes, vinca alkaloids, platinum compounds and bortezomib (Jin et al., 2008;Podratz et al., 2011;Zheng et al., 2011;Zheng et al., 2012). Evidence supports involvement of mitochondria-mediated oxidative and nitrosative stress in the development of peripheral nerve damage, with morphological changes including swollen and vacuolated mitochondria evident in peripheral nerve sections of chemotherapy-treated animals (Areti et al., 2014). Specifically, mitochondrial dysfunction is implicated in oxaliplatin-evoked neuropathic pain and sensory neuropathy and it has been suggested that drugs targeted at improving mitochondrial function may be of use in the treatment and prevention of chemotherapy-induced side-effects (Xiao and Bennett, 2012). Our previous study demonstrated that OXL treatment in mice is associated

with mitochondrial dysfunction and production of mitochondrial superoxide in the enteric neurons (McQuade et al., 2016b). The mechanisms underlying OXL-induced mitochondrial dysfunction in neurons remain unknown, but OXL-induced mitochondrial and energy homeostasis dysregulation in CRC cells may be mediated via direct damage to mitochondrial DNA (mtDNA) (Gourdier et al., 2004). While this effect is of obvious benefit to the induction of cell death pathways in neoplastic cells, suppression of mitochondrial function and the disruption of energy homeostasis would be detrimental to somatic cells (Sorensen et al., 2016) and, more specifically, to enteric neurons as we have found in the present study. Our results demonstrated that co-treatment with BGP-15 reduced levels of mitochondrial superoxide production in the myenteric plexus and increased neuronal survival.

Platinum accumulation has been suggested to contribute to oxaliplatin-induced DNA damage and enteric neuropathy (Stojanovska et al., 2014). Our recent work has shown that the total platinum concentration in longitudinal muscle and myenteric plexus preparations (LMMP) is significantly increased in OXL-treated mice when compared to sham, moreover a significant amount of platinum was found in both the nuclear and mitochondrial fractions of the LMMP preparations (Stojanovska et al., 2016). Increased ROS production is linked to damage to various cellular components and neuronal death (Wei et al., 2000). Mitochondrial dysfunction, particularly at the level of the mitochondrial electron transport chain (mETC), is implicated in ROS production in OXL-induced peripheral sensory neuropathy (Joseph and Levine, 2009). Inhibition of mETC complexes I and III, which are involved in mitochondrial ROS production, attenuates symptoms associated with OXL-induced peripheral neuropathy (Joseph and Levine, 2009). Furthermore, accumulation of dysfunctional mitochondria due to inefficient mitophagy, a cellular process that is required to clear damaged and dysfunctional mitochondria from the mitochondria pool via targeted cell death pathways, loss of anti-oxidant defence systems and accumulation of ROS have also been demonstrated in models of OXL-induced peripheral neuropathy (Melli et al., 2008; Areti et al., 2014).

The mitochondria and mtDNA are particularly susceptible to ROS-induced oxidative damage (Yakes and Van Houten, 1997) due to the fact that mtDNA lacks introns and undergoes transcription at a high rate (Glowacki et al., 2013). Mitochondrial enzymes are especially vulnerable to lipid peroxidation by peroxynitrite, leading to reduced ATP formation and induction of mitochondrial permeability transition via opening of the permeability transition pore, which dissipates the $\Delta\Psi$ (Norenberg and Rao, 2007). Alterations in $\Delta\Psi$ result in cellular stress responses including cessation of electron transport and ATP formation, mitochondrial swelling, and permeabilisation of the outer mitochondrial membrane, allowing the efflux of several pro-apoptotic molecules, including cytochrome *c* (Zoratti and Szabò, 1995;Norenberg and Rao, 2007). Our previous work has demonstrated a significant depolarisation of the mitochondrial membrane in the myenteric plexus of OXL-treated mice, as evidenced by increased JC-10 fluorescence (McQuade et al., 2016b). Here we have shown that co-treatment of BGP-15 with OXL reduced the level of monomeric JC-10 fluorescence suggesting that the $\Delta\Psi$ is maintained in myenteric neurons, this was correlated with neuronal protection in BGP-15 co-treated mice.

Clinical application of BGP-15 has been investigated in a wide variety of pathological conditions and has demonstrated efficacy in pre-clinical studies for the treatment of Duchenne Muscular Dystrophy, heart failure, cardiotoxicity, nephrotoxicity and peripheral neurotoxicity (López et al., 1997;Nanbu et al., 1997;Racz et al., 2002;Volm et al., 2002;Bardos et al., 2003;Hwang et al., 2003;Sarszegi et al., 2012). Nevertheless, this is the first study investigating the effects of BGP-15 on chemotherapy-induced enteric neuropathy and gastrointestinal dysfunction. The diverse clinical efficacy of this nicotinic amidoxime derivative, BGP-15 has been attributed to its multifaceted therapeutic nature as it has PARP inhibitory and HSP72 co-induction activity as well as anti-fibrotic, anti-inflammatory and anti-oxidant properties (Nanbu et al., 1997;Szabados et al., 2000;Racz et al., 2002;Volm et al., 2002). Given the complex pharmacological characteristics of BGP-15, the specific mechanism underlying its beneficial effect on enteric neurons when administered with OXL may be attributed to any or a number

of its therapeutic pathways. Here, we speculate that enteric neuroprotection in mice co-treated with BGP-15 observed in our study might be due to PARP inhibition and/or HSP72 activation.

In the myenteric plexus, NO is a key neurotransmitter released by nNOS-expressing interneurons and inhibitory motor neurons supplying the intestinal smooth muscles (Bornstein et al., 2004). Our previous work has demonstrated an increased proportion of nNOS-IR neurons in the myenteric plexus of the distal colon and concomitant loss of cholinergic neurons associated with colonic dysmotility in OXL-treated mice (Wafai et al., 2013;McQuade et al., 2016b). Our present results are consistent with these data. BGP-15 co-treatment prevented the increase in number and proportion of nNOS neurons in the myenteric plexus and significantly improved colonic motor patterns in OXL-treated mice. Furthermore, we found that BGP-15 alleviated delayed intestinal emptying in OXL-treated mice and improved gastrointestinal transit time and pellet formation. PARP inhibition has exhibited neuroprotective properties in models of cerebral ischemia, Huntington's disease, diabetic neuropathy and chemotherapy-induced peripheral neuropathy (Kaundal et al., 2006;Lupachyk et al., 2011;Cardinale et al., 2015). Although BGP-15 is yet to be investigated as a treatment for gastrointestinal pathologies, our data is consistent with earlier studies of PARP inhibition in models of chemotherapy-induced peripheral neuropathy and diabetic neuropathy (Brederson et al., 2012;Ta et al., 2013). In a rat model of diabetic sensory neuropathy, thermal hyperalgesia, mechanical hyperalgesia and tactile allodynia were associated with increased nitrotyrosine and Poly(ADP-ribose) (PAR) immunoreactivity in the sciatic nerve and increased superoxide formation and nitrotyrosine immunoreactivity in *vasa nervorum* (Ilnytska et al., 2006). PARP inhibition alleviated abnormal sensory responses and markedly reduced PAR and nitrotyrosine abundance in sciatic nerve, as well as superoxide and nitrotyrosine formation in *vasa nervorum* (Ilnytska et al., 2006). In other diabetic rat models a significant reduction in the magnitude of NO mediated muscle relaxation in the gastric fundus was successfully ameliorated following PARP inhibition which was attributed to improved

nitroergic neurotransmission (Gibson et al., 2006). Similarly, in animal models of chemotherapy-induced painful peripheral neuropathy, PARP inhibition successfully reduced symptoms of cisplatin and OXL-induced heat and cold hyperalgesia and mechanical allodynia in mice (Ta et al., 2013) and vincristine-induced mechanical allodynia in rats (Brederson et al., 2012). While we have not investigated PARP activity or expression in the present study, the beneficial effects of BGP-15 observed at the mitochondrial level suggest that inhibition of PARP is a possible therapeutic mechanism.

Induction of HSP72 may be a contributing mechanism to the neuroprotective capacity of BGP-15. Although this was not tested in the current study, there is a body of evidence suggesting the importance of HSP induction in models of peripheral neuropathy and neurodegeneration (Chen et al., 2012;Urban et al., 2012;Chen et al., 2013). Upregulation of HSPs has been associated with reduced neuronal apoptosis (Bienemann et al., 2008) and decreased oxidative stress in neurodegenerative disorders (Chaudhury et al., 2006). HSP72 specifically has been shown to have neuroprotective effects that repair damaged nerves (Chen et al., 2012). In models of diabetic neuropathy, exercise induced HSP72 expression in the spinal cord and peripheral nerves has been found to improve symptoms of peripheral neuropathy (Chen et al., 2013). Moreover, increased HSP72 expression in the sciatic nerve of chronic constriction injury rats has been found to ameliorate neuropathic pain (Chen et al., 2012). Although this is the first study investigating the effects of *in vivo* BGP-15 administration on OXL-induced enteric neuropathy, previous works exploring oral supplementation in OXL-induced peripheral sensory neuropathy have demonstrated that oral glutamine significantly reduces the incidence and severity of peripheral neuropathy in CRC patients receiving OXL (Wang et al., 2007). Whilst the underlying mechanism of glutamine's neuroprotective effect is yet to be established it has been suggested to be attributable to induction of HSPs, particularly HSP72 (Wischmeyer, 2002;Savarese et al., 2003). Whilst the evidence presented above highlights HSP72 induction as a potential neuroprotectant, it must be acknowledged that elevation of HSPs has been

correlated with tumour progression (Gabai et al., 2005). Increased expression of HSP72 in particular has been associated with high invasiveness, metastasis, chemotherapeutic resistance and poor prognostic outlook (Ciocca et al., 1993;Costa et al., 1997;López et al., 1997;Nanbu et al., 1997). In CRC, expression of HSP72 closely correlates with advanced clinical stages and positive lymph node involvement (Volm et al., 2002;Hwang et al., 2003), suggesting that HSP72 induction may be advantageous to tumour cells during cancer progression (Gabai et al., 2005). Therefore, the co-administration of BGP-15 with chemotherapeutics in a CRC model needs to be further investigated to ensure that BGP-15 does not alter the anti-cancer efficacy of chemotherapeutic agents. Moreover, recent studies have indicated a potential interaction between DMSO and platinum based chemotherapeutics, including oxaliplatin (Hall et al., 2014), the degree to which these compounds interact needs to be further investigated.

Results from our previous studies revealed that OXL administration was associated with reduced faecal water content and increased colonic faecal content indicative of constipation. In the current study it was found that BGP-15 administration attenuated these symptoms of constipation in OXL-treated mice, increasing faecal water content. Taken together our data have correlated positive BGP-15 induced changes at the cellular level with physiological improvements in gastrointestinal function at both organ and whole body levels.

5.6 Conclusion

Chemotherapy-induced gastrointestinal symptoms are a serious and somewhat overlooked clinical hurdle that significantly affects the prognostic outcome of CRC patients. Utilization of cytoprotective drugs to reduce gastrointestinal complications of anti-cancer chemotherapies is essential to improving both patient quality of life and outcomes of chemotherapeutic treatment. This study is the first to demonstrate that BGP-15 co-treatment reduces oxidative stress and prevents enteric neuropathy induced by OXL treatment. Neuroprotective efficacy of BGP-15 resulted in improvements to

GI motility at both levels: organ level (*ex vivo* colonic motility) and whole GI transit (*in vivo* x-ray), giving rise to an improvement in symptoms, presenting as increased body weight gain and increased faecal water content. Thus, results of this study indicate that BGP-15 may be considered as a combination therapy with OXL to reduce treatment-related gastrointestinal side-effects.

CHAPTER SIX: GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Comments

Gastrointestinal dysfunction is a common and debilitating side-effect of chemotherapy that has profound impact on clinical efficacy of chemotherapeutic drugs. Despite this, chemotherapy-induced constipation and diarrhoea are frequently overlooked in the clinical setting greatly impacting both patient quality of life and prognostic outlook. The studies contributing to this thesis provide novel insight into the underlying mechanisms of enteric neuropathy and gastrointestinal dysfunction associated with chemotherapeutic agents commonly used in the clinical setting for the treatment of colorectal cancer (CRC), oxaliplatin (OXL), irinotecan (IRI) and 5-fluorouracil (5-FU). Further, this research has shown that the cytoprotectant BGP-15 is a prospective treatment to alleviate gastrointestinal symptoms associated with OXL treatment. The findings of this work have important clinical relevance by providing a new therapeutic target for the treatment of chemotherapy-induced neuropathy and gastrointestinal dysfunction. This chapter aims to integrate the findings presented in this thesis, as well as identify key directions for future research regarding chemotherapy-induced enteric neuropathy and gastrointestinal dysfunction.

6.2 Chemotherapy-Induced Enteric Neuropathy

Enteric neuronal loss has been associated with several pathological conditions including irritable bowel syndrome, slow transit constipation, inflammatory bowel disease, diabetes and diverticulitis (Törnblom et al., 2002; Bassotti and Villanacci, 2006; Wedel et al., 2010; Chandrasekharan et al., 2011; Bernardini et al., 2012). Data presented in Chapters 2, 3 and 4 detail the degree of enteric neuropathy in the distal colon following OXL, IRI and 5-FU administration.

Although all investigated chemotherapeutic agents caused significant neuronal loss in the myenteric plexus following long-term (14 days) administration, the most severe neuronal loss was observed following OXL treatment (42%). IRI treatment induced a 15% neuronal loss, whilst 5-FU-

treated mice lost 12% of neurons. Further to this, reductions of up to 11% were found as early as 7 days following IRI and 5-FU administration. It is well known that the pathophysiology of neuropathy is immensely diverse. The results of this thesis show that the mechanisms underlying chemotherapy-induced neuronal death are agent-specific. Oxidative stress plays an important role in OXL-induced neuronal loss, whereas IRI and 5-FU-induced enteric neuropathy is associated with intestinal inflammation.

Table 6.1 Chemotherapy-induced enteric neuronal loss summary table

| | OXL | IRI | 5-FU |
|---------------------------------------|---|--|--|
| Neuronal loss in the myenteric plexus | 42% loss at day 14 | 11% loss at day 7 15% loss at day 14 | 11% loss at day 7 12% loss at day 14 |
| Mechanism | Oxidative stress | Inflammation | |
| | ↑ MitoSOX level in enteric neurons Mitochondrial membrane potential depolarisation and Cytochrome <i>c</i> release ↑ iNOS level in the longitudinal muscle-myenteric plexus preparations ↑ Number nNOS expressing neurons ↑ Protein nitration | ↑ Mucosal ulceration ↑ CD45-positive immune cells in the mucosa and at the level of myenteric ganglia | ↑ Mucosal ulceration ↑ CD45-positive immune cells in the mucosa and at the level of myenteric ganglia ↑ Faecal lipocalin-2 level |

Oxidative stress has been correlated with neuronal damage and loss in several pathologies including diabetic neuropathy, acrylamide-induced neuropathy, Charcot–Marie neuropathy and chemotherapy-induced peripheral neuropathy (Saifi et al., 2003; Vincent et al., 2004; Chandrasekharan et al., 2011; Areti et al., 2014). In particular, oxidative stress and corresponding mitochondrial dysfunction have been highlighted as key players in the mediation of oxaliplatin-induced peripheral nerve damage (Zheng et al., 2011).

Data presented in Chapter 2 demonstrated that OXL-induced neuronal loss was correlated with increased mitochondrial superoxide production, translocation of nitrated proteins and mitochondrial membrane depolarisation in the myenteric plexus of the distal colon as well increased expression of inducible nitric oxide synthase (iNOS) in the colon. These hallmark characteristics of oxidative stress were further correlated with an increase in the proportion of neuronal nitric oxide synthase (nNOS) immunoreactive (IR) neurons in the distal colon. Similarly, increased proportions of nNOS-IR neurons have been found in rats and mice treated with cisplatin and oxaliplatin (Vera et al., 2011; Wafai et al., 2013). Furthermore, increased levels of mitochondrial superoxide observed in enteric neurons is consistent with findings showing increased mitochondrial reactive oxygen species (ROS) in neuronal cultures from dorsal root ganglia (DRG) of oxaliplatin-treated rats (Kelley et al., 2014). The nervous system is particularly sensitive to oxidative stress due to the enrichment of polyunsaturated fatty acids in many nerve cell membranes. ROS have been found to modify ion transport mechanisms directly via alteration of regulatory proteins or indirectly via peroxidation of membrane lipids. Here, we identified translocation of nitrated proteins in both the submucosal and myenteric plexus of the colon from OXL-treated mice. As well as being an indication of oxidative stress, nitration of structural proteins can have major pathological consequences (Beckman and Koppenol, 1996). Accumulation of nitrotyrosine has been linked to protein misfolding and mitochondrial dysfunction resulting in alteration to membrane potential, ionic gradients, action potential duration and amplitude, spontaneous activity and

excitability (Xiao and Bennett, 2012) and neuronal degeneration (Nakamura and Lipton, 2008). Increased ROS production is also correlated with damage to various cellular components including the mitochondria (Wei et al., 2000).

Mitotoxicity has been observed in the sciatic nerve of oxaliplatin-treated rats resulting in disruption of respiration and ATP production correlated with OXL-induced peripheral neuropathy (Zheng et al., 2011). Mitochondrial dysfunction, particularly at the level of the mitochondrial electron transport chain (mETC), is implicated in ROS production in OXL-induced peripheral neuropathy (Joseph and Levine, 2009).

Inhibition of mETC complexes I and III, which are involved in mitochondrial ROS production, has been found to attenuate symptoms associated with OXL-induced peripheral neuropathy (Joseph and Levine, 2009). Whilst it was outside the scope of this thesis, several studies investigating the effects of various anti-oxidant compounds including silibinin, α -tocopherol, rutin, quercetin and SS-31 in animal models of OXL-induced peripheral pain and neuropathy have demonstrated clear improvements in both oxidative markers and clinical symptoms (Di Cesare Mannelli et al., 2012; Azevedo et al., 2013; Toyama et al., 2014). Similarly, unpublished data from our group has found that *in vivo* co-administration of a potent anti-oxidant, resveratrol, with OXL reduces OXL-induced enteric neuronal loss and improves colonic dysfunction in mice (Donald et al., 2017, *in press*). The aforementioned findings, in combination with the results in Chapter 2, highlight the potential of both anti-oxidants and ROS scavengers in ameliorating OXL-induced enteric neuropathy. Further studies exploring such avenues, although beyond the scope of this thesis, are currently being undertaken in our laboratory.

Other mechanisms might contribute to enteric neuronal death such as platinum accumulation or inflammation induced by OXL treatment. Platinum accumulation has been suggested to contribute to oxaliplatin-induced DNA damage and enteric neuropathy (Stojanovska et al., 2014). Recent work

from our group has shown that the total platinum concentration in longitudinal muscle and myenteric plexus (LMMP) preparations is significantly increased in OXL-treated mice when compared to sham. Moreover, a significant amount of platinum was found in both the nuclear and mitochondrial fractions of the LMMP preparations (Stojanovska et al., 2016). Simultaneous existence of low-grade chronic inflammation and oxidative stress has been implicated in several chronic diseases including diabetes, cardiovascular disease, alcoholic liver disease, chronic kidney disease and neurodegenerative conditions (Biswas et al., 2007; Hald et al., 2007; Cachofeiro et al., 2008; Onyango, 2008; Ambade and Mandrekar, 2012; Biswas, 2016). Both oxidative stress and chronic inflammation may have detrimental effects on neurons (Fischer and Maier, 2015). Preliminary findings from our group have demonstrated that OXL treatment has no effect on immune infiltration in the mouse colon (Stojanovska et al., 2017, *unpublished data*), but whether OXL-induced oxidative stress is correlated with systemic inflammation requires further investigation.

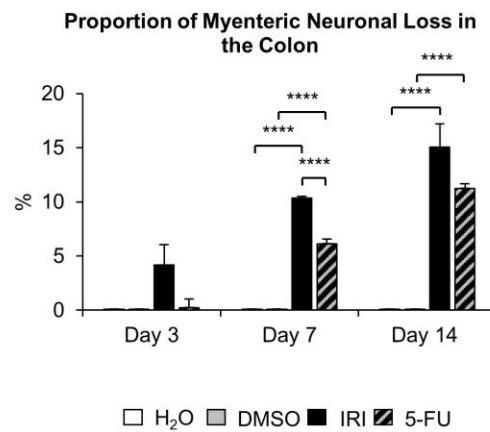
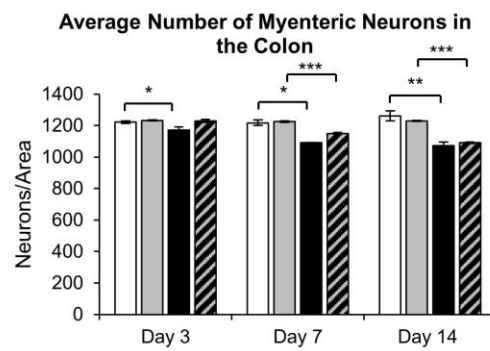
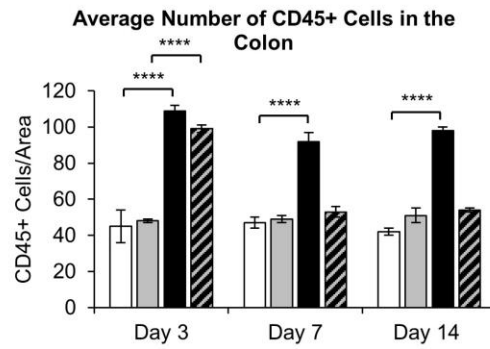
Conversely, data presented in Chapters 3 and 4 demonstrated that both IRI and 5-FU administration are associated with intestinal inflammation. In Chapter 3, results showed severe mucosal ulceration, crypt hypoplasia and morphological disorganisation in the colon following both short and long-term IRI treatment. This is in line with previous studies reporting villus blunting, epithelial atrophy and crypt ablation in the rat jejunum and colon following IRI administration (Logan et al., 2008). However, our results have demonstrated mucosal regeneration in post-treatment mice for the first time. Mucosal regeneration has been confirmed in human studies showing that early histological changes in the gastrointestinal tract following chemotherapeutic administration are resolved within days of treatment cessation, with no abnormal endoscopic findings in patients as early as 16 days post-chemotherapy (Keefe et al., 2000). Changes in colonic mucosal structure demonstrated in Chapter 3 were correlated with increases in CD45 positive leukocytes in the colon. There was a significant increase in CD45 positive leukocytes following 3 days of IRI administration, which persisted throughout the course of treatment as well as post-treatment. In Chapter 4,

however, our results showed that short-term 5-FU treatment was associated with destruction of the epithelial brush border and severe loss of colonic crypts and goblet cells, but chronic treatment did not exacerbate epithelial damage. In fact, regeneration of mucosa was evident during chronic 5-FU treatment, although crypts still appeared shorter, distended and disorganised. These results are consistent with previous findings where loss of crypt architecture, shortening of villi and inflammatory infiltration in the lamina propria of the rat duodenum were reported after 3 days of 5-FU treatment, followed by restoration of intestinal villi and crypts after 15 days of 5-FU treatment (Soares et al., 2008). The results presented in Chapter 4 show that, like IRI, 5-FU-induced changes to mucosal architecture were associated with an increased number of CD45 positive leukocytes in the colon and an amplified concentration of neutrophil gelatinase-associated protein, lipocalin-2, in faecal samples. However, unlike IRI treatment, these changes were transient with no significant difference in the number of CD45 positive leukocytes or lipocalin-2 concentration found after 7 or 14 days of 5-FU treatment.

Specific populations of inflammatory mediators involved in IRI and 5-FU-induced intestinal inflammation were not identified in this thesis. However, previous studies have shown that *in vitro* and *in vivo* treatment with both IRI and 5-FU promotes the attraction of inflammatory cells (Logan et al., 2008;Cottone et al., 2015), which may be associated with enteric neuronal damage and death. Although previous research has demonstrated that co-administration of the anti-inflammatory agent St John's Wort with IRI inhibits the expression of pro-inflammatory markers in the intestines and improves symptoms of diarrhoea in rats (Hu et al., 2006), no research has been undertaken to investigate the effects of anti-inflammatory administration on enteric neuronal survival and gastrointestinal function in models of chemotherapy-induced diarrhoea. The work within this thesis has provided the basis for future studies exploring the effects of specific and potent anti-inflammatory agents in models of IRI and 5-FU-induced diarrhoea.

Although it has been shown that histological damage resolves within days following chemotherapeutic treatment, long-term diarrhoea persists up to 10 years following chemotherapy (Schneider et al., 2007;Denlinger and Barsevick, 2009;Numico et al., 2015). While the underlying mechanisms of delayed onset and long-term diarrhoea remain unclear, early mucosal damage and acute intestinal inflammation can lead to death and damage of enteric neurons resulting in long-term gastrointestinal dysfunction (Boyer et al., 2005;Linden et al., 2005;Nurgali et al., 2007;Nurgali et al., 2011). In particular, chronic intestinal inflammation has been associated with damage to the ENS with neuronal losses of up to 50% reported in the myenteric plexus (Boyer et al., 2005;Gulbransen et al., 2012), which may result in downstream effects on gastrointestinal function. I found that persistent and severe mucosal disorganisation and inflammation following IRI administration were associated with substantial neuronal loss in the myenteric plexus, whilst transient changes in mucosal structure and inflammation following 5-FU administration were associated with a lesser degree of neuronal loss (**Figure 6.1**). Neuronal loss has previously been correlated with altered colonic and intestinal motility (Chandrasekharan et al., 2011).

Figure 6.1 Summary figure of IRI and 5-FU-induced inflammation and neuronal loss (based on data presented in Chapters 3 and 4). Average number of CD45+ cells in the colon (**A**). Average number of myenteric neurons in the colon (**B**). Proportion of neuronal loss in the colon (**C**).



□ H₂O □ DMSO ■ IRI ▨ 5-FU

6.3 Chemotherapy-Induced Gastrointestinal Dysfunction

This thesis provides novel insight into the association between chemotherapy-induced enteric neuropathy and gastrointestinal dysfunction. Following OXL, IRI and 5-FU administration, enteric neuronal loss was associated with significant alterations in both whole gut transit and colonic motor patterns. OXL administration was associated with altered inhibitory neurotransmission correlating with a reduction in the total number of colonic contractions and symptoms of constipation. In contrast, both IRI and 5-FU administration were associated with an increased proportion of cholinergic myenteric neurons correlated with increased total numbers of colonic contractions and increased faecal water content associated with diarrhoea (**Table 6.2**).

Table 6.2 Chemotherapy-induced changes in gastrointestinal function summary table

| | OXL | IRI | 5-FU |
|------------------------------|--|--|--|
| Gastrointestinal dysfunction | ↓ Total number of contractions ↓ %CMMCs ↑ %FCs ↓ %SCs ↓ GI Transit speed ↓ Pellet formation speed | ↑ Total number of contractions ↓ %CMMCs ↑ %FCs ↔ GI Transit speed ↔ Pellet formation speed ↔ Food consumption | ↑ Total number of contractions ↓ %CMMCs ↑ %FCs ↑ %SCs ↓ GI Transit speed ↓ Pellet formation speed |
| Neuronal changes | ↑ %nNOS-IR myenteric neurons | ↑ %ChAT-IR myenteric neurons ↑ VChT fibre density | ↑ %ChAT-IR myenteric neurons |
| Faecal changes | ↓ Faecal output ↓ Faecal water content | ↑ Pellet length ↑ Faecal water content | |

Data presented in Chapter 2 demonstrated delayed gastrointestinal transit in OXL-treated mice and a reduction in the total number of colonic contractions. Analysis of spatiotemporal maps revealed that OXL treatment induced significant reductions in the proportion and frequency of CMMCs, as well as reductions in the frequency of short contractions (SCs). These findings are consistent with previous studies demonstrating inhibition of CMMCs in the colon of OXL-treated mice (Wafai et al., 2013) and inhibition of intestinal transit in cisplatin-treated rats (Vera et al., 2011). These changes in gastrointestinal transit, colonic motor patterns and circular muscle tone resulted in reduced faecal output and reduced faecal water

content in OXL-treated mice, consistent with clinical symptoms of constipation. This is in line with previous clinical studies, with severe gastrointestinal toxicity commonly documented in patients receiving combination chemotherapies including OXL and other platinum compounds (Muggia et al., 2000;Bano, 2013).

We hypothesised that gastrointestinal dysfunction associated with OXL treatment is due to the morphological and functional changes in the enteric neurons. The results presented in Chapter 2 support this hypothesis. OXL treatment induced phenotypic changes in myenteric neurons of the colon. The proportion of nNOS expressing neurons was increased following OXL administration. Nitric oxide (NO) released from nNOS expressing motor and interneurons is an integral neurotransmitter in the gastrointestinal tract (Lecci et al., 2002;Takahashi, 2003). Excessive NO production and consequent nitrosylation has been linked to inhibition of dynamin-related protein 1, resulting in synaptic impairment and disruption to synaptic transmission (Savidge, 2011). Intracellular recordings from colonic circular muscles presented in Chapter 2 revealed changes in neuromuscular transmission with an increase in amplitude of NO-mediated slow inhibitory junction potentials (sIJPs) in OXL-treated mice, these changes in NO-mediated transmission correlated with decreased basal tone and, thus, a larger resting diameter of the distal colon compromising smooth muscle relaxation in response to NO donor, sodium nitroprusside.

Both IRI and 5-FU (Chapters 3 and 4) induced an increase in the total number of colonic contractions, a reduction in the proportion of CMMCs alongside an increase in proportion of fragmented contraction (FCs). In 5-FU-treated mice, this was also coupled with an increase in the proportion of SCs. Results presented in Chapter 3 show that although food consumption was not changed at any point throughout the course of IRI treatment, faecal water content was significantly increased at days 3, 14 and post-treatment, suggestive of secretory diarrhoea. This is in line with previous work suggesting that delayed onset and long-term diarrhoea is multifaceted,

resulting from a primarily secretory mechanism with an inflammatory component (Bleiberg and Cvitkovic, 1996; Saliba et al., 1998).

Results presented in Chapters 3 and 4 have demonstrated that increases in the proportion of choline acetyltransferase (ChAT)-IR neurons and/or vesicular choline acetyltransferase (VACht)-IR fibres in IRI and 5-FU-treated mice were associated with an increase in a total number of contractions. Acute diarrhoea experienced within the first 24 hours of IRI treatment has been attributed to cholinergic syndrome in CRC patients (Hecht, 1998). Acetylcholine is a major excitatory neurotransmitter responsible for the contractions of circular and smooth muscle (Furness, 2012) and mucosal secretion (Cooke, 2000). Thus, cholinergic neurons are a vital signalling component in excitatory motor innervation of the colon (Furness, 2012).

Given the importance of cholinergic transmission in the generation of healthy colonic motor patterns and overall muscle function, three different transport systems participate in choline uptake; high-affinity choline transporter 1 (CHT1), choline transporter-like 1 (CTL1) and organic cation transporters (OCTs) such as OCT1, OCT2 and OCT3. CHT1 and OCT-1 immunoreactivity has been observed in the submucosal and myenteric plexi as well as in the mucosal epithelium in the small and large intestines (Chen et al., 2001; Harrington et al., 2010). In human intestine, various OCTs have been shown to participate in the absorption of anti-cancer chemotherapeutics including IRI (Shnitsar et al., 2009; Koepsell, 2015). Thus, future studies investigating the relationship between IRI and 5-FU treatment and CHT1, CTL1 and OCT function are warranted.

Post-inflammatory dysmotility is a well-recognised clinical entity both at the site of inflammation and at distant non-inflamed sites (Moreels et al., 2001; Akiho et al., 2005; Demedts et al., 2006; O'Hara et al., 2007; Hughes et al., 2009; Qin et al., 2011). Both humoral and cell-mediated inflammatory responses are associated with increased excitability of enteric neurons (Frieling et al., 1994; Palmer et al., 1998; Linden et al., 2003) and synaptic

facilitation (Palmer et al., 1998;Linden et al., 2003). Changes to the electrophysiological properties of enteric neurons following inflammation have been demonstrated in the ileum (Nurgali et al., 2007;Nurgali et al., 2011), jejunum (Palmer et al., 1998) and colon (Frieling et al., 1994;Linden et al., 2003;Lomax et al., 2005;Lomax et al., 2006;Lomax et al., 2007). Motor disturbances and neuronal changes persist long after the resolution of inflammation (Demedts et al., 2006). It has been suggested that these changes to enteric neurons contribute to disorders of motility, secretion and hypersensitivity during and following gastrointestinal inflammation (Sharkey and Kroese, 2001;De Giorgio et al., 2004b). Further, several inflammatory mediators have been implicated in both short- and long-term smooth muscle contractility changes (Demedts et al., 2006). Therefore, the effects of both IRI and 5-FU administration on electrophysiological properties of enteric neurons, neuromuscular transmission and muscle contractility require further investigation.

Whilst this thesis aimed to correlate myenteric neuronal loss following IRI and 5-FU administration with colonic inflammation, the identity of specific inflammatory mediators involved in this response remains unclear. Future experiments investigating the inflammatory mediators involved in IRI and 5-FU-induced immune response need to be undertaken.

The mechanisms underlying chemotherapy-induced gastrointestinal dysfunction are complex and most likely multifaceted. Previous research has demonstrated the capacity of microbiota to influence the ENS, in particular probiotic organisms enhance excitability of enteric sensory neurons while decreasing intestinal motility (Kunze et al., 2009;Wang et al., 2010). Thus, future studies should investigate the role of microbiota in chemotherapy-induced enteric neuropathy and the relationship with gastrointestinal dysfunction.

An interesting finding from this thesis was the presentation of persistent post-treatment gastrointestinal side-effects alongside IRI-induced myenteric neuronal loss (Chapter 3). Whilst significant in demonstrating the long-term functional consequences of myenteric neuronal loss, due to the time

constraints of candidature these experiments could not be replicated in the other chemotherapy-treated cohorts. Future research should investigate the persistence of post-treatment gastrointestinal side-effects following both OXL and 5-FU administration.

6.4 BGP-15 Co-Treatment

Given the severity of neuronal loss following OXL administration demonstrated in Chapter 2, the effects of BGP-15 co-treatment with OXL on neuronal survival and gastrointestinal dysfunction were examined in Chapter 5.

Growing research supports the notion of enteric neuroprotection as a therapeutic target for gastrointestinal dysfunction in a variety of conditions. The novel cytoprotectant BGP-15 is a pharmacological modulator of the cellular response to stress, acting as a poly(ADP-ribose) polymerase (PARP)1 inhibitor (Sarszegi et al., 2012), heat shock protein (HSP) co-inducer (Ciocca et al., 1993;Costa et al., 1997) and a mild anti-oxidant (Racz et al., 2002) in a variety of tissues. Clinical application of BGP-15 has been investigated in a wide variety of pathological conditions and has demonstrated efficacy in pre-clinical studies for the treatment of Duchenne muscular dystrophy, heart failure, cardiotoxicity, nephrotoxicity and peripheral neurotoxicity (López et al., 1997;Nanbu et al., 1997;Racz et al., 2002;Volm et al., 2002;Bardos et al., 2003;Hwang et al., 2003;Sarszegi et al., 2012). In particular BGP-15 is suggested to have neuroprotective properties in animal models of both cisplatin- and taxol-induced peripheral sensory neuropathy (Bardos et al., 2003). Therefore, I hypothesised that BGP-15 co-administration may protect against chemotherapy-induced neuropathy and thus ameliorate symptoms of gastrointestinal dysfunction. The results presented in Chapter 5 indicate that co-treatment with BGP-15 reduced levels of mitochondrial superoxide production and alleviated mitochondrial depolarisation in the myenteric plexus. These findings were in line with previous studies, highlighting mitochondrial dysfunction as a key player in the pathogenesis of platinum chemotherapy-induced sensory neuronal death and dysfunction (Podratz et al., 2011;Zheng et al.,

2011;Areti et al., 2014). Several studies in animal models suggest that mitochondrial dysfunction and axonal mitotoxicity associated with chemotherapeutic administration contribute to neuropathic symptoms produced by various chemotherapeutic agents such as taxanes, vinca alkaloids, platinum compounds and bortezomib (Jin et al., 2008;Podratz et al., 2011;Zheng et al., 2011;Zheng et al., 2012). The results presented in Chapter 2 and from other studies support involvement of mitochondria-mediated oxidative and nitrosative stress in the development of enteric neuropathy and peripheral sensory nerve damage (Areti et al., 2014). Mitochondrial dysfunction has been implicated in oxaliplatin-evoked neuropathic pain and neuropathy and, therefore, it has been suggested that drugs targeted at improving mitochondrial function may be of use in the treatment and prevention side-effects of chemotherapy (Xiao and Bennett, 2012).

This was confirmed by the data presented in Chapter 5 which demonstrate that treatment with BGP-15 alleviates mitochondrial dysfunctions leading to better survival of enteric neurons and a reduced proportion of nNOS-immunoreactive neurons. Significant delays in gastrointestinal transit, gastric and intestinal emptying and pellet formation, as well as impaired colonic motor activity, reduced faecal water content and lack of weight gain associated with OXL treatment were restored to sham levels in mice co-treated with BGP-15, suggesting that BGP-15 may relieve the gastrointestinal side-effects associated with chemotherapy.

Whilst this thesis has demonstrated that BGP-15 elicited beneficial neuroprotective effects when administered in combination with OXL, the mechanisms underlying these favourable effects remain unclear. Given the complex pharmacological characteristics of BGP-15, the specific mechanism underlying its beneficial effect on enteric neurons when administered with OXL may be attributed to any number of its therapeutic pathways, including PARP inhibition and/or HSP72 activation. Previous studies have demonstrated that both PARP-1 inhibition and HSP induction reduce the incidence and severity of peripheral neuropathy symptoms

following chemotherapeutic administration (Wang et al., 2007; Brederson et al., 2012; Ta et al., 2013). Thus, given the multifaceted therapeutic nature of BGP-15 further investigation should focus on exploring the effects of potent PARP inhibitors and HSP inducers given in combination with chemotherapeutic administration. Further to this, given the positive effects demonstrated following BGP-15 administration in combination with OXL, further studies investigating the effects of BGP-15 in combination with 5-FU and IRI also need to be undertaken.

6.5 Limitations and Future Directions

Although this work is amongst the first aimed understanding mechanisms underlying chemotherapy-induced enteric neuropathy and providing a comprehensive analysis of both whole gastrointestinal transit and colonic motility, there are several limitations that may impact the clinical relevance of the findings. This might include, but are not limited to, studies of the effects of combination therapies, use of animal models of cancer and human studies.

6.5.1 Combination Therapies

This thesis has demonstrated that isolated administration of chemotherapeutic agents OXL, IRI and 5-FU induced significant enteric neuropathy correlated with gastrointestinal dysfunction. Current clinical guidelines for the treatment of CRC involve primarily combination therapies such as FOLFOX, FOLFIRI and FOLFOXIRI (Falcone et al., 2002; Grothey et al., 2004; Souglakos et al., 2006; Van Cutsem et al., 2010). Whilst the effects of combination chemotherapeutic treatment on the ENS is yet to be investigated, several studies have validated the anti-cancer efficacy of combination regimes FOLFOX and FOLFIRI in mouse models of CRC (Ishihara et al 2010, Robinson et al 2013). Although the present work was essential to provide the necessary background/context, further experiments exploring the effects of clinically relevant combination therapies on enteric neuronal survival and gastrointestinal dysfunction are needed.

6.5.2 CRC Models

The present study is limited in its resemblance to the clinical setting in which only patients afflicted with cancer are administered chemotherapeutic agents. As a result, the experiments contained within this thesis do not take into consideration the complex interactions between cancer/tumour-mediated gastrointestinal disturbance and chemotherapy-induced gastrointestinal dysfunction. Previous studies from human CRC patients have confirmed damage to the innervation of the large intestines in the course of CRC invasion (Godlewski, 2010). Changes in the proportion of vasoactive intestinal peptide, neuropeptide Y, calcitonin gene regulating peptide and substance P-IR neurons and neuronal fibre density have also been reported in both the submucosal and myenteric plexus of the large intestines of human CRC patients (Godlewski, 2010). Given this, future research needs to be conducted to determine the combined effects of chemotherapeutic administration in a CRC model. Further, the chemotherapeutic and chemo-potentiating capacity of PARP inhibitors such as iniparib and olaparib are currently under clinical investigation in breast, ovarian, prostate cancer with promising results (Gelmon et al., 2011; O'Shaughnessy et al., 2011; Liu et al., 2013; Mateo et al., 2015). I acknowledge the complex therapeutic nature of BGP-15 and recognise that it has been associated with several intra and extracellular activities including HSP72 induction and anti-oxidant behaviour. Therefore, co-administration of BGP-15 with chemotherapeutic agents in a CRC model needs to be further investigated to ensure that BGP-15 does not alter the anti-cancer efficacy of these agents.

6.5.3 Human Studies

Finally, while this thesis has provided substantial evidence that enteric neuronal loss may underlie chemotherapy induced-gastrointestinal dysfunction in mice, limited studies on enteric neurons in human tissues from chemotherapy-treated patients have demonstrated changes in electrophysiological properties of myenteric neurons (Carbone et al., 2016)

and translocation of proteins from the cytoplasm to the nuclei (Stojanovska et al., 2014;Carbone et al., 2016) indicative of neuronal damage. However, the mechanisms underlying these changes have not been investigated. Prospective studies need to examine the effects of both chemotherapy administration as well as the efficacy and safety of BGP-15 co-treatment in CRC patients.

6.6 General Conclusions

Although the median overall survival of patients with metastatic CRC has increased from 12 months to approximately 24 months over the past decade as a result of improvements and implementation of new cytotoxic therapies, including irinotecan and oxaliplatin, the 5-year survival of patients remains poor (Meyerhardt and Mayer, 2005;Balko and Black, 2009;Bathe et al., 2009;Fletcher, 2009). Optimization of already available chemotherapeutic agents to ameliorate dose-limiting symptoms is a promising avenue of research that may improve the prognostic outcome of CRC. While future research needs to rigorously evaluate the use of BGP-15 within a CRC cohort alongside chemotherapeutic treatment before efficacy and safety can be determined, it is clear from the studies contained within this thesis, and previously published observations (Bardos et al., 2003;Argyriou et al., 2006;Wang et al., 2007;Jin et al., 2008;Melli et al., 2008;Pace et al., 2010;Pachman et al., 2011;Brederson et al., 2012;Ta et al., 2013;Piccolo and Kolesar, 2014) that pharmacologically protecting enteric neurons could be a potentially powerful tool in alleviating chemotherapy-induced neuropathy and gastrointestinal dysfunction.

CHAPTER SEVEN: REFERENCES

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APPENDIX A: PUBLICATIONS FROM THIS THESIS

Colorectal Cancer Chemotherapy: The Evolution of Treatment and New Approaches

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ABSTRACT

Colorectal cancer (CRC) is one the greatest contributors to cancer related mortality. Although 5 year survival rate for patients at the early stage of CRC (stages I and II) is above 60%, more than 50% of patients are diagnosed at or beyond stage III when distant metastasis has already occurred, in which case 5 year survival rate drops to 10%. Chemotherapeutic intervention coupled with surgery is the backbone of metastatic CRC treatment and the only means of enhanced survival. For decades following its discovery, an antimetabolite 5-fluorouracil (5-FU) was the only chemotherapeutic agent available to successfully improve 12 month survival in CRC patients. Treatment of metastatic CRC has been considered palliative for many years; aiming to increase the duration and quality of the patient's remaining life, with little hope of cure, highlighting the need for novel DNA and RNA targeted therapies in the treatment of CRC. Over the last several decades, combinations of several chemotherapeutic agents have been incorporated into routine clinical practice. Combination regimes incorporating irinotecan, a semisynthetic inhibitor of topoisomerase, oxaliplatin, a third-generation platinum compound that causes mitotic arrest via the formation of DNA adducts, and capecitabine, a 5-FU prodrug, are now all established options for use as first-line, second-line and sequential treatment of CRC. This review provides a brief overview of the evolution of CRC chemotherapy as well as new and emerging treatment options.

Key words: (6-8 words)

Colorectal cancer; chemotherapy; 5-fluorouracil, capecitabine and leucovorin; cisplatin and oxaliplatin; combination chemotherapy; targeted therapies and anti-Inflammatories; ruthenium; PARP inhibitors

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide [1]. With a global incidence of approximately 1.4 million and mortality rates approaching 700,000 people per year, it is one of the highest contributors to cancer related death [1, 2]. Globally the incidence of CRC is highly variable, with rates fluctuating up to 10-fold between countries [3, 4], ranging from more than 40 per 100,000 people in the United States, Australia, New Zealand, and Western Europe to less than 5 per 100,000 in Africa and some parts of Asia [5]. Lower rates of CRC in developing countries may be due to lack of screening and diagnostics of population over 50 years of age, which is implemented in many western countries.

Several risk factors are associated with the incidence of CRC. Non modifiable risk factors include those that an individual cannot control including age and hereditary factors [6]. Additionally, a substantial number of environmental and lifestyle factors, considered as modifiable risk factors, may play an important role in the development of CRC [6, 7]. CRC is widely considered to be an environmental disease and is associated with a diverse range of ill-defined cultural, social, and lifestyle factors including, abdominal obesity [8, 9], alcohol consumption [10, 11] and smoking [12-14]. Thus, many CRC cases may theoretically be preventable [15, 16].

The histopathogenesis of CRC revolves heavily around development of mucosal colonic polyps. The most common histological types of colonic polyps are hyperplastic and adenomatous [17]. Approximately 75% of colorectal tumours do not appear to be due to inherited genetic mutations and are referred to as non-familial or sporadic tumours, however a small proportion of colorectal tumours have been correlated with inherited mutations in specific oncogenes such as adenomatous polyposis coli (APC) and defective DNA mismatch

repair genes MLH1, MSH2, PMS2 and MSH6 [18]. Approximately 30-50% of colorectal tumours are known to have a mutated V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) gene. A further 10% of CRC patients are characterized by a mutation in the B-Raf proto-oncogene serine/threonine kinase (BRAF) gene resulting in a valine-to-glutamate change at the residue 600 (V600E) [19]. Presence or absence of KRAS and BRAF mutations has been found to greatly impact the efficacy of certain anti-cancer agents; furthermore disruption of several signalling pathways is associated with disease progression. Transforming growth factor- β (TGF β) has been found to promote tumour growth via Smad-independent mechanisms [20], and is involved in crosstalk with several other pathways including the mitogen-activated protein kinases (MAPK) and Wnt signalling pathway. Deregulation of Wnt and T-cell factor (Tcf) signalling and accumulation of β -catenin contributes to accretion of undifferentiated cells in colonic crypts which when combined with subsequent mutations, involving genes, such as KRAS and BRAF, may eventually lead to carcinoma [21].

The clinical manifestations of CRC are highly dependent on the location and characteristics of the tumour regardless of preceding condition. Approximately 65% of colon cancers are distal to the splenic flexure and thus may be detectable via sigmoidoscopy, conversely 35% of primary tumours are located proximal to the sigmoid and are essentially imperceptible [22, 23]. Exacerbating this diagnostic dilemma, the discernible symptoms of CRC are non-specific and not well defined, with a recent review of literature indicating that rectal bleeding and weight loss are the only definitive symptoms associated with CRC [24]. Whilst the 5 year survival rate for patients at the early stage of CRC (I and II) currently sits at 63%, that rate drops to below 10% in patients with metastatic form of the disease (stage III) [6, 25-27]. Currently, patients with chemotherapy-refractory colorectal liver metastases have a median

survival of only 4–6 months with optimal supportive care [28]. Approximately 60% of CRC sufferers are diagnosed at or beyond stage III resulting in prognostic outlook relying heavily on the successful application of chemotherapeutic treatment [29].

Approximately two thirds of CRC patients undergo surgical resectioning with or without adjuvant chemotherapy [30]. Unfortunately, 30-50% of patients develop recurrent disease, with greater than 90% of recurrences in the first 5 years following surgery [31]. Advances in preoperative chemotherapeutic therapies over the last several decades have led to development of several promising regimes resulting in improvement of 5 year survival rates for locally resected stage III CRC [32, 33]. Progress in the area of CRC chemotherapeutics over the last 70 years has, however, been limited with initial treatments such as 5-fluorouracil (5-FU) still representing the foundation of chemotherapeutic regimens. While modest, the evolution of CRC management has seen a transition in treatment structure and administration from singular cytotoxic agents to targeted and multi-agent regimes. Incorporation of platinum agents, plant alkaloids and antibiotics to adjuvant and neoadjuvant chemotherapy combination regimens resulted in an improvement in both 5 and 10 year survival rates for CRC sufferers [34], highlighting the importance of continual advancement of CRC treatments [35-40].

2. The Evolution of Colorectal Cancer Chemotherapeutics

Since the introduction of 5-FU ($C_4H_3FN_2O_2$) into clinical treatment of CRC in 1957, progress in the management of advanced CRC has been modest [41]. Trends in treatment for stage III and IV metastatic CRC over the last several decades may be categorised into several eras based on induction of new chemotherapeutics and novel combinations.

2.1. 5-Fluorouracil and Leucovorin

For many years following fluoropyrimidine discovery, 5-FU remained the backbone of therapy for CRC and until recently was considered the standard first-line treatment for metastatic CRC [25]. Fluoropyrimidines were developed following the observation that rat hepatomas utilised pyrimidine uracil more rapidly than normal tissues [42], signifying that uracil metabolism may be a potential target for antimetabolite chemotherapy. 5-FU enters the cell using the same facilitated transport mechanism as uracil [43] and is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). These active metabolites disrupt RNA synthesis triggering mis-incorporation of fluoro nucleotides into RNA and DNA alongside inhibition of the enzyme thymidylate synthase [44]. Thymidylate synthase-catalysed reactions provide the sole source of thymidylate, a key molecule necessary for DNA replication and repair [44] (Fig. 1).

5-FU has been used for more than 50 years in the treatment of CRC. Many early randomized studies of 5-FU in the adjuvant setting failed to show significant improvement in patient survival [45-47], with the overall response rate as a single agent in advanced CRC reported as approximately 10–15% [48]. However, over the past 30 years important modulation strategies have been developed to reduce cytotoxicity, increase anti-cancer activity and overcome clinical resistance associated with 5-FU. Side-effect of 5-FU include stomatitis, esophagopharyngitis, diarrhoea, nausea, vomiting, dermatologic changes, alopecia, hematopoietic depression, fever, neurotoxicity, cardiotoxicity and death [49-51]. Leukopenia is recognised as a major dose-limiting toxicity of 5-FU monotherapy with rates as high as 93% reported in some studies [48, 52]. However, dose reduction from 15 mg/kg/day to 12 mg/kg/day has resulted in tolerable toxicity [48], allowing 5-FU to remain a key agent for the treatment of both advanced and early-stage CRC [44].

Leucovorin (LV) ($C_{20}H_{23}N_7O_7$) is a folinic acid derivative that enhances the cytotoxic effects of 5-FU by inhibiting the production of thymidylate synthase [44]. Thymidylate synthase catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), using reduced folate 5, 10-methylenetetrahydrofolate (CH_2THF) as the methyl donor [49] (Fig. 1). This reaction provides the sole *de novo* source of thymidylate, which is necessary for DNA replication and repair [44].

High intracellular levels of the reduced folate CH_2THF are necessary for optimal binding of the 5-FU metabolite FdUMP to thymidylate synthase. Leucovorin increases intracellular concentrations of CH_2THF and has been shown to increase both *in vitro* [53] and *in vivo* [54] toxicity of 5-FU in many cancer cell lines. Synergistic administration of 5-FU/LV has been shown to improve response rates in CRC by up to 37% when compared to a single agent 5-FU bolus as well as increasing median and overall survival [55, 56]. Several studies have found the dose-limiting toxicity of both high and low dose 5-FU/LV regimens to be gastrointestinal [52, 55] including severe ulcerative stomatitis occurring at rates as high as 30% of patients [51,52]. Although it has been reported that severe diarrhoea is not more frequent in patients treated with 5-FU/LV compared to patients treated with 5-FU alone [48], severe diarrhoea specifically has been described as a major treatment-related toxicity affecting approximately 40% of patients receiving 5-FU/LV, of these patients 52% not only required a dose reduction of 5-FU, but also hospitalization for intravenous hydration [55]. Moreover, treatment related diarrhoea has been correlated with the demise of 5% of the patient cohort [52].

Although modest, the improvements resulting from the combination of 5-FU with LV offered the possibility of enhancing the anti-cancer efficacy of 5-FU. Increased understanding of the

mechanisms of action of 5-FU in the years to come led to development of strategies to successfully increase its anti-cancer activity [44], with 5-FU now used in clinical combinations with several new generation anti-cancer chemotherapeutics.

2.2 Capecitabine

Capecitabine ($C_{15}H_{22}FN_3O_6$) is an oral prodrug that is enzymatically converted to 5-FU by thymidine phosphorylase. Derived from a predecessor prodrug of 5-FU, doxifluridine, capecitabine is metabolized to FU through 3 activation steps: i) capecitabine is absorbed through the intestine and converted to 5'-deoxy-S-fluorocytidine (5'-DFCR) by carboxylesterase and then ii) to 5'-deoxy-S-fluorouridine (5'-DFUR) by cytidine deaminase, in the liver; iii) thymidine phosphorylase then converts 5'-DFUR to the active drug, FU (Fig. 1). This occurs in both neoplastic and healthy tissues. However, thymidine phosphorylase is present at higher levels in tumour cells than in healthy tissues, allowing for selective activation of the drug and less systemic toxicity [57-59].

Initially approved in 1998 for use in patients with metastatic breast cancer whose tumours were resistant to standard chemotherapy with paclitaxel (Taxol) and an anthracycline-containing regimen, capecitabine was first trialled for the use in metastatic CRC in the early 2000's. Results demonstrated preferential conversion of capecitabine to 5-FU in colorectal tumours after oral administration to patients [60]. Several clinical trials have demonstrated that single agent capecitabine administration has a significantly superior response rate compared to 5-FU/LV, even in patient subgroups with poor prognostic indicators. Capecitabine, compared with bolus 5-FU/LV treatment also demonstrated improved safety producing a significantly lower incidence of diarrhea, stomatitis, nausea, alopecia and myelosuppression [61-63]. However, severe hand-foot syndrome and hyperbilirubinemia

were significantly more frequent in capecitabine-treated patients compared to those treated with 5-FU/LV [62]. Regardless of this, capecitabine patients required substantially fewer hospital visits for drug administration than 5-FU/LV patients and spent fewer days in hospital for the management of treatment-related adverse events than did patients treated with 5-FU/LV [61, 62].

2.3 Platinum-based chemotherapeutics for CRC

The serendipitous rediscovery of biologically active platinum complexes in the early 1960's, [64] triggered a resurrection in inorganic chemistry and re-evaluation of thousands of platinum analogues as potential chemotherapeutic agents [65]. As a result, platinum-based agents, cisplatin and oxaliplatin, are now used to treat a broad range of malignant diseases.

2.3.1 Cisplatin

Following *in vivo* administration in murine sarcoma in the late 1960's cisplatin ($\text{Cl}_2\text{H}_6\text{N}_2\text{Pt}$) was the first member in the class of platinum-containing anti-cancer drugs to enter phase I clinical trials in 1971 [65]. The main biochemical mechanism of action of cisplatin involves the formation of mono-adducts as well as intra- and interstrand crosslinks at the nucleophilic site of the DNA, resulting in interference with normal transcription and/or DNA replication mechanisms [66-68]. These adducts cause distortions in DNA, including unwinding and bending (Fig. 2), and are recognized by several cellular proteins; some of which are involved in DNA-repair pathways [69]. These disruptions trigger cytotoxic processes generally leading to apoptotic cell death [70], although the pathways from platinum–DNA binding to apoptosis remain incompletely elucidated [65]. There is continued debate as to which of the various platinum–DNA adducts is more biologically significant in response to cisplatin treatment.

Early trials evaluating the efficacy of cisplatin in combination with 5-FU showed no significant improvement in median survival time when compared to 5-FU alone [71-73], and although effective against some tumours, the administration of cisplatin was hindered by high rates of drug resistance and severe side-effects of nephrotoxicity, neurotoxicity and ototoxicity that limited its therapeutic potential [66, 74]. Although still regularly used for the treatment of lung cancer, bladder cancer and various gastrointestinal cancers, cisplatin alone and in combination with 5-FU has not shown any significant cytotoxic effect in CRC [73]. However, the discovery of platinum-DNA adducts through cisplatin stimulated the search for other anti-tumor active platinum complexes with improved pharmacological properties, resulting in the discovery of oxaliplatin.

2.3.2 Oxaliplatin

Oxaliplatin ($C_8H_{14}N_2O_4Pt$), is a third generation platinum-based agent currently used as the first line of treatment for metastatic CRC [37, 75]. It exerts its cytotoxic effects via formation of platinum-DNA adducts that trigger immobilization of the mitotic cell cycle and stimulates apoptosis of dividing cells [76, 77] (Fig. 2). At clinically recommended doses, it is reported to be less toxic for the auditory, hematologic and renal systems than predecessor drugs like cisplatin and carboplatin [78]. However, oxaliplatin appears to have a unique pattern of side-effects unrelated to those observed with other platinum-derived chemotherapeutics. During the course of oxaliplatin clinical trials, commonly reported side-effects included hematologic toxicity, gastrointestinal toxicity and peripheral sensory neuropathy [79]. Although these side-effects are prominent in oxaliplatin-treated patients, treatment with oxaliplatin has significantly improved efficacy for many tumours resistant to the first and second generation platinum-based agents [80, 81]. Several key studies undertaken in the early 2000's evaluated the effectiveness of oxaliplatin, as both a first and a second-line treatment in combination

with 5-FU/LV for CRC uncovering great therapeutic potential [80, 82-84]. Incorporation of oxaliplatin into treatment regimens led to the innovation of a novel therapeutic pathway in which patients may undergo a secondary potentially curative surgery.

2.4 Irinotecan

Irinotecan ($C_{33}H_{38}N_4O_6$) is a semi-synthetic analog of a naturally occurring quinoline alkaloid, camptothecin, which exerts its cytotoxicity through inhibition of topoisomerase I (Top I) [85]. Top I is essential for DNA transcription and acts to cut, relax and reanneal DNA strands. Irinotecan's active metabolite SN-38 binds to Top I and its DNA complex resulting in the formation of a stable ternary structure that prevents DNA re-ligation and promotes DNA damage and apoptosis (Fig. 3). Cellular toxicity from irinotecan administration is primarily a result of conversion from single-strand breaks into double-strand breaks during the S-phase of the cell cycle when the replication fork collides with the cleavage complexes formed by DNA and SN-38 [86, 87]. Phase II trials investigating the overall benefit of irinotecan in the late 1990's highlighted the positive impact of irinotecan as a second line therapy in 5-FU refractory patients with advanced CRC [40]. These studies uncovered that patients, whose disease did not respond to first line 5-FU therapy, showed increased survival times when administered sequential irinotecan compared to patients receiving supportive care [88] and 5-FU/LV [89].

Common side-effects associated with irinotecan administration include cholinergic syndrome, nausea, vomiting, constipation, dyspnoea and neutropenia [88, 89]; however, the major dose limiting side-effect is diarrhoea. Acute diarrhoea experienced within the first 24 hours following irinotecan administration, occurs in approximately 60-80% of patients [90]. This type of diarrhoea is thought to be primarily secretory with attenuation of symptoms by administration of atropine suggesting a cholinergic nature [91, 92]. Delayed onset diarrhoea,

experienced 24-48 hours following chemotherapeutic treatment occurs in approximately 80% of patients [93]. Irinotecan-induced diarrhoea has been linked to the enzymatic activity of β -glucuronidase in the intestinal microflora. Detoxified SN-38 (SN-38 glucuronide) is hydrolysed by β -glucuronidase in the lumen to reform an active metabolite SN-38, potentially contributing to delayed onset diarrhoea [94]. Treatment with the broad spectrum antibiotic neomycin has been found to reduce fecal β -glucuronidase activity to undetectable levels and decreases fecal concentrations of the active SN-38. Although neomycin had no significant effect on haematological toxicity, it was found to ameliorate diarrhoea in 86% patients, indicating that bacterial β -glucuronidase plays a crucial role in irinotecan-induced diarrhoea [95].

2.5 Combination and sequential therapies

Moving forward almost a decade, the most effective current anti-CRC agents remain 5-FU, cisplatin, oxaliplatin, irinotecan, and capecitabine. Although proven to be individually effective, multi-agent regimens including leucovorin (LV) given in combination or sequentially are now the standard line of chemotherapeutic treatment and are the most promising approaches to curative and non-curative CRC management [37-39, 81, 96-98]. A detailed analysis of outcomes and survival rates of the most pivotal studies for single agent and combination therapies for CRC has been published in a recent review [99].

2.5.1 FOLFOX, FOLFIRI and FOLFOXIRI

Originally intended as salvage therapies for patients failing to respond to single agent 5-FU treatment, combination therapies containing oxaliplatin and irinotecan are now clinically established first-line, second-line and sequential treatment options for advanced CRC [25, 37-40, 100]. Several key trials conducted in the late 1990's established improved efficacy and

overall survival of both FOLFOX (FOL = folinic acid (Leucovorin) + F = 5-fluorouracil (5-FU) + OX = oxaliplatin (Eloxatin)) and FOLFIRI (FOL = folinic acid (Leucovorin) + F = 5-fluorouracil (5-FU) + IRI = irinotecan (CPT-11) combinations over 5-FU/LV alone in metastatic CRC [39, 101].

Combination of oxaliplatin with 5-FU/LV (FOLFOX), in particular, has shown great therapeutic potential, improving progression-free survival by approximately 20% and overall survival rates by approximately 6% of stage II and III CRC patients [84, 98, 102]. Addition of oxaliplatin to 5-FU/LV has proven to significantly enhance antitumor efficacy, improving median progression free survival from 6.1 to 8.7 months in 5-FU/LV treated and FOLFOX treated patients respectively [103]. In a further study FOLFOX treatment compared to 5-FU/LV was found to increase response rates by almost 30% [37], additionally 20-30% of patients from various trials were referred for surgery, the median survival of this group was 3-4 years [104-106]. It was found, however, that in comparison to 5-FU/LV, FOLFOX had higher frequencies of grade 3/4 neutropenia (5.3% v 41.7% of patients), grade 3/4 diarrhea (5.3% v 11.9%), and grade 3 neurosensory toxicity (0% v 18.2%) [37].

Several phase III trials have demonstrated a significant survival advantage for irinotecan combined with 5-FU/LV (FOLFIRI), compared to 5-FU/LV alone in patients with metastatic CRC [38, 39]. A multicentre trial evaluating FOLFIRI as a first line therapy for metastatic CRC found that patients receiving FOLFIRI combination had significantly higher response rates when compared to 5-FU/LV (49% vs 31% respectively) and average time to progression was significantly longer in the FOLFIRI group (6.7 vs 4.4 months) [38]. These studies established irinotecan-based chemotherapy as the preferred comparator regimen and

FOLFIRI was approved as a first-line therapy for advanced CRC by the United States Food and Drug Administration in 2000, replacing 5-FU/LV as the standard of care [33, 40].

Given increased efficacy demonstrated by FOLFOX and FOLFIRI when compared with 5-FU/LV alone in randomized studies [37-39, 103] the notion of a triple combination therapy FOLFOXIRI (FOL = folinic acid (Leucovorin) + F = 5-fluorouracil (5-FU) + OX = oxaliplatin (Eloxatin) + IRI = irinotecan (CPT-11) emerged. Initial phase I-III studies found FOLFOXIRI treatment was well tolerated; with a response rate of 72%, and a median progression free survival of 10.8 and overall survival of 28.4 months. Furthermore, it was found that resection of residual metastatic disease could be performed in 26% of initially unresectable patients, with the 4 year survival of these patients recorded as 37% [107-109]. A phase III trial comparing infusional FOLFOXIRI to FOLFIRI demonstrated superior response rates as well as significantly improved progression free survival and overall survival in FOLFOXIRI treated patients [100].

2.5.2 XELOX and XELIRI

Capecitabine's equivalent efficacy and clinically significant safety advantage compared to 5-FU treatment underpinned the rationale for capecitabine inclusive combination and sequential treatments [62, 110, 111]. Early studies confirmed that the combination of capecitabine with oxaliplatin (XELOX) was feasible and established with the recommended dose regimen that safety was predictable and similar to the FOLFOX regimen [112]. Phase II studies combining capecitabine with either irinotecan (XELIRI) (XEL = capecitabine (Xeloda) + IRI = irinotecan (CPT-11)) or oxaliplatin (XELOX) (XEL = capecitabine (Xeloda) + OX = oxaliplatin (Eloxatin)) have shown efficacy and toxicity comparable with combination schedules involving 5-FU [113, 114]. XELOX has shown to be a highly effective first-line

treatment for metastatic CRC with response rates, time to progression and overall survival similar to those observed with FOLFOX combinations. Studies comparing XELOX with 5-FU/LV as adjuvant therapy for stage III CRC demonstrated that XELOX therapy improved disease free survival 70.9% when compared to 66.5% with FU/LV and had an increased overall survival rate of 77.6% compared to 5-FU/LV (74.2%) [35]. XELOX treatment was associated with lower rates of neutropenia and stomatitis compared to patients treated with 5-FU/LV. However, it was associated with higher rates of hand-foot syndrome and thrombocytopenia than those seen in 5-FU/LV-treated patients [35].

Although XELIRI showed promise in early trials as an active first line treatment for metastatic CRC [115], further studies showed little benefit of XELIRI over FOLFIRI [116, 117]. No significant difference between XELIRI and FOLFIRI in grade 3 or 4 adverse events, including diarrhoea, neutropenia, ischemic stroke and acute coronary syndrome, was found [117]. It was concluded the XELIRI regimen showed similar progression free survival and overall survival with acceptable toxicity compared to the FOLFIRI regime. It has been reported that upfront combination chemotherapy is more toxic, but not more effective than the sequential use of the same cytotoxic drugs in patients with advanced, non-resectable CRC [118].

3. Targeted therapies

Although advances in chemotherapeutic therapies have led to improved outcomes for patients with CRC, toxicity and lack of specificity are major limitations in clinical application. Greater understanding of cancer signalling pathways has led to considerable progress in generating therapies to target specific elements of these pathways in a bid to improve therapeutic efficacy and specificity [119]. Receptor tyrosine kinases involved in regulating

cell proliferation, apoptosis and angiogenesis have been strongly implicated in tumour growth and progression [120]. Much research has been undertaken over the last decade investigating the role that these receptors may play in the management of cancer. Targeted drugs approved for metastatic CRC may be divided into 3 categories i) monoclonal antibodies against VEGF and EGFR, ii) recombinant fusion proteins against angiogenic factors and iii) molecules that inhibit tyrosine kinase receptors [121].

3.1 Bevacizumab, Cetuximab and Panitumumab

Epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) are members of the receptor tyrosine kinase family, and have been implicated in processes involved in tumour growth and progression. Both epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) over expression has been found in a wide range of solid tumours, including CRC [122]. As a result, considerable research has been undertaken to investigate the potential of EGF and VEGF inhibition in anticancer treatment [123, 124]. Between 2004 and 2006, three novel monoclonal antibodies directed against EGFR and VEGF, bevacizumab, cetuximab and panitumumab, were approved by the US Food and Drug Administration for the treatment of metastatic CRC [125, 126].

Bevacizumab (Avastin) ($C_{6538}H_{10034}N_{1716}O_{2033}S_{44}$) is a humanised monoclonal antibody developed against VEGF that binds to soluble VEGF, preventing receptor binding and inhibiting endothelial cell proliferation and vessel formation [127]. Major roles of VEGF include stimulation of endothelial cell proliferation and mediation of blood vessel permeability making it a key facilitator in angiogenesis [128, 129]. In randomised trials in patients with metastatic CRC, bevacizumab improved response rates, overall survival and progression-free survival when combined with 5-FU/LV, FOLFIRI and FOLFOX or

(XELOX) compared with chemotherapy alone [130-134]. However, in a phase III trial comparing XELIRI-bevacizumab and FOLFIRI-bevacizumab as first-line treatment in patients with previously untreated metastatic CRC no significant differences were found when comparing progression-free and overall patient survival [116]. A limitation is that bevacizumab is ineffective on its own [121, 135].

Cetuximab (C₆₄₈₄H₁₀₀₄₂N₁₇₃₂O₂₀₂₃S₃₆) is a recombinant human/mouse chimeric monoclonal antibody that binds to the extracellular domain of human EGFR [120]. Cetuximab competitively inhibits the binding of epidermal growth factor (EGF) and other ligands to EGFR, blocking receptor phosphorylation and activation of receptor-associated kinases consequently inhibiting downstream signal transduction [124, 136, 137]. Cetuximab is approved as a monotherapy or in combination with irinotecan for the treatment of EGFR-expressing metastatic CRC carcinoma in patients intolerant or refractory to irinotecan-based chemotherapy [120]. Clinical trials involving cetuximab have found that FOLFIRI plus cetuximab significantly improves overall survival, progression free survival and response rate in patients with KRAS wild-type tumours when compared to FOLFIRI alone [138, 139], however BRAF tumour mutation has been associated with poor prognosis [139].

Panitumumab (C₆₃₉₈H₉₈₇₈N₁₆₉₄O₂₀₁₆S₄₈) is a recombinant human monoclonal antibody that binds with high affinity to EGFR. Panitumumab blocks the binding of EGFR ligands to various EGFR-expressing human cancer cell lines, and inhibits EGF dependent tumour-cell activation [140, 141]. Panitumumab is approved as a monotherapy for patients with EGFR-expressing refractory metastatic CRC [125], its use is associated with an equivalent overall survival benefit to that of cetuximab [142]. Panitumumab has been evaluated in first-line setting combined with standard FOLFOX regimen in patients with metastatic CRC [143]. A

modest advantage in progression-free survival was found only in KRAS wild-type tumour bearing patients, however no improvements in overall survival were found [143]. It has been suggested that panitumumab should have a lower efficacy than cetuximab [143], however further studies have found that panitumumab is not inferior to cetuximab and that both agents provide similar overall survival benefits in patients with chemotherapy-refractory KRAS mutant patients with metastatic colorectal cancer [142]. Notably, detrimental effects on progression-free survival have been observed in KRAS mutant patients, who received panitumumab in combination with FOLFOX and FOLFIRI in second-line treatment [121, 144].

3.2 Aflibercept

Aflibercept (C₄₃₁₈H₆₇₈₈N₁₁₆₄O₁₃₀₄S₃₂) is a recombinant fusion protein containing VEGF-binding portions from the extracellular domains of human VEGF receptors 1 and 2, fused to human immunoglobulin (Ig)G1 [145]. Acting as a high-affinity ligand trap, aflibercept blocks the activity of VEGF-A primarily involved in angiogenesis, vasculogenesis and vascular permeability, VEGF-B involved in embryonic and pathological angiogenesis, and placental growth factor by preventing ligands from binding to their endogenous receptors [146]. In non-clinical studies, aflibercept demonstrated tumour growth inhibition *in vivo* with a potent antiangiogenic action [147, 148], however results from clinical trials with various chemotherapeutics are conflicting [145, 149]. A phase II study investigating aflibercept combined with modified FOLFOX6 as a first-line treatment of metastatic CRC found no significant difference in progression-free survival at 1 year follow up [149]. A further phase III trial investigating aflibercept combined with FOLFIRI reported significantly improved overall survival, progression-free survival and response rate when compared to FOLFIRI/placebo [145]. But pre-treatment appeared to have no impact on overall survival

and progression free survival with a consistent trend across all pre-specified subgroup analyses, including bevacizumab pre-treated patients [145, 150]. Moreover, adverse effects including diarrhoea, asthenia, stomatitis and ulceration, infections, hypertension, gastrointestinal and abdominal pain, neutropenia and neutropenic complications, and proteinuria reported with aflibercept/FOLFIRI resulted in increased incidence of some chemotherapy-related toxicities [145].

3.3 Regorafenib

Regorafenib ($C_{21}H_{15}ClF_4N_4O_3$) (Fig. 4) is an oral multi-kinase inhibitor that targets angiogenic, stromal and oncogenic receptor tyrosine kinases (RTKs) [151]. RTKs have been implicated in various signalling pathways in the development and progression of CRC as well as in downstream signalling cascades [152]. Regorafenib blocks the activity of several protein kinases, including those involved in the regulation of tumour angiogenesis and is the first small-molecule multi kinase inhibitor to demonstrate a survival benefit in metastatic CRC that has progressed after all standard therapies [153]. In a recent phase III study, regorafenib showed significant overall survival benefit in patients with treatment refractory metastatic CRC. Patients receiving best supportive care and regorafenib demonstrated an improved overall survival 1.4 months longer than a comparable group receiving best supportive care and placebo [153]. As a result, regorafenib is now considered to be a standard treatment option in pre-treated patients [154]. However side-effects of regorafenib include a specific hand-foot-skin reaction, fatigue and elevated liver enzymes, limiting the benefit to only those patients with a good performance status and adequate organ function [154].

Table 1. Current and emerging treatments for colorectal cancer

| Agent | Mechanism | Key studies |
|----------------------------|--|-----------------|
| Current Treatments | | |
| 5-Fluorouracil | Fluoropyrimidine: fluoro nucleotide mis-incorporation into RNA and DNA alongside inhibition of the enzyme thymidylate synthase. | [52, 55] |
| Leucovorin | Folinic acid derivative | [155] |
| Capecitabine | Fluoropyrimidine: oral prodrug for 5-fluorouracil | [156] |
| FOLFOX | Combination regime containing: FOL = folinic acid (Leucovorin) + F = 5-fluorouracil (5-FU) + OX = oxaliplatin (Elotaxin) | [84, 157] |
| FOLFIRI | Combination regime containing: FOL = folinic acid (Leucovorin) + F = 5-fluorouracil (5-FU) + IRI = irinotecan (CPT-11) | [39, 158] |
| FOLFOXIRI | Combination regime containing: FOL = folinic acid (Leucovorin) + F = 5-fluorouracil (5-FU) + OX = oxaliplatin (Elotaxin) + IRI = irinotecan (CPT-11) | [33, 107] |
| XELOX | Combination regime containing: XEL = capecitabine (Xeloda) + OX = oxaliplatin (Elotaxin) | [112, 113, 159] |
| XELIRI | Combination regime containing: XEL = capecitabine (Xeloda) + IRI = irinotecan (CPT-11) | [115, 160] |
| Bevacizumab | VEGF inhibitor | [133, 134] |
| Cetuximab | EGFR inhibitor | [138, 139, 161] |
| Panitumumab | EGFR inhibitor | [143] |
| Aflibercept | VEGF inhibitor | [145, 149] |
| Regorafenib | Oral multi-kinase inhibitor | [153, 154] |
| Emerging Treatments | | |
| Celecoxib | COX 2 inhibitor | [162, 163] |
| Ruthenium | Transition metal complex | [164-166] |
| Olaparib | PARP inhibitor | [167-169] |

4. Potential Chemotherapeutics for Colorectal Cancer

The last decade has seen significant advancement in the treatment of CRC. A plethora of promising emerging treatments exploring various mechanisms are currently under investigation or in clinical trials for the treatment of CRC (Table 1). Although there are numerous emerging treatments, this review will focus on anti-inflammatory therapies, metal complex chemotherapeutics and PARP inhibitors.

4.1 Anti-inflammatories

Administration of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin has been shown to significantly reduce CRC mortality [170, 171]. NSAIDs appear to work by preventing the production of prostaglandins in inflammatory or neoplastic tissue through inhibition of the cyclooxygenase (COX) pathways [172]. COX enzymes are required for the conversion of arachidonic acid to prostaglandins. COX-1 is constitutively expressed in all tissues; whereas COX-2 is predominantly expressed in inflammatory or neoplastic tissue [173, 174]. COX-2 mediates the inflammatory effects of the COX enzyme activity, and is induced by a wide spectrum of growth factors and pro-inflammatory cytokines. Overexpression of COX-2 has been demonstrated in numerous premalignant and malignant lesions including CRC [171]. Selective COX-2 inhibition has shown great promise as both a chemopreventative and a chemotherapeutic. However addition of COX-2 inhibitors to various chemotherapeutic regimes appears to yield contradictory results.

Administration of celecoxib ($C_{17}H_{14}F_3N_3O_2S$) (Fig. 4), a selective COX 2 inhibitor, in randomised, double blinded, placebo controlled studies has been found to significantly reduce duodenal polyposis [172] and occurrence of rectal adenomas [175]. Celecoxib combined with FOLFOX7 was reported to have an acceptable safety profile; it produced response rates and progression-free survival in the lower range of those obtained with FOLFOX alone,

progression free survival at 6 months appeared lower than in patients receiving FOLFOX7 with simplified LV/5-FU2 [162]. In contrast, addition of celecoxib to the FOLFIRI regime in patients with advanced CRC appeared to have no effect on patient outcome [163].

4.2 Metal Complex Chemotherapeutics

Based on the clinical success of platinum-based anti-cancer drugs, a variety of metal-based anti-cancer compounds are currently under investigation in clinical trials. In particular, several ruthenium-based compounds exhibiting unique biochemical properties and reduced toxicity profiles compared to the clinically used platinum-based drugs have been identified [176, 177]. The idea of studying ruthenium compounds as anti-tumor agents was originally inspired by the observation that certain ruthenium complexes preferentially localize in tumour tissue [178]. Ruthenium is a transition metal of the platinum group and is believed to exert its cytotoxic effects similarly to platinum-based drugs via direct interaction with DNA [179]. However, in contrast to platinum-based drugs, ruthenium appears to accumulate preferentially in neoplastic tissues whilst sparing healthy tissue [180, 181].

In early pharmacokinetic studies, a ruthenium indazole analogue, KP1019 ($C_{21}H_{19}C_{14}N_6Ru$) (Fig. 5) produced up to 95% reduction of tumour volume without any mortality or considerable weight loss in rat models of CRC [178, 182]. In addition, it was found to be superior to 5-FU [182]. Several ruthenium agents have entered clinical trials producing promising results to date [164-166, 183].

4.3 PARP inhibitors

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear protein with DNA binding domains that play a key role in DNA repair. Discovered more than 50 years ago, PARP inhibitors, originally engineered to elucidate the function of the PARP enzyme, were serendipitously

found to inhibit DNA repair and increase the potency of anti-cancer cytotoxic agents [64]. PARP inhibitors of increasing strength were then developed and entered clinical trials in cancer patients in 2003 as chemo- and radio-sensitizers [184]. However in 2005, the finding that PARP inhibitors are lethal in cells with DNA repair defects led to major interest in this class of drug as a potential chemotherapeutic [184]. Several PARP inhibitors have since entered clinical trials and have shown promising activity in breast, ovarian and other cancers associated with defects in homologous recombination DNA repair.

PARP inhibitors have been evaluated in clinical trials as single agents as well as in combination with DNA damaging therapies [185]. In animal models, PARP-1 knockout has shown to significantly impair DNA repair following damage via radiation [186] and cytotoxic insult [187]. Furthermore combining low-dose radiotherapy with PARP inhibition has been shown to enhance anti-tumour efficacy through potentiating DNA damage [167]. In phase I clinical trials enhanced DNA damage has been identified in tumor biopsies and circulating tumor cells following PARP inhibition in patients with chemotherapy-refractory solid tumours already receiving topoisomerase I inhibitor, topotecan [169]. In phase II trials the PARP inhibitor, olaparib ($C_{24}H_{23}FN_4O_3$) (Fig. 6), demonstrated single agent activity in both breast and ovarian cancer with an overall response rate of approximately 40% in patients with germline mutations in BRCA1/2 [168, 188].

Although early cell culture studies concluded that microsatellite instable (MSI) colorectal tumours deficient in double strand break repair showed higher sensitivity to PARP-1 inhibition [189], phase II trials using PARP inhibitor olaparib in previously treated MSI and microsatellite stable (MSS) CRC patients failed to show any anti-cancer activity [190]. Following treatment with olaparib, median progression free survival for all patients was 1.84 months with no statistically significant differences MSS and MSI groups [190]. The use of

DNA-damaging chemotherapy and/or radiation therapy in combination with PARP inhibitors should be tested in further studies.

However, interestingly PARP inhibitors have also been shown to restore sensitivity of resistant tumours to methylating agents or topoisomerase I inhibitors, drugs presently used for the treatment of primary and secondary brain tumours or malignancies refractory to standard chemotherapy [191]. Furthermore, PARP inhibitors may also provide protection from dose-limiting side-effects exerted by certain anti-cancer drugs, which trigger oxidative stress and consequent PARP over-activation [191].

5. Conclusion

No treatment options are currently available for patients with metastatic CRC that progresses after all approved standard therapies, although many patients maintain a good performance status and could be candidates for further therapy. Therefore, the search for novel chemotherapeutics and molecular targets is imperative to improving overall patient survival and curability of CRC. Because of the prolonged chemotherapy treatment of patients with CRC and the approach of continuous chemotherapy until progression, the development of novel methods or drugs that ameliorate treatment toxicity is of major importance.

CONFLICT OF INTERESTS

The authors declare they have no competing interests.

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Figure Legends

Figure 1. 5-Fluorouracil, Capecitabine and Leucovorin

Capecitabine is an oral prodrug that is converted to its only active metabolite, 5-Fluorouracil (5-FU) by thymidine phosphorylase. The chief mechanism of 5-FU activation is conversion of 5-FU to fluorouridine monophosphate (FUMP). FUMP is phosphorylated to fluorouridine diphosphate (FUDP), which can either be further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or be converted to fluorodeoxyuridine diphosphate (FdUDP). In turn, FdUDP can either be phosphorylated or dephosphorylated to generate the active metabolites fluorodeoxyuridine triphosphate (FdUTP) and fluorodeoxyuridine monophosphate (FdUMP), respectively. An alternative activation pathway involves the thymidine phosphorylase catalysed conversion of 5-FU to fluorodeoxyuridine (FdUR), which is then phosphorylated to FdUMP. Thymidylate synthase (TS) catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5,10-methylene tetrahydrofolate (CH₂THF) as the methyl donor. Active metabolite FdUMP binds to TS and CH₂THF, blocking access of dUMP to the nucleotide-binding site and inhibiting dTMP synthesis. This results in imbalances to deoxynucleotide (dNTP) levels and accumulation of deoxyuridine triphosphate (dUTP). Accumulating dUTP triggers misincorporation of uracil into the DNA via DNA polymerase. Although the DNA repair mechanism can excise the misincorporated DNA, the existing dUMP is just re-phosphorylated to dUTP and re-incorporated back into the DNA. This process is known as futile cycling. DNA strand breakage and damage occur as repair mechanisms ultimately fail.

Figure 2. Cisplatin and Oxaliplatin

In aqueous condition cisplatin is converted into active metabolites mono-aqua and diaqua. These metabolites bind with DNA to form 1,2-intrastrand cross-links with purine bases resulting in changes in the conformation of DNA that affect DNA replication.

Oxaliplatin is a third generation coordination complex of platinum II with a 1,2-diaminocyclohexane (DACH) core. Oxaliplatin is activated through conversion to monochloro, dichloro and diaquo via non-enzymatic hydrolysis with the displacement of the oxalate group. Cytotoxicity of oxaliplatin is attributed to the formation of inter- and intra-strand cross links in DNA which prevent DNA replication and transcription.

Figure 3. Irinotecan

Irinotecan is converted into its active metabolite SN38 through hydrolysis by enzyme carboxylesterase. SN38 forms a complex with DNA and topoisomerase I (Top I). (Top I) is responsible for cleaving and unwinding the DNA for replication and transcription. Formation

of SN38 and Top I complexes inhibits the activity of Top I causing obstruction of the re-ligation of DNA and blocking the movement of DNA polymerase resulting in inhibition of DNA replication and transcription. SN38 is deactivated through glucuronidation by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) to form SN38 glucuronide (SN38-G), but can be reactivated in the presence of β -glucuronidase to SN38.

Figure 4. Targeted Therapies and Anti-Inflammatories

Chemical structures of Regorafenib and Celecoxib.

Figure 5. Ruthenium

Ruthenium (Ru) seems to accumulate preferentially in neoplastic tissue rather than healthy tissue, it has been suggested that transferrin receptor may be responsible for this phenomenon, by actively transporting transferrin–Ru complexes into neoplastic tissues which contain high transferrin receptor densities. Once bound by the transferrin receptor, it is believed that ruthenium is liberated and internalized by the tumour where it is “activated by reduction” from inactive Ru (III) to reactive Ru (II) due to the lower oxygen content and higher acidity within the cell. The exact mechanism of Ru-induced DNA damage remains unclear, however it has been hypothesized that Ru exert its anticancer effects by direct interaction with DNA as observed with platinum. Ru compound KP1019 induces DNA damage leading to cell cycle delay and cell death.

Figure 6. PARP inhibitor Olaparib

Poly(ADP-ribose) polymerase (PARP) is a critical enzyme in the repair of DNA strand breaks. PARP-1 uses Nicotinamide Adenine Dinucleotide (NAD^+), comprised of an ADP Ribose molecule bound to Nicotinamide (Nam), as a substrate to synthesize Poly-ADP Ribose Polymerase (PAR). DNA damage results in the recruitment and binding of PARP-1 to the site of damage, triggering the formation of long branched Poly-ADP Ribose (PAR) chains. PAR promotes recruitment of DNA repair proteins involved in the base excision repair pathway to the site of DNA damage, and facilitates removal of PARP-1 from damage sites, allowing access to other repair proteins. Olaparib is a potent inhibitor of both PARP-1 and PARP-2.



Chemotherapy-Induced Constipation and Diarrhea: Pathophysiology, Current and Emerging Treatments

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Gastrointestinal (GI) side-effects of chemotherapy are a debilitating and often overlooked clinical hurdle in cancer management. Chemotherapy-induced constipation (CIC) and Diarrhea (CID) present a constant challenge in the efficient and tolerable treatment of cancer and are amongst the primary contributors to dose reductions, delays and cessation of treatment. Although prevalence of CIC is hard to estimate, it is believed to affect approximately 16% of cancer patients, whilst incidence of CID has been estimated to be as high as 80%. Despite this, the underlying mechanisms of both CID and CIC remain unclear, but are believed to result from a combination of intersecting mechanisms including inflammation, secretory dysfunctions, GI dysmotility and alterations in GI innervation. Current treatments for CIC and CID aim to reduce the severity of symptoms rather than combating the pathophysiological mechanisms of dysfunction, and often result in worsening of already chronic GI symptoms or trigger the onset of a plethora of other side-effects including respiratory depression, uneven heartbeat, seizures, and neurotoxicity. Emerging treatments including those targeting the enteric nervous system present promising avenues to alleviate CID and CIC. Identification of potential targets for novel therapies to alleviate chemotherapy-induced toxicity is essential to improve clinical outcomes and quality of life amongst cancer sufferers.

Keywords: chemotherapy, chemotherapy-induced constipation, chemotherapy-induced diarrhea, pathophysiology, treatments

INTRODUCTION

Cancer is a leading cause of death worldwide (Jemal et al., 2011; Torre et al., 2015) with approximately 14.1 million new cancer cases and 8.2 million cancer deaths in 2012 alone (Ferlay et al., 2015). Although advances in modern medicine have improved scanning and cancer detection techniques, the burden for global health of cancer is expected to intensify in decades to come particularly in low and middle income families and economically developed countries (Jemal et al., 2010a,b). Population aging and growth coupled with the adoption of high risk lifestyle choices

Abbreviations: CIC, chemotherapy-induced constipation; CID, chemotherapy-induced diarrhea; ENS, enteric nervous system; GI, gastrointestinal.

such as smoking, physical inactivity, and westernization of diets have been identified as underlying factors contributing to the increasing incidence of cancer worldwide (Jemal et al., 2011). It is now anticipated that by 2025 more than 20 million people will be affected by cancer (Ferlay et al., 2015).

Most cancer patients receive curative or palliative chemotherapeutic intervention throughout the course of treatment (Louvét et al., 2002; Benson et al., 2004b; Kaufmann et al., 2006; Wagner et al., 2006; Goffin et al., 2010; Okines et al., 2010). Although chemotherapy has greatly improved overall survival in many types of cancer, cytotoxic side-effects are a significant hurdle greatly impeding the clinical application of otherwise beneficial therapies (Xue et al., 2011; Iwamoto, 2013). GI side-effects such as nausea, vomiting, ulceration, bloating, constipation and, in particular, diarrhea are major obstacles causing delays, adjustments, and discontinuation of treatment whilst greatly impacting quality of life in many cancer patients (Benson et al., 2004a; Stringer et al., 2007, 2009d; Denlinger and Barsevick, 2009; Peterson et al., 2011). Although specific chemotherapeutic agents have been correlated with heightened incidence of GI side-effects (Table 1), incidences as high as 40% in patients receiving standard dose chemotherapy and 100% in patients receiving high dose chemotherapy have been reported (McQuade et al., 2014). Furthermore, the incidence of chronic post-treatment constipation and diarrhea amongst cancer survivors has been estimated to be as high as 49% with episodes persisting up to 10 years after the cessation of treatment (Schneider et al., 2007; Denlinger and Barsevick, 2009; Kim et al., 2012). The underlying mechanisms of CIC and diarrhea (CID) remain unclear. Although mucositis presenting as inflammation and ulceration of the intestinal epithelium is a significant contributing factor, the pathophysiology of CID and CIC is likely to be complex, involving several overlapping inflammatory, secretory and neural mechanisms.

CHEMOTHERAPY-INDUCED DIARRHEA

Diarrhea is a frequently under-recognized clinical issue that significantly affects morbidity and mortality of cancer patients worldwide (Maroun et al., 2007). Prevalence and severity of CID vary greatly depending on chemotherapeutic regime administration and dosage. A direct correlation between cumulative dose and severity of CID has been recognized, with high dose regimens associated with heightened incidence of CID (Verstappen et al., 2003). Certain regimens, especially those containing 5-fluorouracil and irinotecan are associated with rates of CID of up to 80% (Benson et al., 2004a; Richardson and Dobish, 2007) with one third of patients experiencing severe (grade 3 or 4) diarrhea (Table 2) (Maroun et al., 2007).

Chemotherapy-induced diarrhea severely interferes with anti-cancer treatment, resulting in treatment alterations in approximately 60% of patients, dose reductions in 22% of patients, dose delays in 28% of patients and complete termination of treatment in 15% of patients (Arbuckle et al., 2000; Dranitsaris et al., 2005). Moreover, CID has been

reported to last as long as 10 years post-treatment (Denlinger and Barsevick, 2009). Persistent and severe chemotherapy-associated Diarrhea is correlated with significant malnutrition and dehydration resulting in concomitant weight loss (cachexia), fatigue, renal failure, hemorrhoids, and perianal skin breakdown (Mitchell, 2006; Shafi and Bresalier, 2010). CID related dehydration is linked to early death rates in roughly 5% of patients undergoing anti-cancer treatment (Rothenberg et al., 2001). Further to this, chemotherapeutic administration may also prompt severe intestinal inflammation, bowel wall thickening and ulceration (Kuebler et al., 2007) contributing to clinical disruptions with potentially life-threatening ramifications (Rothenberg et al., 2001; Benson et al., 2004a; Stein et al., 2010).

For over 30% of CID sufferers it interferes with their daily activities (Stein et al., 2010), with detrimental effects on the mental and social health of cancer survivors. Persistent and uncontrollable CID has been linked to anxiety, depression, social isolation, and low self-esteem (Viele, 2003), emphasizing the importance of both elucidating the underlying mechanisms of CID and improving treatment efficacy (Carelle et al., 2002).

Pathophysiology of Chemotherapy-Induced Diarrhea

Although several chemotherapy regimens have been associated with Diarrhea to varying degrees (Table 1), most basic research into the mechanisms underlying CID has focused on irinotecan and its active metabolite SN38 (Gibson and Keefe, 2006). As diarrhea is a well-recognized side-effect of irinotecan treatment, the histological changes that occur throughout the GI tract in response to irinotecan administration have been examined in several animal studies (Araki et al., 1993; Ikuno et al., 1995; Takasuna et al., 1996; Gibson et al., 2003). Pronounced crypt ablation, villus blunting and epithelial atrophy in the small and large intestines have been reported (Logan et al., 2008), resulting in mucosal damage and degeneration being a major theme throughout the literature surrounding CID. Although patients do not routinely have imaging or endoscopy to diagnose the chemotherapy-induced mucosal inflammation (Toucheffeu et al., 2014), CID is still largely believed to be a form, or by-product, of GI mucositis. Mucositis is defined as mucosal injury presenting as inflammation and ulceration, resulting in alterations of intestinal microflora and GI secretion (Stringer, 2009; Stringer et al., 2009a,b). The basic pathophysiology of mucositis can be broken into 5 sequential phases: (i) initiation; (ii) up-regulation; (iii) signaling and amplification; (iv) ulceration and inflammation; and (v) healing (Sonis et al., 2004; Lee et al., 2014).

Initiation of mucositis is believed to result from direct or indirect effects of cytotoxic chemotherapeutics on the rapidly dividing epithelial cells in GI tract, triggering apoptosis. This leads to reductions in crypt length and villus area, coupled with activation of nuclear factor-kappa B (NFκB) and subsequent up-regulation of pro-inflammatory cytokines including interleukin 1 (Lawrence, 2009), which contribute to ulceration and inflammation in the mucosal epithelium (Gibson et al., 2003; Stringer et al., 2007, 2008, 2009a,d; Logan et al., 2008). Intestinal microbiota is known to play an integral role in

intestinal homeostasis and are now believed to play a key role in the development of mucositis (van Vliet et al., 2010; Touchefeu et al., 2014). Recent studies have revealed that chemotherapeutic administration has effects on intestinal microbial composition (Stringer et al., 2009a,b), and fecal microbiota (Touchefeu et al., 2014).

Much of the research investigating the effects of chemotherapeutic administration on microbiota has focused primarily on topoisomerase I inhibitor, irinotecan, due to the involvement of microbiota in its metabolism (Stringer, 2013). Upon metabolism in the liver, irinotecan is converted to its active metabolite SN-38 by enzyme carboxylesterase, before being deactivated through glucuronidation by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) to form SN38 glucuronide (SN38-G). However, SN38G may be reactivated to SN38 in the presence of enzyme β -glucuronidase, which may be produced by the intestinal microbiome. Several studies have shown a shift in commensal bacteria, in particular

Bifidobacterium spp. toward *Salmonella* spp. and *Escherichia coli* following irinotecan administration (Stringer et al., 2009b). Of the β -glucuronidase-producing bacteria, *Bacteroides* spp. has been shown to decrease following irinotecan treatment, concurrently *Staphylococcus* spp., *Clostridium* spp. and *E. coli* have been found to be increased, whilst presence of beneficial bacteria, *Lactobacillus* spp. and *Bifidobacterium* spp. was decreased following irinotecan treatment (Stringer et al., 2007). When given in combination with antimetabolite 5-fluorouracil, both *Clostridium cluster XI* and *Enterobacteriaceae* presence was found to be increased, whilst treatment with 5-fluorouracil alone has also been found to increase the presence of *Clostridium* spp. and *Staphylococcus* spp. at 24 h post-treatment (Stringer et al., 2009c).

These changes in microbiota are believed to play an important role not only in maintaining intestinal homeostasis and integrity but in the modulation of inflammatory responses through interaction with Toll-like receptors and the nucleotide

TABLE 1 | Gastrointestinal side-effects of chemotherapy.

| Mechanisms | Chemotherapeutic agents | Cancer type | GI side-effects |
|--------------------|-------------------------|--|--|
| Alkylating Agents | Cisplatin | Lung, Breast, Stomach, Colorectal, Liver | Nausea, Vomiting, Diarrhea, Constipation (Ilson et al., 1999; Ardizzoni et al., 2007) |
| | Cyclophosphamide | Breast | Nausea, Vomiting, Abdominal Pain, Diarrhea (Fraiser et al., 1991; Boussios et al., 2012) |
| | Oxaliplatin | Colorectal, Breast, Stomach | Nausea, Vomiting, Diarrhea, Constipation (Edra et al., 1990; Kim et al., 2003) |
| Antimetabolites | 5-Fluorouracil | Breast, Colorectal, Stomach, Liver | Nausea, Vomiting, Abdominal Pain, Diarrhea (Douillard et al., 2010; Boussios et al., 2012) |
| | Capecitabine | Colorectal, Breast, Stomach | Nausea, Vomiting, Diarrhea (Walko and Lindley, 2005; Stathopoulos et al., 2007; Boussios et al., 2012) |
| | Gemcitabine | Lung, Breast | Nausea, Vomiting, Abdominal Pain, Constipation, Diarrhea (Walf et al., 2001; Mutch et al., 2007; Boussios et al., 2012) |
| | Methotrexate | Breast | Nausea, Vomiting, Abdominal Pain, Diarrhea (Boussios et al., 2012) |
| Anthracycline | Doxorubicin | Breast, Lung, Liver | Nausea, Vomiting, Abdominal pain, GI Ulceration, Diarrhea (Boussios et al., 2012; Tecar et al., 2013) |
| | Thalidomide | Myeloma, Kidney | Nausea, Vomiting, Diarrhea, Constipation (Smith et al., 2008) |
| Mitotic inhibitors | Cabazitaxel | Prostate | Nausea, Vomiting, Abdominal pain, Diarrhea (Nightingale and Ryu, 2012; Dieras et al., 2013) |
| | Docetaxel | Prostate, Breast, Lung, Stomach | Nausea, Vomiting, Diarrhea (Boussios et al., 2012) |
| | Paclitaxel | Lung, Stomach, Prostate, Breast | Nausea, Vomiting, Diarrhea (Boussios et al., 2012) |
| | Vincristine | Breast, Lung | Constipation , Abdominal Pain (Holland et al., 1973) |
| | Irinotecan | Colorectal, Breast, Stomach, Lung | Nausea, Vomiting, Acute and Delayed Diarrhea (Hecht, 1998) |

TABLE 2 | Common toxicity criteria for diarrhea and constipation grading (adapted from the National Cancer Institute).

| Toxicity | Grade 1 | Grade 2 | Grade 3 | Grade 4 | Grade 5 |
|--------------|--|--|--|---|---------|
| Diarrhea | Increase of <4 stools per day over baseline. | Increase of 4–6 stools per day over baseline. | Increase of >7 stools per day over baseline. Incontinence. Hospitalization. | Life threatening consequences. Urgent intervention indicated. | Death |
| Constipation | Occasional or intermittent symptoms; occasional use of stool softeners, laxatives, dietary modification, or enema. | Persistent symptoms with regular use of laxatives or enemas indicated. | Symptoms interfering with activities of daily living; obstipation with manual evacuation indicated | Life-threatening consequences (e.g., obstruction, toxic megacolon). | Death |

oligomerization domain receptors that activate NF κ B (van Vliet et al., 2010). In the healing phase, proliferation and differentiation of the GI epithelium return approximately 2 weeks post-chemotherapy (Sonis et al., 2004; Lee et al., 2014), but functional changes persist after recovery of morphological changes (Keefe et al., 2000; Rubenstein et al., 2004). The pathophysiology underlying these persistent changes in GI functions includes several overlapping secretory, osmotic, inflammatory, and neurogenic mechanisms (McQuade et al., 2014).

Disruption to water and electrolyte balance within the GI tract is a key component in the pathophysiology of all types of diarrhea. Direct mucosal damage has been suggested as a major contributor to malabsorption and hypersecretion associated with CID (Richardson and Dobish, 2007; Stringer et al., 2007, 2009b; Stein et al., 2010). Studies using animal models of CID have demonstrated increased apoptosis in the crypts of both the jejunum and colon, resulting in metaplasia of goblet cells and excessive mucous secretion (Ikuno et al., 1995; Gibson et al., 2003). Hyperplasia of the rapidly dividing crypt cells in the epithelium of the gut probably results in heightened proportions of immature secretory cells, leading to increased secretion and decreased absorptive capacity of the villi, thereby contributing to the onset of diarrhea (Castro-Rodríguez et al., 1997). Retention of non-absorbable compounds within the lumen triggers an osmotic shift of water into the lumen (Castro-Rodríguez et al., 1997; Richardson and Dobish, 2007; Stringer et al., 2007). This reduced absorptive capacity and increased secretion in the small intestines results in increased fluid and solutes in the intestinal lumen and overwhelms the absorptive capacity of the colon resulting in diarrhea (Gibson and Keefe, 2006).

Secondary to mucosal damage, CID has been associated with mucosal inflammation throughout the GI tract (Logan et al., 2008). Increased expression of cyclooxygenase (COX)-2, associated with increased release of prostaglandin E₂ (PGE₂), is seen in rat colon following irinotecan administration (Yang et al., 2005). PGE₂ stimulates colonic secretion and hyperperistalsis of the gut, whilst inhibiting sodium, potassium and adenosine triphosphatase, and triggering excessive chloride secretion, all of which further contribute to the onset of diarrhea (Kase et al., 1997a,b; Leahy et al., 2002; Yang et al., 2005). Further, irinotecan stimulates the production of thromboxane A₂, a potent physiological stimulant of chloride and water secretion in the colon (Sakai et al., 1997; Suzuki et al., 2000) as well as tumor necrosis factor- α (TNF- α) a pro-inflammatory cytokine and a primary mediator of immune regulation associated with CID (Yang et al., 2005).

Chemotherapy can induce damage to the ENS (Vera et al., 2011; Wafai et al., 2013) which may also underlie GI secretory disturbances involved in pathophysiology of CID. Innervation of the GI tract is primarily from the ENS, sometimes referred to as “the second brain” due to its ability to function autonomously of the central nervous system (Phillips and Powley, 2007). The ENS is comprised of ganglia, primary interganglionic fiber tracts as well as secondary and tertiary fibers which project to many of the effector systems of the gut including muscle cells, glands, and blood vessels (Hansen, 2003). The ENS is divided into two major ganglionated plexi, the myenteric (Auerbach’s), and submucosal

(Meissner’s), which are responsible for controlling gut functions including motility, secretion, absorption and vascular tone. Enteric neuropathy has been linked to a variety of GI pathologies, in part due to its regulation of intestinal epithelial function and colonic motility (De Giorgio et al., 2000, 2004; De Giorgio and Camilleri, 2004; Chandrasekharan et al., 2011; Furness, 2012). However, effects of chemotherapeutics on enteric neurons and GI dysfunction have been largely overlooked until recently. It has been shown that chronic treatment with cisplatin results in myenteric neuronal loss, increase in amplitude of the neurally induced contractions of the gastric fundus strips in mice and occasional diarrhea (Pini et al., 2016). Thus enteric neuropathy may be an underlying cause of chemotherapy-induced GI dysmotility.

Movement of fluid between the lumen of the intestine and the body fluid compartments is a complex and tightly regulated process involving neural, endocrine, paracrine, and autocrine systems that act via the enteric neurons within the submucosal plexus (Lundgren et al., 2000; Johnson et al., 2012). Situated superficially to the mucosa, the submucosal plexus lies between the circular muscle and muscularis mucosa layer of the mucosa and derives innervation from neurons in the myenteric plexus as well as direct innervation from branches of the sympathetic and parasympathetic nervous systems. The submucosal plexus innervates the mucosal epithelium and submucosal arterioles to control and maintain water and electrolyte balance, secretion and vascular tone (Furness, 2012). Fluid is absorbed from the lumen containing nutrients via ion-coupled transporters and returned through secretomotor reflexes. Through activation of secretomotor neurons, water and electrolytes are moved from the interstitium of the lamina propria to the lumen, drawn from both the circulation and the absorbed fluids. Neural control of secretion and absorption of water and electrolytes occurs on multiple interacting levels. While there are secretomotor circuits confined to the submucosal plexus, they can be directly controlled by circuitry within the myenteric plexus. Despite the important role of the ENS in controlling secretory function, very little research has been undertaken to elucidate the relationship between the ENS and CID. Enteric neuropathy and/or neuronal dysfunction may be a contributing factor in chemotherapy-induced secretory dysfunction.

Current Treatments for Chemotherapy-Induced Diarrhea

Chemotherapy-induced diarrhea may be classified as uncomplicated (grade 1–2 with no complications) or complicated (grade 3–4 with one or more complicating signs or symptoms), early onset (<24 h after administration) or late onset (>24 h after administration) and may be categorized as persistent (present for >4 weeks) or non-persistent (present for <4 weeks) according to the National Cancer Institute’s Common Terminology Criteria for Adverse Effects grading system (Stein et al., 2010). Although uncomplicated CID may be managed by modification of the diet and administration of standard anti-diarrheal drugs such as loperamide, octreotide and tincture of opium, complicated diarrhea requires aggressive high dose anti-diarrheal

administration and hospitalization (McQuade et al., 2014). The recommendations on the management of CID were published in 1998 and updated in 2004 (Wadler et al., 1998; Benson et al., 2004a), providing guidelines for evaluation and management of CID. These guidelines have not been updated since 2004. Currently the only drugs recommended in the updated treatment guidelines are opioid derivatives such as loperamide and deodorized tincture of opium (DTO), and octreotide.

Loperamide

Loperamide is a non-analgesic agonist that acts at μ -opioid receptors at the level of the myenteric plexus to decrease intestinal motility (Regnard et al., 2011). High dose loperamide alleviates diarrhea associated with chemotherapeutic administration (Stein et al., 2010). However, its use leads to a range of side-effects including severe constipation, abdominal pain, dizziness, rashes as well as worsening of already present bloating, nausea and vomiting (Lenfers et al., 1999; Stein et al., 2010). High dose loperamide is reported to increase incidents of paralytic ileus, in association with abdominal distension (Sharma et al., 2005; Richardson and Dobish, 2007). Despite these severe side-effects, loperamide remains the standard first line therapy for CID.

Octreotide

Octreotide is a synthetic somatostatin analog that promotes absorption by inhibiting specific gut hormones to increase intestinal transit time (Högenauer et al., 2002; Mitchell, 2006) as well as hyperpolarizing enteric secretomotor neurons (Högenauer et al., 2002). Octreotide is administered to treat both complicated diarrhea and loperamide-refractory diarrhea and is generally reserved as a second line treatment for patients who are unresponsive to loperamide after 48 h, despite loperamide dose escalation (Regnard et al., 2011). Although octreotide decreases CID effectively, severe side-effects including slow and/or uneven heartbeat, severe constipation, stomach pain, enlarged thyroid, vomiting, nausea, headache and dizziness occur in over 10% of patients (Bhattacharya et al., 2008).

Deodorised Tincture of Opium

Deodorized tincture of opium (DTO) is another widely used antidiarrheal agent, despite the absence of literature to support its use in CID treatment (Stein et al., 2010). Similar to loperamide, DTO activates μ -opioid receptors within the GI tract inhibiting intestinal peristalsis, increasing intestinal transit time and promoting fluid reabsorption (Richardson and Dobish, 2007). The efficacy of DTO in treatment of CID has not been reported, however, it is a commonly used anti-diarrheal drug and may be considered as a second-line therapy for persistent and uncomplicated diarrhea (Richardson and Dobish, 2007). DTO contains 10 mg/ml of morphine and is one of the most potent forms of orally administered morphine available by prescription. DTO induces many side-effects including euphoria, nausea, vomiting, painful/difficult urination, stomach and abdominal pain, seizures and allergic reactions. Further, DTO administration associates with psychological and physical dependence, miosis, respiratory depression (Benson et al., 2004a; Richardson and Dobish, 2007) and constipation, with

continued/prolonged opioid use linked to severe constipation (Benyamin et al., 2008).

CHEMOTHERAPY-INDUCED CONSTIPATION

Constipation is a frequent, and underestimated, complication in patients with advanced cancer (Mancini and Bruera, 1998). As constipation is a subjective sensation, there is difficulty surrounding acceptance of a universal definition, although it is broadly recognized clinically as a mixture of reduced frequency of bowel action and increased stool consistency (Connolly and Larkin, 2012). Constipation occurs in 50–87% of advanced cancer patients (Abernethy et al., 2009). Constipation is the third most common symptom in patients receiving cytotoxic chemotherapy with an overall prevalence of 16%, with 5% classified as severe and 11% classified as moderate (Yamagishi et al., 2009; Anthony, 2010).

The mechanisms underlying CIC are poorly defined with minimal clinical studies existing. Distinguishing true CIC from secondary constipation from drugs given to control other chemotherapy or cancer-induced symptoms (such as anti-emetics for nausea and vomiting and opioids for pain) is a major issue hindering investigation (Gibson and Keefe, 2006). Given the scarcity of literature concerning CIC it is hard to estimate accurate incidence and severity among all chemotherapy-treated cancer sufferers, but specific chemotherapeutic agents such as thalidomide, cisplatin and vinca alkaloids such as vincristine, vinblastine, and vinorelbine induce true CIC in up to 80–90% of patients (Ghobrial and Rajkumar, 2003; Pujol et al., 2006; Stojanovska et al., 2015).

Constipation is not deemed to be of clinical importance until it causes physical risks or impairs quality of life. Constipation can cause a number of significant symptoms. Severely constipated patients experience abdominal distension usually accompanied by severe abrupt episodes of abdominal pain (Falcón et al., 2016). Furthermore, rectal tearing, hemorrhoids and rectal fissures caused by passing hard, dry stool are frequent complications of constipation (Leung et al., 2011). Untreated constipation may progress to obstipation, severe persistent constipation, which can have life threatening complications associated with fecal impaction and bowel obstruction (Leung et al., 2011). Fecal impaction, the presence of unpassable masses of stool, and increases intraluminal pressure within the bowel can lead to ischaemic necrosis of the mucosa, pain, bleeding, and perforation. Fecal impaction is also well recognized as a factor in urinary incontinence in the elderly (MacDonald et al., 1991). Constipation can also cause confusion, increase retroperitoneal or liver pain, trigger rapid onset nausea with or without vomiting in the presence of intestinal blockage and lead to inadequate absorption of oral drugs (Mancini and Bruera, 1998), greatly affecting the tolerability and efficacy of chemotherapeutic administration. There is accumulating evidence that self-reported constipation and functional constipation lead to significant impairment of quality of life, with the implication that this is a serious condition in the majority of people afflicted (Talley, 2003;

Dennison et al., 2005), however, little work has been undertaken to elucidate prevalence and mechanisms.

Pathophysiology of Chemotherapy-Induced Constipation

Normal bowel function requires the coordination of motility, mucosal transport, and defecation reflexes (Mancini and Bruera, 1998). Broadly constipation can be classified into three categories: normal-transit constipation, defecatory disorders and slow-transit constipation (Lembo and Camilleri, 2003). Normal-transit constipation is the most common form of constipation, where frequency of colonic evacuation is normal, yet patients believe they are constipated due to a perceived difficulty with evacuation or the presence of hard stools. Symptoms of normal-transit constipation include bloating and abdominal pain or discomfort, as well as increased psychosocial distress (Ashraf et al., 1996). Constipation resulting from defecatory disorders is most commonly due to dysfunction of the pelvic floor or anal sphincter. Defecatory disorders may result from prolonged avoidance of the pain associated with the passage of a large, hard stool or painful, anal fissure or hemorrhoid (Loening-Baucke, 1996). Structural abnormalities, such as rectal intussusception, rectocele, obstructing sigmoidocele, and excessive perineal descent, are less common causes of defecatory disorders (Lembo and Camilleri, 2003). Slow-transit constipation is associated with infrequent urge to defecate, bloating, and abdominal pain or discomfort.

Though little clinical research has been undertaken to elucidate the underlying pathology in CIC, it has been hypothesized that CIC may result from effects of chemotherapy on nerve endings in the gut (Ghobrial and Rajkumar, 2003). The GI tract is innervated by the ENS together with fibers from extrinsic sympathetic, parasympathetic (vagus nerve) and sensory afferent neurons (Phillips and Powley, 2007). Both the extrinsic and intrinsic innervation play an important role in the motor activity of the GI tract. The internal circular smooth muscle layer and the external longitudinal smooth muscle are controlled by two main mechanisms: non-neural pacemaker cells, interstitial cells of Cajal (ICCs), which generate myogenic activity and enteric neurons which provide neurogenic supply. Neuronal terminals are closely associated with ICCs which are linked to smooth muscle cells via gap junctions. Within the ENS, three main neuronal classes of myenteric neurons govern the complex motor reflex pathways: sensory neurons, interneurons, and motor neurons. The integration of inputs from these neurons and ICCs to smooth muscle cells in the colon allows expression of various motor patterns including phasic contractile activity and tonic contractile activity which contribute to colonic motor activity and the peristaltic reflex (Gwynne et al., 2004; Dinning et al., 2009; Huizinga and Lammers, 2009; Kuizenga et al., 2015).

Subtle changes to the ENS, not evident in conventional histological examination, have been suggested as a potential underlying mechanism for abnormal colonic motor function leading to constipation (Bassotti and Villanacci, 2011). For instance, alterations in the number of myenteric neurons expressing the excitatory neurotransmitter substance P, as well

as abnormalities in the inhibitory neurotransmitters, vasoactive intestinal peptide and nitric oxide, and a reduction in the number of ICCs (Cortesini et al., 1995; Tzavella et al., 1996; He et al., 2000) have been observed in patients with slow-transit constipation. However, the effects of chemotherapeutics on ENS and GI dysfunction have been largely overlooked until recently. A study investigating the effects of 5-fluorouracil-induced dysmotility in mice uncovered myenteric neuronal loss alongside delayed GI transit and inhibition of propagating colonic contractions (McQuade et al., 2016). Similar results have been demonstrated following oxaliplatin administration in mice, and cisplatin administration in rats, where enteric neuronal loss was associated with a reduction in colonic motor activity and reduced GI transit time, respectively (Vera et al., 2011; Wafai et al., 2013). Loss of enteric neurons following administration of cisplatin and oxaliplatin has been correlated with an increase in a population of the myenteric neurons expressing neuronal nitric oxide synthase (Vera et al., 2011; Wafai et al., 2013) and changes in glial cell populations (Robinson et al., 2016). These studies emphasize the importance of enteric neuronal integrity in GI function whilst suggesting neuroprotection as a potential therapeutic pathway for the treatment of chemotherapy-induced GI disorders.

Opioid-Induced Constipation

As previously mentioned, a major limitation in the estimation and evaluation of true CIC is the onset of secondary constipation, namely opioid-induced constipation produced by opioid analgesia. Whilst opioid analgesics are the gold standard in pain relief for cancer patients, adverse effects such as opioid-induced bowel dysfunction (OIBD) and opioid-induced constipation (OIC) severely compromise their therapeutic potential (Gonzalez and Halm, 2016). Incidence of OIC ranges from 50 to 87% in terminally ill cancer patients and is positively associated with chronic opioid treatment (Abernethy et al., 2009; Abramowitz et al., 2013). Opioid receptors are located throughout the central and peripheral nervous system and are involved in pain transmission (Camilleri, 2011). In the GI tract, μ -receptors are widely distributed throughout the ileum, stomach and proximal colon where they contribute to the control of fluid and electrolyte transport as well as motility (McKay et al., 1981; Fickel et al., 1997; Garg et al., 2016). Opioid analgesics interfere with GI motility by delaying transit, stimulating non-propulsive motility and altering GI segmentation and tone through their effects on enteric neurons (De Schepper et al., 2004; Wood and Galligan, 2004). These changes coupled with activation of mucosal sensory receptors that trigger a reflex arc facilitate excessive fluid reabsorption, resulting in OIC (Panchal et al., 2007; Camilleri, 2011).

Whilst administration of laxatives remains the first-line treatment option for OIC, this intervention alone is frequently ineffective (Gatti and Sabato, 2012). Selective μ -opioid receptor antagonists are emerging as a promising first line treatment for OIC, in particular treatment with methylnaltrexone bromide (methylnaltrexone) has been found to improve GI transit in chronically ill patients and has been recommended for use in cancer patients (Gatti and Sabato, 2012). Methylnaltrexone

has demonstrated efficacy in improving opioid-induced delay in the oral–caecal transit time and inducing laxation in both healthy subjects and advanced illness patients (Culpepper-Morgan et al., 1992; Thomas et al., 2005; Thomas et al., 2008). Similarly, treatment with peripheral μ -opioid receptor antagonist Alvimopan has been found to increase the frequency of spontaneous bowel movements in non-cancer patients with opioid induced bowel dysfunction (Webster et al., 2008).

Current Treatments for Chemotherapy-Induced Constipation

The management of constipation can be divided into general interventions and therapeutic measures. The general interventions involve increasing physical exercise, fluid intake and fiber consumption, availability of comfort, privacy and convenience during defecation as well as elimination of medical factors that may be contributing to constipation (Mancini and Bruera, 1998). Therapeutic interventions for the management of constipation, including CIC involve the administration of both oral and/or rectal bulk-forming, emollient, osmotic/saline, stimulant, and lubricant laxatives (Connolly and Larkin, 2012). Laxative compounds may fall into one of several categories depending on their mechanism of action.

Bulk-Forming Laxatives

Bulk-forming laxatives such as methylcellulose, psyllium, and polycarbophil most closely mimic the physiologic mechanisms involved in promoting GI evacuation. Available as natural or semisynthetic hydrophilic polysaccharides, cellulose derivatives, or polyacrylic resins, bulk forming laxatives work by either dissolving or swelling in the intestines to form a viscous liquid that provides mechanical distension. This facilitates the passage of intestinal contents by stimulating peristalsis and reducing GI transit time. Although typically recommended as initial therapy for most forms of mild constipation (Kirschenbaum, 2001), bulk-forming agents can take up to 72 h to exert their effects and therefore are not ideal for the initial management of symptomatic constipation in cancer patients (Avila, 2004; Connolly and Larkin, 2012). Bulk forming laxatives require the patients to drink extra fluids as otherwise a viscous mass may form and aggravate a partial bowel obstruction. In addition, significant allergy to these substances has been reported, and their effectiveness in severe constipation is doubtful (Klaschik et al., 2003). Though they are considered safe, some patients' experience suggests that they may worsen symptoms, causing distension, bloating, and abdominal pain (Costilla and Foxx-Orenstein, 2014).

Osmotic Laxatives

Osmotic laxatives such as lactulose, sorbitol, polyethylene glycol compounds, and saline laxatives (magnesium hydroxide), attract and retain fluid within GI tract (Twycross et al., 2012). Osmotic laxatives include salts of poorly absorbable cations (magnesium), anions (phosphate, sulfate) as well as molecules that are not absorbed in the small bowel but are metabolized in the colon (lactulose and sorbitol) and metabolically inert compounds such as polyethylene glycol. The presence of these molecules in the lumen results in

water retention to maintain normal osmolarity of the stool (Costilla and Foxx-Orenstein, 2014). The laxative effect of these agents depends on the extent to which they remain in the lumen with the onset between 24 and 72 h (Xing and Soffer, 2001). Adverse effects such as abdominal pain, flatulence, cramping and distension can arise shortly after ingestion, although side-effects may subside after several days of treatment, higher lactulose doses can induce bloating and colic (Ford and Soares, 2011; Costilla and Foxx-Orenstein, 2014). Excessive use of osmotic laxatives may result in hypermagnesemia, hyperphosphatemia, hypercalcemia, hypernatremia, hypokalemia, and hypoalbuminemia (Xing and Soffer, 2001; Kurniawan and Simadibrata, 2011).

Emollient (Stool Softener) Laxatives

Emollient laxatives, also known as stool softeners, are anionic surfactants increasing efficiency of intestinal fluids and facilitating the mixing of aqueous and fatty substances within the feces; this softens the feces allowing them to move more easily through the GI tract (Avila, 2004). Stool softeners are of little value when administered unaccompanied in the treatment of long-term constipation as they do not stimulate peristalsis and evacuation, but concurrent administration with bulk-forming agents and dietary fiber provides beneficial effect reducing straining (O'Mahony et al., 2001; Avila, 2004). Increased fluid intake essential during treatment with emollient laxatives to facilitate stool softening and so are not ideal for chronic constipation in cancer patients. Docusate is the most widely used emollient laxative produced as docusate calcium, docusate sodium, and docusate potassium. The onset of action is 1–2 days after administration but might be up to 5 days. However, docusates have been found to enhance GI or hepatic uptake of other drugs, increasing the risk of hepatotoxicity (Xing and Soffer, 2001). There is also some evidence that docusates cause significant neuronal loss in the myenteric plexus (Fox et al., 1983) and cause structural changes in the gut mucosa of humans (Xing and Soffer, 2001), but the clinical significance of this remains unclear.

Stimulant Laxatives

Stimulant laxatives such as diphenylmethane derivatives (phenolphthalein, sodium picosulfate, anthranoids (senna and cascara), ricinoleic acid (castor oil), and surface-acting agents directly stimulate myenteric neurons to increase peristalsis resulting in reduced net absorption of water and electrolytes from the intraluminal contents (Twycross et al., 2012). Stimulant laxatives are more potent than bulk-forming and osmotic laxatives and appear to be more effective than enemas (Dosh, 2002; Scarlett, 2004). They are amongst the most commonly administered laxatives for opioid-induced constipation (Ruston et al., 2013). Although short-term use is safe, overuse can cause dehydration and long-term ingestion may result in laxative dependence. This dependence also known as 'laxative bowel' is thought to result from damage to the myenteric plexus and smooth muscles cells in the colon (Xing and Soffer, 2001; Kurniawan and Simadibrata, 2011).

Lubricant Laxatives

Lubricant laxatives emulsify themselves into the fecal mass, coating the feces and rectum for easier passage whilst retarding colonic water absorption to simultaneously soften stool (Avila, 2004). Liquid paraffin, also known as mineral oil, is the major lubricant laxative in use although seed oils from croton and arachis are also available (Xing and Soffer, 2001). These laxatives can be administered orally or rectally and are useful for patients who complain of excess straining, but long-term use is associated with malabsorption of fat soluble vitamins and minerals, as well as anal leakage (Costilla and Foxx-Orenstein, 2014). Lubricant laxatives are not routinely recommended for long-term use due to possible inflammatory conditions such as lipid pneumonia (Schiller, 1999).

Rectal Laxatives

Rectal laxatives such as bisacodyl (stimulant), sodium phosphate (saline), glycerin (osmotic), and mineral oil (lubricant) (Avila, 2004) generally accepted not to be regularly used for CIC treatment (Fallon and O'Neill, 1997), but may be necessary alongside digital stimulation for treating fecal impaction or constipation associated with neurogenic bowel dysfunction. Rectal suppository of bisacodyl (stimulant) is most commonly utilized when evacuation of soft stools is needed, while glycerin suppositories are more appropriate when a hard stool needs to be softened (Fallon and O'Neill, 1997). Acute severe constipation might require an administration of rectal laxatives by enema, however, rectal suppositories or enemas cannot be used in patients with neutropenia and thrombocytopenia (O'Mahony et al., 2001).

EMERGING AND POTENTIAL TREATMENTS FOR CID AND CIC

As current therapies for CID and CIC have limited efficacy and a plethora of adverse effects, a search for and use of novel anti-diarrheal and laxative agents is essential to improve quality of life and chemotherapeutic efficacy for cancer patients. Several emerging and already existing therapies used for treatment of other conditions such as diarrhea predominant irritable bowel syndrome (IBS-D), constipation predominant irritable bowel syndrome (IBS-C) and chronic idiopathic diarrhea and constipation could be employed for the treatment of CID and CIC.

Chloride Channel Inhibition and Activation

Chloride is an essential ion in intestinal secretion and absorption. Secretory diarrhea, such as that experienced in irinotecan-treated patients, results from a combination of excessive secretion and reduced absorption in the intestinal lumen (Thiagarajah and Verkman, 2012). Excessive fluid secretion is driven by active chloride secretion, followed by secondary movement of water and sodium into the intestine. Although there is a lack of selective potent inhibitors of voltage gated chloride channels, inhibition of calcium-activated chloride channels throughout the intestines

successfully reduced secretion of chloride into the intestinal lumen (Thiagarajah and Verkman, 2013; Thiagarajah et al., 2015). In a mouse model of rotavirus-induced severe secretory diarrhea, inhibition of calcium-activated chloride channels with a red wine extract reduced intestinal fluid secretion, diminishing the symptoms of diarrhea (Ko et al., 2014).

Conversely, chloride channel activation has been used in the management of chronic idiopathic constipation and constipation related to irritable bowel syndrome (IBS-C). Lubiprostone is a bicyclic fatty acid derived from prostaglandin E1 that specifically activates chloride channels in the intestine, whilst having no effect on smooth muscle contraction (Jun, 2013). The underlying mechanism of lubiprostone involves stimulation of electrogenic chloride secretion through activation of chloride channel type-2 (Lacy and Levy, 2007) and cystic fibrosis transmembrane conductance regulator chloride channels (Bijvelds et al., 2009) in the apical membrane of intestinal epithelial cells. Activation of these epithelial channels results in active secretion of chloride into the intestinal lumen followed by a passive secretion of electrolytes and water increasing the liquidity of the luminal contents (June, 2013). Resulting luminal distension from increased intestinal fluid content promotes GI motility and increases intestinal and colonic transit. In healthy volunteers, daily lubiprostone delays gastric emptying, increases fasting gastric volume, reduces maximum tolerated gastric volume, and accelerates small bowel and colon transit (Camilleri et al., 2006). In randomized trials involving patients with IBS-C, lubiprostone twice daily reduced abdominal pain and increased complete spontaneous bowel movement and improved stool consistency, straining, and bloating (Schey and Rao, 2011). Currently, oral lubiprostone is approved for IBS-C at 8 µg twice daily and CIC at doses of 24 µg twice daily, but approval for CIC is limited to only women who have not responded to laxatives (Davis and Gamier, 2015). At present there are no studies investigating the efficacy of lubiprostone for CID.

Cannabinoid Receptor Inhibition and Activation

Cannabinoids mediate their effects via binding to two main G-protein coupled receptors, CB₁ and CB₂, widely expressed in the GI tract (Abalo et al., 2012). Although the activity of the endocannabinoid system varies between species and different regions of the GI tract within the same species, activation of CB₁ receptors coupled to cholinergic motor neurons has been found to inhibit excitatory neuromuscular transmission in human colonic circular muscle (Hinds et al., 2006) and inhibit colonic propulsion in mice and rat (Pinto et al., 2002; Abalo et al., 2015). In recent human trials, dronabinol, a non-selective cannabinoid receptor agonist, was found to inhibit colonic motility in both healthy subjects (Esfandyari et al., 2006, 2007) and patients with IBS-related diarrhea (IBS-D) (Wong et al., 2011). Conversely, a CB₁ receptor inverse agonist, taranabant, has been shown to improve symptoms related to slow GI motility and abdominal pain when administered *in vivo* in mice (Fichna et al., 2013). Taranabant increased the number of bowel movements after systemic and oral administration and significantly increased fecal pellet output in mice with constipation induced by ipratropium

(Fichna et al., 2013). It has been demonstrated that a low dose of a non-selective cannabinoid agonist WIN55,212-2 reduced the severity of 5-fluorouracil-induced diarrhea in rats (Abalo et al., 2016).

Guanylate Cyclase C Activation

Guanylate cyclase C is the principal receptor for heat-stable enterotoxins and plays a major role in *E. coli*-induced secretory diarrhea (Camilleri, 2010). Enterotoxins and endogenous peptides bind to guanylate cyclase C and stimulate the production of intracellular cyclic guanosine monophosphate (cGMP). Increased levels of cGMP activate the secretion of chloride ions through the cystic fibrosis transmembrane conductance regulator. Linaclotide is a minimally absorbed 14-amino acid peptide that selectively stimulates intestinal epithelial cell guanylate cyclase C receptors, resulting in increased intracellular and extracellular cGMP leading to accelerated stool transit and laxation (Harris and Crowell, 2007). In phase II and III placebo-controlled studies in chronically constipated and IBS-C patients, linaclotide was found to accelerate colonic transit and improve abdominal pain and symptoms of constipation (Andresen et al., 2007; Johnston et al., 2009, 2010; Lembo et al., 2010). Linaclotide is particularly interesting in that it is both a laxative and analgesic, reducing visceral hypersensitivity with very few drug interactions, it is presently licensed for chronic idiopathic constipation and IBS-C in the USA (Davis and Gamier, 2015), but no trials on CIC have been reported to date.

Probiotics, Antibiotics, and β -glucuronidase Inhibitors

With the recognition that intestinal microbiota play key roles in the pathophysiology of mucositis and development of CID/CIC, both antibiotics and probiotics have emerged as promising therapeutic options. Administration of probiotics have been shown to prevent CID in both 5-fluorouracil and irinotecan-treated animals (Bültzingslöwen et al., 2003; Bowen et al., 2007). Similarly, a combination of *Lactobacillus rhamnosus* and fiber has been found to reduce the severity of grade 3/4 5-fluorouracil/leucovorin-induced diarrhea by 15% in a randomized study of patients treated for colorectal cancer (Österlund et al., 2007). Administration of oral antibiotics, such as fluoroquinolone, has also been recommended for aggressive treatment of CID (Benson et al., 2004a; Maroun et al., 2007).

The selective inhibition of bacterial β -glucuronidase has recently been shown to alleviate drug-induced GI toxicity in mice (Wallace et al., 2015). A low-potency β -glucuronidase

inhibitor showed promise in reducing the GI toxicity associated with irinotecan in rats (Fittkau et al., 2004). Similarly, oral administration of potent bacterial β -glucuronidase inhibitors has been found to reduce the severity of irinotecan-induced toxicity (Wallace et al., 2010). In clinical trials, Kampo medicine Hangeshashinto (TJ-14) which contains baicalin, a β -glucuronidase inhibitor, has been found to successfully reduce both the incidence and duration of chemotherapy-induced oral mucositis in colorectal cancer patients when compared to placebo patients (Matsuda et al., 2015). In non-small-cell lung cancer patients TJ-14 alleviated irinotecan-induced diarrhea (Mori et al., 2003). Compared with control patients, the TJ-14-treated patients showed a significant improvement in both diarrhea grade, as well as a reduced frequency of grade 3 and 4 diarrhea (Mori et al., 2003).

CONCLUSION

Chemotherapy-induced diarrhea and CIC are amongst the most common chemotherapy-induced GI toxicities, heavily contributing to treatment delays, dose reductions and in some cases cessation of anti-cancer treatment, greatly affecting management and clinical outcomes. Current treatments for CID and CIC are limited and come with a profuse amount of concomitant symptoms; however, novel therapies present a promising avenue of treatment for CID and CIC. Identification of potential targets and the development of novel treatments alleviating chemotherapy-induced toxicity are essential to improve clinical outcomes and quality of life amongst cancer sufferers.

AUTHOR CONTRIBUTIONS

RM: conception and manuscript writing; VS, RA, JB, and KN: critical revision of the manuscript. All authors approved final version of the manuscript to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Anti-Colorectal Cancer Chemotherapy-Induced Diarrhoea: Current Treatments and Side-Effects

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Abstract

Chemotherapy-induced diarrhoea (CID) is a common side-effect experienced by patients being treated with a variety of antineoplastic agents. Approximately 80% of patients undergoing chemotherapeutic treatment for colorectal and other gastrointestinal cancers present with CID; moreover, about 5% of early deaths associated with combination anti-cancer chemotherapy are due to CID. Chronic post-treatment diarrhoea amongst cancer survivors can persist for more than 10 years greatly effecting long-term quality of life. Gastrointestinal toxicities such as diarrhoea and vomiting are amongst the primary contributors to dose reductions and delays throughout anti-cancer treatment, presenting a significant hurdle in clinical management of anti-cancer regimes and often result in sub-optimum treatment. However, little is known about pathophysiological mechanisms underlying CID. This work provides a review of chemotherapy-induced diarrhoea, current management guidelines, and shortcomings of current treatments as well as emerging and already existing anti-diarrhoeal treatments potentially suitable for CID.

Keywords

Oxaliplatin; Irinotecan, 5-Fluorouracil, Cisplatin, Carboplatin, Chemotherapy, Colorectal Cancer, Chemotherapy-Induced Diarrhoea

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality in the western world [1] [2].

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With global incidence and mortality rates of approximately 1.2 million and 610,000 respectively per year [3], and consequently low 5 and 10 year survival rates, CRC is projected to account for more than 1.6 million deaths annually by 2020 [4], placing it amongst the highest contributors to cancer related deaths globally [3] [5]–[7].

Surgical resection of the tumour-bearing and adjacent segments of intestines is used in an attempt to eliminate all cancer cells. However, about half of patients are diagnosed beyond stage III, where penetration of the intestinal wall and metastasis to surrounding tissues have already occurred; therefore, chemotherapeutic intervention has been implemented as a mainstream adjuvant therapy, broadening the avenues of treatment [7] [8].

Advances in medicine over the last 50 years have widened the prospects of CRC treatment allowing it to be approached and administered in a variety of ways. Surgical resection, radiotherapy and/or chemotherapy depending on the location of tumour may now be combined and tailored for targeted treatment. Furthermore, chemotherapeutics may be manipulated and modified to be delivered as adjuvants, to complement and/or improve the efficacy of an already delivered drug, as well as being delivered in combination, merging two or more chemotherapeutic agents in a single infusion.

Current combinations of chemotherapeutic agents include 5-fluorouracil (5-FU)/leucovorin with oxaliplatin (FOLFOX), 5-FU/leucovorin and irinotecan (FOLFIRI), capecitabine and oxaliplatin (CAPEOX/XELOX) and 5-FU/leucovorin/oxaliplatin and irinotecan (FOLFOXIRI) [9]–[11]. Several key trials undertaken in 2000 established improved efficacy of FOLFOX and FOLFIRI combinations in metastatic CRC. As a result, FOLFOX and FOLFIRI have become the standard of care throughout most of the world, showing moderate superiority when compared to previous chemo-doublet 5-FU/LV, promoting higher overall survival rates and progression-free survival by approximately 12 months. Although proven to be effective in the treatment of CRC, with mortality rates decreasing by approximately 50% in most parts of the world since 1950 [12]–[14], these cytotoxic chemotherapies are notorious for prompting undesirable neurological and gastrointestinal side-effects (Table 1).

Chronic side-effects often result in dose limitations, and in severe circumstances cessation of anti-cancer treatment, presenting a constant challenge in efficient and tolerable treatment of CRC [15]–[21]. Amongst the most common of these dose-limiting side-effects is chemotherapy-induced diarrhoea (CID) affecting approximately 80% of patients undergoing chemotherapeutic treatment for colorectal and other gastrointestinal cancers [20] [22]. Moreover, about 5% of early deaths associated with combination anti-cancer chemotherapy are due to CID [23]. Although there are now established and well-structured guidelines to navigate management of CID, numerous attempts to prevent peripheral and gastrointestinal neurotoxicity have proven ineffective thus far [18] and avenues of treatment are still limited and carry an excessive amount of side-effects including worsening of already chronic gastrointestinal symptoms.

Investigation of the mechanisms underlying adverse effects caused by anti-cancer treatment is imperative for the development of new therapies to target such pathologies as CID, to increase effectiveness of current treatments and improve CRC patients' quality of life.

2. Chemotherapy-Induced Diarrhoea

About 40% of patients receiving standard dose chemotherapy and all patients receiving high dose chemotherapy for gastrointestinal cancers exhibit pain, ulceration, bloating, vomiting and diarrhoea [30] [31]. Recent statistics on colorectal cancer and its treatments indicate that CID occurs at rates as high as 80% in patients [32]. Diarrhoea is a frequently under-recognised clinical hurdle that significantly affects morbidity and mortality of cancer

Table 1. Neurotoxic and gastrointestinal side-effects associated with anti-cancer chemotherapeutic agents used for the treatment of CRC [24]–[29].

| Drug | Side-effects |
|----------------|---|
| Carboplatin | Cortical blindness, peripheral sensory neuropathy, nausea, vomiting, constipation and diarrhoea . |
| Cisplatin | Encephalopathy, headache, stroke, seizures, sensory peripheral neuropathy, Lhermitte's sign, muscle cramps, nausea, vomiting and diarrhoea . |
| Oxaliplatin | Paraesthesia, sensory peripheral neuropathy, nausea, vomiting, weight loss, constipation and diarrhoea . |
| 5-Fluorouracil | Cerebellar dysfunction, inflammatory leucoencephalopathy, peripheral neuropathy, nausea, vomiting, severe diarrhoea and anorexia. |
| Irinotecan | Dysarthria, peripheral sensory neuropathy, palmar-plant erythrodysesthesia, nausea, vomiting, severe diarrhoea , constipation and anorexia. |

patients worldwide [22]. Chemotherapeutic agents most commonly associated with CID include 5-FU, capecitabine, irinotecan and oxaliplatin [32] [33]. Although prevalence and severity of chemotherapy-induced diarrhoea varies greatly based on the combination of chemotherapeutics, certain regimens, especially those containing 5-FU in combination with irinotecan are associated with rates as high as 87% [22] [34] with one third of patients experiencing severe (grade 3 or 4) (Table 2) diarrhoea [35].

Though CID is a clearly established side-effect of gastrointestinal cancer treatment, little research is underway to determine its underlying mechanisms. CID is believed to be a form, or by-product, of alimentary mucositis; that is, inflammation and ulceration of the mucous membranes of the digestive tract [37]. Alimentary mucositis occurs as a result of altered intestinal microflora and mucin secretion, and is a highly probable contributor to the development of CID; however the pathophysiology behind CID is extensive and complex and probably results from several overlapping mechanisms [37].

Specific anti-cancer treatments can be directly linked to a variety of different types of diarrhoea including secretory, osmotic, malabsorptive, exudative, motility related and inflammatory; all of which may be differentially diagnosed from varying symptomology [37].

Disruption to water and electrolyte balance within the gastrointestinal tract is a key component in the pathophysiology of all types of diarrhoea. Intestinal water balance involves many complex processes and pathways involving inflammatory mediators, hormones and neuropeptides that are strictly regulated to maintain the integrity of the intestinal wall, efficiency of the circulatory and enteric nervous systems [38] [39].

Prominent intestinal mucosal damage is a theme throughout the literature surrounding CID. Toxicity to the rapidly dividing crypt cells of the intestinal epithelium coupled with destruction and depletion of intestinal enzymes is believed to be a major contributor to the decrease in absorption and increase in fluid secretion seen in CID [30]-[34] [40]. Direct harm to the villi and mature cells of the intestinal wall results in a higher proportion of immature secretory cells, this increase in secretion and decrease in absorptive capacity of the villi alters the osmotic gradient within the gut contributing to the onset of diarrhoea [41]. The osmotic component is believed to be attributable to cytotoxic agent-induced damage to colonic crypts, thereby reducing chloride absorption and causing water to be released into the intestinal lumen [41]. Chemotherapy-induced disturbance to intestinal absorptive capacity causes an increase in solutes within the intestinal lumen; this triggers an osmotic shift of water into the lumen resulting in osmotic diarrhoea [30] [34] [41]. When non-absorbable compounds are retained within the lumen, active transport of water and electrons is initiated to maintain luminal potential as well as to flush foreign matter from the gastrointestinal tract. Coupled with this, inflammation throughout the intestines leads to secretion of factors such as prostaglandins, leukotrienes and cytokines further stimulating secretion and increasing damage to the epithelium and disrupting intestinal water and ion transport resulting in hypersecretion [34] [40]. It has been suggested that acute diarrhoea experienced 24 - 96 hours post-chemotherapeutic infusion is primarily secretory [32]. However, whether all chemotherapy-induced diarrhoeas are due to changes in epithelial surface area unknown. Damage to the enteric nervous system caused by chemotherapeutic treatments [42] [43] might underlie gastrointestinal secretory and motility disturbances involved in pathophysiology of CID. It is also unclear whether different chemotherapeutic regimens are associated with different types of diarrhoea. Moreover, the pathophysiological mechanisms underlying chronic diarrhoea persisting long after the chemotherapy have not been investigated.

Although the likelihood of CID is typically unpredictable, occurrence of CID has been linked to a variety of patient and treatment-associated risk factors (Table 3). It has been found that age >65, gender and low performance status are common patient-associated risk factors as well as associated bowel pathologies and genetic polymorphisms that may affect drug metabolism [34]. Accompanying this, certain chemotherapeutic drugs and administration regimens have been found to increase susceptibility to CID. Most prominently the addition of 5-FU and leucovorin to treatments, and combination of 5-FU with irinotecan and oxaliplatin increase both severity and prevalence of CID [43] [44].

Table 2. Common terminology criteria for diarrhoea [36].

| Toxicity | Grade 1 | Grade 2 | Grade 3 | Grade 4 | Grade 5 |
|-----------|--|---|---|---|---------|
| Diarrhoea | Increase of <4 stools per day over baseline. | Increase of 4 - 6 stools per day over baseline. | Increase of >7 stools per day over baseline. Incontinence. Hospitalization. | Life threatening consequences. Urgent intervention indicated. | Death |

Table 3. Risk factors for chemotherapy-induced diarrhoea (modified from [34] [45]–[48]).

| | |
|-----------------------------------|--|
| Patient-associated risk factors | Elderly (>65) |
| | Female |
| | Genetic polymorphisms affecting drug metabolism and distribution such as Gilbert's syndrome, Crigler-Najjar syndrome |
| | Biliary obstruction |
| | Associated bowel pathology such as inflammatory bowel disease or malabsorption |
| Treatment-associated risk factors | Irinotecan & 5-FU |
| | Weekly chemotherapy schedule |
| | Infusional chemotherapy |
| | Bolus 5-FU |
| | Prior history of CID |
| | Prior or concomitant radiotherapy |

3. Impact of CID

Persistent and severe diarrhoea in combination chemotherapy-treated patients often causes malnutrition and dehydration leading to rapid weight loss (cachexia), fatigue, renal failure, haemorrhoids, perianal skin breakdown [49] [50]. In some cases, chemotherapy causes severe intestinal inflammation, bowel wall thickening and ulceration [51]. Thus, CID is a key contributor to the interruptions in optimal clinical outcomes and may lead to life-threatening sequelae [22] [23] [32].

CID is the primary contributor to approximately 60% of treatment alterations, with about 22% of patients receiving dose reductions, 28% of patients having dose delays and complete cessation of treatment in approximately 15% of patients due to severe diarrhoea during the course of treatment [48] [52]. A direct correlation between cumulative dose and severity of CID has been recognised, with high dose regimens associated with higher reported incidence of CID [20] [29]. Early death rates, occurring in approximately 5% of patients undergoing anti-cancer treatment, are primarily due to CID leading to severe dehydration [23].

About 53% of anti-CRC treated patients that receive adjuvant therapies experience grade 3 or 4 CID at some point during the course of treatment and current guidelines recommend aggressive treatment and hospitalization [53]. Thus, CID is fast becoming a major drain on healthcare resources. A recent cost of illness analysis on the economic impact of grade 3 - 4 CID revealed a median hospital stay of 8 days, indicating that CID is a debilitating and costly complication of CRC chemotherapy [48]. The incidence of chronic post-treatment diarrhoea amongst cancer survivors varies from 14% to 49% and episodes of diarrhoea can persist for more than 10 years [54] greatly effecting long-term quality of life.

Long-term psychological effects of treatment-related chronic persistent diarrhoea are rarely recognised. Mental and social health of CID sufferers has been shown to be greatly compromised with known psychological effects of uncontrolled CID including anxiety, depression, social isolation and low self-esteem [55]. More than 30% of CID sufferers experience interference to their daily activities [32].

4. Treatment of CID

Current management of CID is based heavily on the nature and progression of the diarrhoea itself. CID may be classified as complicated or uncomplicated, late or early onset and categorised as persistent or non-persistent depending on the National Cancer Institute's (2009) Common Terminology Criteria for Adverse Effects grading system [32] [36].

Uncomplicated CID can be managed through diet modification and standard dose of recommended drugs such as loperamide, octreotide and tincture of opium in an outpatient setting, however, complicated diarrhoea requires aggressive treatment and hospitalisation where patients are recommended to take anti-diarrhoeal drugs and intravenous fluids [34].

The recommendations of a consensus conference on the management of CID were published in 1998 and updated in 2004 [40] [45]. These publications provide guidelines for evaluation and classification of symptoms of CID (Figure 1). Opioid derivatives loperamide and deodorized tincture of opium (DTO), and octreotide are the only drugs recommended in the updated treatment guidelines, due to lack of efficacy and insufficient evidence for benefits of other therapeutic approaches such as prophylactic treatments [22]. Although the National Cancer Institute's Common Terminology Criteria for Adverse Effects grading system has been updated on several occasions, CID management and treatment guidelines have not been changed since 2004. A consensus working statement published in 2007 offered several clinical recommendations and improvement; however no changes to the treatment of CID have been made [35].

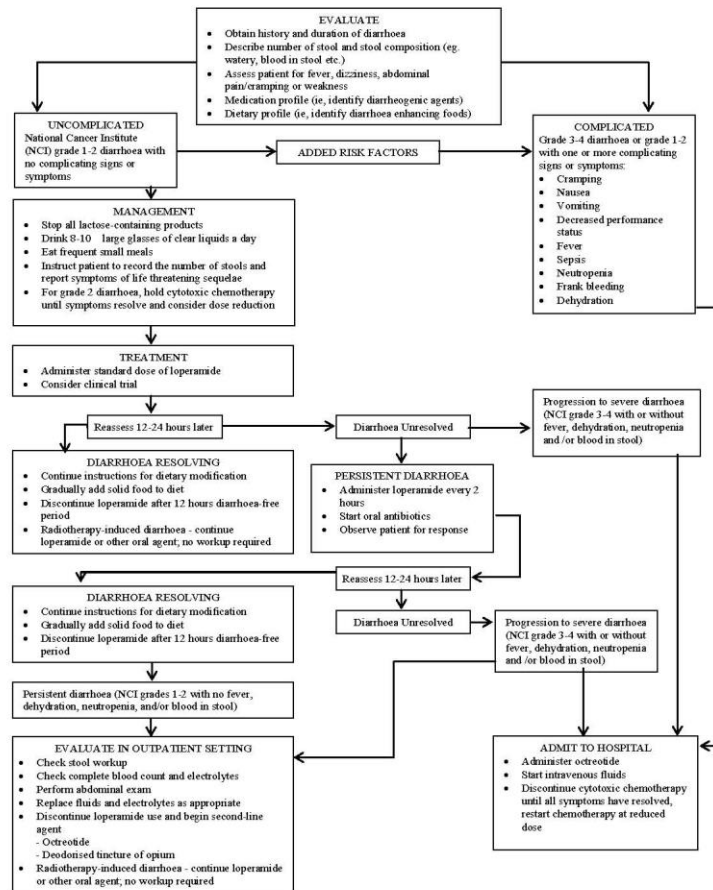


Figure 1. Guidelines for treatment of chemotherapy-induced diarrhoea (modified from [22] [32]).

Until recently the significance of chemotherapy-induced diarrhoea and its impact on regimen designation and treatment efficiency has been greatly underestimated. As a result current treatments available are limited and carry a wide variety of adverse effects including further gastrointestinal symptoms (Table 4).

5. National Cancer Institute Guideline-Based Treatment for CID

5.1. Loperamide

Loperamide is a non-analgesic agonist that decreases intestinal motility through an action at μ -opioid receptors of the myenteric plexus [32]. High dose loperamide alleviates diarrhoea associated with chemotherapeutic administration and is the standard first line therapy for CID [22]. However, it is associated with high levels of resistance and failure, specifically when used with irinotecan, and has a variety of side-effects including severe constipation, abdominal pain, dizziness, and rashes and worsening of already present bloating, nausea and vomiting [32] [56]. Clinical studies have also reported incidents of paralytic ileus, in association with abdominal distension occurring in patients administered a very high dose of loperamide [34] [57].

5.2. Octreotide

Loperamide-refractory and complicated diarrhoea are both treated with octreotide [22], an indirect anti-motility substance. Octreotide is a synthetic somatostatin analogue that inhibits specific gut hormones to increase intestinal transit time promoting absorption [38] [49] [58] as well as hyperpolarizing secretomotor neurons within the enteric nervous system to inhibit secretion and promote net absorption [58]. Octreotide has shown to be effective in decreasing CID in clinical trials [38]. However severe adverse effects such as slow and/or uneven heartbeat, severe constipation, severe stomach pain, enlarged thyroid, vomiting, nausea, headache and dizziness occur in more than 10% of patients treated with octreotide [59]. Octreotide is generally reserved as a second line treatment for patients who are unresponsive to loperamide after 48 hours, despite loperamide escalation [32].

5.3. Deodorised Tincture of Opium

Deodorized tincture of opium (DTO) similarly to loperamide, works by activating μ -opioid receptors within the

Table 4. Current treatments and their side-effects for chemotherapy-induced diarrhoea.

| Treatment | Mechanism | Side-Effects | References |
|-------------------|--|--|---|
| Loperamide | Synthetic opiate derivative μ -opioid receptor agonist that slows intestinal peristalsis, increases intestinal transit time to promote fluid reabsorption. Also exhibits anti-secretory effects through inhibition of thromboxane-A ₂ . | Stomach pain and bloating, ongoing or worsening of diarrhoea, watery and bloody diarrhoea, severe skin reactions (blistering and peeling), nausea, vomiting, dizziness, drowsiness, severe constipation and paralytic ileus. | [32] [34] [38] [47] [56] [80] |
| Octreotide | Synthetically engineered somatostatin analogue designed to bind to somatostatin receptors; inhibits adenylyl cyclase to reduce secretion of pancreatic and intestinal hormones, increases intestinal transit time and promotes reabsorption. | Easy bruising and unusual bleeding (nose, mouth, vagina and rectum), purple/red pinpoint spots under skin, slow and/or uneven heart beats, severe constipation, severe stomach pain, enlarged thyroid, vomiting, nausea, headache and dizziness. | [22] [32] [38] [47] [48] [59] [80] |
| Tincture of opium | Similar to loperamide, acts by activating μ -opioid receptors to slow intestinal peristalsis thereby increasing intestinal transit time and promoting fluid reabsorption. | Vomiting, nausea and light headedness, stomach/abdominal pain, painful/difficult urination, seizures, itching and swelling of the face, tongue and throat, psychological dependence, physical dependence, miosis and respiratory depression. | [22] [34] |
| Budesonide | Orally administered synthetic steroid with anti-inflammatory effects. Helps to restore mucosal function and improve intestinal fluid reabsorption. Has inhibitory effect on mucosal prostaglandins. | Increased blood pressure, headache, thinning of the skin, easy bruising, muscle pain, stomach pain, indigestion, mild skin rash, changes to menstrual cycle. | [46] [61] [63] |
| Atropine | Competitive antagonist at muscarinic receptors. Helps to suppress the cholinergic effects of irinotecan. | Allergic reactions (swelling of the lips, tongue and face), irregular or fast heart rate, rash or flushing, eye pain, headache, dizziness, blurred vision, bloating, nausea, heartburn, constipation, weakness, difficulty urinating. | [60]–[62] |

gastrointestinal tract to slow intestinal peristalsis, thereby increasing intestinal transit time and promoting fluid reabsorption [34]. Currently there are no studies demonstrating the efficacy of DTO in treatment of CID, but it is still a widely used anti-diarrheal drug and may be considered as a second-line therapy for persistent and uncomplicated diarrhoea [34]. DTO contains 10 mg/ml of morphine, making it one of the most potent forms of orally administered morphine available by prescription. Side-effects of DTO range from euphoria or dysphoria to vomiting, nausea, light headedness, stomach/abdominal pain, painful/difficult urination as well as seizures, itching and swelling of the face, tongue and throat. DTO has also been linked to psychological and physical dependence, meiosis and respiratory depression [22] [34].

6. Prophylactic Measures

6.1. Atropine

Atropine is a competitive antagonist at muscarinic receptors that helps to suppress the cholinergic effects of secretory diarrhoea. Atropine functions by inhibiting the muscarinic actions of acetylcholine released by cholinergic nerve fibres innervating gastrointestinal smooth muscles, as well as inhibiting the actions of cholinergic secretomotor neurons innervating the mucosa. Dosage ranges from 0.25 - 1 mg and can be delivered intravenously or subcutaneously as either prophylaxis or treatment for CID, to a maximum cumulative dose of 1.2 mg [60]. It has been found that subcutaneous administration of atropine prior to chemotherapeutic treatment was effective at reducing CID [60] [61]. However atropine administration is linked to swelling of the lips, tongue and face, irregular or fast heart rate, rash or flushing, eye pain, headache, dizziness, blurred vision, bloating, nausea, heartburn, constipation, weakness, difficulty urinating [60]-[62].

6.2. Budesonide

Budesonide is an orally administered synthetic steroid with anti-inflammatory effects, commonly used in patients with inflammatory bowel disease. It works to restore mucosal function and improve intestinal fluid reabsorption as well as having an inhibitory effect on mucosal prostaglandins. Suppression of inflammation throughout the bowel could potentially contribute to reductions in the occurrence of CID; however published data revealed no significant decreases in incidence of CID from concurrent budesonide and loperamide treatment [63]. Furthermore budesonide has been linked to increased blood pressure, headache, thinning of the skin, easy bruising, muscle pain, stomach pain, indigestion, mild skin rash, and changes to menstrual cycle [46] [61] [63].

6.3. Antibiotics

Several studies have investigated the effectiveness of antibiotic therapy to combat irinotecan-induced diarrhoea [64]. Results surrounding antibiotic use in irinotecan-induced diarrhoea remain controversial. Several studies indicated that elimination of β -glucuronidase producing microflora through the use of antibiotics namely penicillin, streptomycin, cefixime, ceftriaxone, neomycin and levofloxacin may decrease incidence and severity of CID [65]-[67]. In contrary, non-significant reductions in severity and frequency of diarrhoea accompanied by substantially higher rates of grade 2 diarrhoea were found [68]. Moreover, contention over the use of antibiotics in immune compromised patients for risk of *Clostridium difficile* (*C. difficile*) infection causing both nosocomial and antibiotic-associated diarrhoea is evident throughout literature [46] [65] [69]. *C. difficile* is a prominent and potentially life threatening infection that results from disturbance of normal bacterial flora in the colon, triggering release of toxins that cause mucosal inflammation and damage. *C. difficile* infection is most frequent amongst hospitalized patients and it is well known that colorectal cancer, abdominal surgery, as well as a weakened immune system as a result of chemotherapy, increases susceptibility to *C. difficile* infection [70] [71].

6.4. Glutamine

Glutamine is an amino acid found within the gastrointestinal tract, it acts as a major energy source for enterocytes as well as playing an important role in gut integrity and immune responses [72]. It is believed that glutamine can stimulate intestinal mucosa growth, and therefore may reduce gastrointestinal toxicity [32] [73] [74]. Several studies over the last decade have investigated the incidence and severity of CID in patients receiving

prophylactic oral and intravenous glutamine [75]–[79]. It was reported that intravenous administration of 20 g glutamine reduced plasma endotoxin levels and decreased severity of CID [77]. However a more recently conducted meta-analysis concluded that, although glutamine may reduce the duration of CID, it does not reduce its severity [78]. Furthermore, a trial involving high-dose chemotherapy and glutamine-containing intravenous solutions showed significantly higher incidence of relapse and death in patients receiving glutamine [79].

7. Emerging & Potential Treatments for CID

Given that current treatments for CID have limited efficacy and a wide range of adverse effects, the search for and use of alternative anti-diarrhoeal agents with few or no side-effects is essential. Several emerging and already existing treatments for other conditions associated with acute and chronic diarrhoea, such as irritable bowel syndrome (IBS), travellers' diarrhoea and other conditions could be considered and tested for the treatment of CID.

7.1. Adsorbents

Adsorbent substances are emerging as promising avenues in the treatment of CID. Adsorbents work by collecting excess materials on their surface and transporting them through the excretion process. Activated charcoal has long been used in the treatment of acute poisoning and is under investigation as a remedy for the late onset diarrhoea experienced after 5-FU and irinotecan infusions [81]. AST-120 also known as kremazin, a carbon-based adsorbent that is believed to absorb uremic toxins, such as indoxyl sulphate, in the gut has been explored in both cancer patients with irinotecan-induced diarrhoea and nonconstipated IBS sufferers [82] [83]. AST-120 has been found to be safe and well-tolerated, effective in reducing pain and bloating in nonconstipated IBS sufferers and to ameliorate irinotecan-induced diarrhoea without disrupting clearance of irinotecan metabolites.

7.2. Chloride Channel Inhibitors

Chloride is an essential ion in intestinal secretion and absorption. Secretory diarrhoea, such as that experienced in irinotecan-treated patients, results from a combination of excessive secretion and reduced absorption in the intestinal lumen [84] [85]. Excessive fluid secretion is driven by active chloride secretion, followed by secondary movement of water and sodium into the intestine. Deliberate inhibition of calcium-activated chloride channels located on enterocytes throughout the intestines reduces excretion of chloride into the intestinal lumen and consequently decreases the presence of water and sodium, diminishing the symptoms of diarrhoea [86].

7.3. Kappa Opioid Receptor Agonists

Opioid receptors (μ (mu), δ (delta) and κ (kappa)) located throughout the peripheral and central nervous systems participate in inhibition of perception of noxious stimuli from the gastrointestinal tract [87]. Although effective as anti-diarrhoeal agents both μ and δ agonists have many adverse effects such as constipation and opioid dependence, which impede their general use in clinical practice [87]. However, κ -opioid receptor agonists seem to be devoid of these symptoms.

Asimadoline is a highly selective and potent κ -opioid receptor agonist that is predominantly peripherally restricted at doses below 5 mg [88]. In clinical trials conducted in IBS patients it displayed a significant improvement in frequency/urgency of stools and pain free days and adequate relief of pain scores [89] [90]. Although there was no significant improvement in primary end point (number of months accrued with adequate relief of pain), asimadoline appeared to be well tolerated and produced overall improvement of gastrointestinal symptoms, including diarrhoea [90] highlighting that it could be used as a potential therapeutic for CID given that pain relief is not a primary concern in CID sufferers.

7.4. Enkephalinase Inhibitors

Enkephalins are endogenous neurotransmitters found throughout the enteric nervous system. Known to be pro-absorptive and anti-secretory in nature, enkephalins act on δ -opioid receptors, increasing chloride absorption and inhibiting adenylate cyclase to reduce intestinal secretion. Endogenous enkephalins are rapidly degraded by

enkephalinase. Inhibition of enkephalinase has recently been tested for anti-secretory effects against numerous secretagogues including cholera toxin and prostaglandins. Racecadotril is a powerful enkephalinase inhibitor with its active metabolite thiorphan being effective in clinical management of acute diarrhoea in both adults and children [91] [92]. Clinical trials of racecadotril have primarily been undertaken in patients with acute diarrhoea of presumed infectious origin and chronic HIV-related diarrhoea. Racecadotril significantly decreases mean stool output and duration of acute diarrhoea in both adults and infants when compared to placebo [93] [94], and has a similar efficacy to loperamide [91] [95] [96], but appears to be better tolerated than loperamide in both adults and children [97].

7.5. Cannabinoids

The effects of cannabinoids are primarily mediated by cannabinoid receptors, a widely expressed class of G-protein coupled receptors. Currently known two receptor subtypes, CB₁ and CB₂, are the main constituent of the endocannabinoid system. Recent studies have found that endocannabinoids acting on myenteric cannabinoid receptors can inhibit colonic propulsion [98] [99]. Dronabinol, a non-selective cannabinoid receptor agonist inhibits colonic propulsion in healthy subjects [98] [99] and patients with IBS-related diarrhoea [100].

8. Conclusion

Combination therapies now represent the standard first-line of treatment in CRC worldwide, with statistics showing great potential to increase overall survival in locally resected stage III CRC. Chemotherapy-induced gastrointestinal toxicity persists as a major contributor to dose delays, reductions and treatment terminations. Chemotherapy-induced diarrhoea is one of the most common disruptions in clinical management of CRC, affecting a vast majority of patients being treated with combination or adjuvant therapies containing 5-FU and irinotecan. Current treatments for CID are limited and have an abundance of concomitant symptoms; but novel and emerging anti-diarrhoeal treatments may present an additional unexplored avenue of treatments for CID sufferers. Ongoing investigation to identify potential targets and innovative treatment agendas to decrease chemotherapy-related toxicity is essential to improve clinical outcomes and quality of life amongst CID sufferers.

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Abbreviations

5-FU: 5-Fluorouracil
 CAPEOX: Combination of Capecitabine and Oxaliplatin. Also known as XELOX.
 CID: Chemotherapy-Induced Diarrhoea
 CRC: Colorectal Cancer
 DTO: Deodorized Tincture of Opium
 FOLFIRI: Combination of 5-Fluorouracil, Irinotecan and Leucovorin.
 FOLFOX: Combination of 5-Fluorouracil, Oxaliplatin and Leucovorin.
 FOLFOXIRI: Combination of 5-Fluorouracil, Oxaliplatin, Irinotecan and Leucovorin
 HIV: Human Immunodeficiency Virus
 IBS: Irritable Bowel Syndrome
 NCI: National Cancer Institute
 XELOX: Combination of Capecitabine and Oxaliplatin. Also known as CAPEOX.

RESEARCH PAPER

Role of oxidative stress in oxaliplatin-induced enteric neuropathy and colonic dysmotility in mice

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BACKGROUND AND PURPOSE

Oxaliplatin is a platinum-based chemotherapeutic drug used as a first-line therapy for colorectal cancer. However, its use is associated with severe gastrointestinal side-effects resulting in dose limitations and/or cessation of treatment. In this study, we tested whether oxidative stress, caused by chronic oxaliplatin treatment, induces enteric neuronal damage and colonic dysmotility.

EXPERIMENTAL APPROACH

Oxaliplatin (3 mg·kg⁻¹ per day) was administered *in vivo* to Balb/c mice intraperitoneally three times a week. The distal colon was collected at day 14 of treatment. Immunohistochemistry was performed in wholemount preparations of submucosal and myenteric ganglia. Neuromuscular transmission was studied by intracellular electrophysiology. Circular muscle tone was studied by force transducers. Colon propulsive activity studied in organ bath experiments and faeces were collected to measure water content.

KEY RESULTS

Chronic *in vivo* oxaliplatin treatment resulted in increased formation of reactive oxygen species (O₂⁻), nitration of proteins, mitochondrial membrane depolarisation resulting in the release of cytochrome c, loss of neurons, increased inducible NOS expression and apoptosis in both the submucosal and myenteric plexuses of the colon. Oxaliplatin treatment enhanced NO-mediated inhibitory junction potentials and altered the response of circular muscles to the NO donor, sodium nitroprusside. It also reduced the frequency of colonic migrating motor complexes and decreased circular muscle tone, effects reversed by the NO synthase inhibitor, N^ω-Nitro-L-arginine.

CONCLUSION AND IMPLICATIONS

Our study is the first to provide evidence that oxidative stress is a key player in enteric neuropathy and colonic dysmotility leading to symptoms of chronic constipation observed in oxaliplatin-treated mice.

Abbreviations

CMMC, colonic migrating motor complex; EJPs, excitatory junction potentials; ENS, enteric nervous system; fIJPs, fast inhibitory junction potentials; iNOS, inducible NOS; IR, immunoreactive; L-NNA, N^ω-nitro-L-arginine; nNOS, neuronal NOS; sIJPs, slow inhibitory junction potentials; SNP, sodium nitroprusside

Tables of Links

| TARGETS |
|----------------------------|
| GPCRs^a |
| Muscarinic receptors |
| P2Y ₁ receptors |
| Enzymes^b |
| nNOS, neuronal NOS |

| LIGANDS |
|---|
| Atropine |
| Carbachol |
| Nicardipine |
| L-NNA, N ^G -nitro-L-arginine |
| MRS2500 |
| Oxaliplatin |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (^aAlexander *et al.*, 2015a,b).

Introduction

Oxaliplatin, usually administered together with 5-fluorouracil and leucovorin, is a third generation platinum-based agent used as the first line of treatment for tumours resistant to the first and second generation platinum-based agents, cisplatin and carboplatin (Raymond *et al.*, 1998). Combinations containing oxaliplatin have shown great therapeutic potential, improving progression-free and overall survival rates of patients with colorectal cancer (André *et al.*, 2004; De Gramont *et al.*, 2007).

Oxaliplatin exerts its cytotoxic effects via formation of platinum-DNA adducts that trigger immobilization of the mitotic cell cycle and stimulate apoptosis of dividing cells (Graham *et al.*, 2000; Goodisman *et al.*, 2006). At clinically recommended doses, oxaliplatin is reported to be less toxic for the auditory, haematological and renal systems than cisplatin and carboplatin (Raymond *et al.*, 1998). However, oxaliplatin-induced neurotoxicity differs from that induced by other platinum compounds, triggering both acute and delayed peripheral neuropathies as well as gastrointestinal dysfunctions (Stojanovska *et al.*, 2015).

Gastrointestinal side-effects are a predominant cause of dose limitation, presenting a constant challenge for efficient and tolerable treatment of cancer (Verstappen *et al.*, 2003; McQuade *et al.*, 2014). About 40% of patients receiving standard dose chemotherapy and nearly all patients receiving high dose chemotherapy exhibit pain, ulceration, bloating, vomiting, diarrhoea and/or constipation throughout the course of treatment (McQuade *et al.*, 2014). Platinum-based agents specifically are linked to a heightened incidence of gastrointestinal side-effects with up to 90% of patients experiencing vomiting, nausea and/or diarrhoea during the course of treatment (Sharma *et al.*, 2005). Addition of platinum-based chemotherapeutics to combination regimes (such as FOLFOX – a combination of 5-fluorouracil, leucovorin and oxaliplatin) increases the incidence of chronic diarrhoea and treatment-related death (Souglakos *et al.*, 2006). Gastrointestinal side-effects can persist up to 10 years after the treatment has ceased (Denlinger and Barsevick, 2009).

Gastrointestinal functions are controlled by the enteric nervous system (ENS) embedded in the wall of the

gastrointestinal tract (Furness, 2012). Damage to the ENS underlies gastrointestinal dysfunction in many pathophysiological conditions (Furness, 2012). To date, few studies have investigated the effects of platinum-based chemotherapeutics on enteric neurons in animal models (Vera *et al.*, 2011; Wafai *et al.*, 2013; Pini *et al.*, 2016). These studies reported significant reductions in the number of myenteric neurons in the colon and stomach, alongside altered expression of neuronal NOS (nNOS) following *in vivo* treatment with cisplatin (Vera *et al.*, 2011; Pini *et al.*, 2016) and oxaliplatin (Wafai *et al.*, 2013). These changes in the ENS are correlated with reductions in gastrointestinal transit and colonic propulsive activity in oxaliplatin-treated animals, which may be related to symptoms of constipation or diarrhoea.

NO, synthesized by the NOS enzymes, is a highly reactive and widely distributed transmitter found throughout both the central and peripheral nervous systems. In the gastrointestinal tract, NO is a well-established mediator of vasodilation and gastrointestinal relaxation (Takahashi, 2003; Bornstein *et al.*, 2004). Two types of NOS participate in normal physiological responses, nNOS localized to neurons and endothelial NOS (eNOS) localized to the endothelium. Increased expression of inducible NOS (iNOS) occurs during times of cellular stress. Neurons containing nNOS are the primary source of NO in the ENS and represent approximately 30% of the neuronal population in the myenteric plexus of the mouse small intestine and colon (Qu *et al.*, 2008; Wafai *et al.*, 2013). In enteric ganglia, nNOS is expressed by descending interneurons and by inhibitory motor neurons supplying the intestinal smooth muscle (Lecci *et al.*, 2002). Moreover, a myogenic nNOS isoform is expressed by gastrointestinal smooth muscle cells (Daniel *et al.*, 2000). NO released from both neurons and smooth muscle is essential for sphincter relaxation and generation of complex gastrointestinal motor patterns, which contribute to propulsion during digestion (Sarna *et al.*, 1993; Roberts *et al.*, 2007; Roberts *et al.*, 2008). Altered expression of nNOS in the ENS has been linked to several pathological conditions, and increased levels of nNOS in the ENS can be indicative of oxidative stress (Rivera *et al.*, 2011a). The effects of oxidative stress on post mitotic cells, including enteric neurons, can be cumulative, resulting in neuronal loss and deterioration of neuronal function impairing gastrointestinal motility

patterns (Chandrasekharan *et al.*, 2011). The role of oxidative stress in chemotherapy-induced gastrointestinal dysfunction has not been explored. This study investigated the role of oxidative stress in enteric neuronal damage and colonic dysmotility caused by *in vivo* oxaliplatin treatment.

Methods

Animals

All animal care and experimental procedures were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Male Balb/c mice aged 6–8 weeks (18–25 g) supplied from the Animal Resources Centre (Perth, Australia) were used for all experiments. Mice had free access to food and water and were kept under a 12 h light/dark cycle in a well-ventilated room at an approximate temperature of 22°C. Mice acclimatized for a minimum of 5 days and a maximum of 7 days prior to the commencement of *in vivo* intraperitoneal injections. A total of 70 mice were used for this study.

In vivo oxaliplatin injections

Mice were randomly assigned into two groups: oxaliplatin-treated and sham-treated. Mice received intraperitoneal injections of oxaliplatin (Tocris Bioscience, UK), 3 mg·kg⁻¹ per dose, 3 times a week via a 26 gauge needle. Oxaliplatin was dissolved in sterile water in order to make 10⁻² M·L⁻¹ stock solutions and refrigerated at –20°C. The stock was then defrosted and further diluted with sterile water to make 10⁻³ M·L⁻¹ solutions for injections. The oxaliplatin dose was calculated to be equivalent to standard human dose per body surface area (Renn *et al.*, 2011). Sham-treated mice received sterile water via intraperitoneal injection 3 times a week via a 26 gauge needle. The volume injected did not exceed 200 µL per injection. Mice were killed via cervical dislocation 14 days after the first injection. Colons were collected for *in vivo* experiments.

Assessment of mitochondrial superoxide production

MitoSOX™ Red M36008 (Invitrogen, Australia) was used to visualize mitochondrially-derived superoxide in whole-mount preparations of submucosal and myenteric ganglia of the distal colon. Freshly excised distal colon preparations were dissected to expose submucosal and myenteric ganglia. Preparations were incubated in oxygenated physiological saline with MitoSOX™ Red M36008 (5 µM) in a gently shaking incubator Unimax 1010 (Heidolph Instruments, Germany) at a constant temperature of 37°C for 40 min. Tissues were washed (2 × 30 min) with oxygenated physiological saline (composition in mM: NaCl 118, KCl 4.6, CaCl₂ 3.5, MgSO₄ 1.2, NaH₂PO₄ 1, NaHCO₃ 25 and d-Glucose 11; bubbled with 95%O₂ and 5% CO₂) and fixed in 4% paraformaldehyde overnight at 4°C. The following day tissues were washed

(2 × 30 min) with physiological saline and mounted on glass slides with DAKO fluorescent mounting medium for imaging. All images were captured at identical acquisition exposure-time conditions, calibrated to standardized minimum baseline fluorescence, converted to binary and changes in fluorescence from baseline were measured in arbitrary units (arb. units) using Image J software (NIH, MD, USA). The corrected total fluorescence was calculated as previously described (Burgess *et al.*, 2010) in 32.5 × 5 µm² boxes within myenteric ganglia from each preparation to exclude fluorescence outside the ganglia.

Mitochondrial membrane potential assay

Mitochondrial membrane potential changes in cells may be detected with the cationic, lipophilic JC-10 dye. In normal cells, JC-10 concentrates in the mitochondrial matrix where it forms red fluorescent aggregates (JC aggregates). In contrast, apoptotic cells stain in green fluorescent colour (JC monomeric form) due to the JC-10-labelled release of cytochrome *c* diffusing out of the mitochondria as a result of mitochondrial depolarisation and increased permeability. JC-10 fluorescent mitochondrial membrane potential microplate assay kit (Abcam, MA, USA) was used to detect mitochondrial membrane potential changes, and the release of cytochrome *c* from damaged mitochondria in the myenteric ganglia of the distal colon. Freshly excised distal colon preparations from sham and oxaliplatin-treated mice were bathed in oxygenated physiological saline and dissected to expose the myenteric ganglia. Immediately following dissection, preparations were incubated for 20 min with 500 µL of JC-10 dye solution (buffer A) in a gently shaking incubator Unimax 1010 (Heidolph Instruments, Germany) at a constant temperature of 37°C. After 20 min, 500 µL of buffer B solution was added to tissue preparations and allowed to incubate for another 20 min in a gently shaking incubator at a constant temperature of 37°C. Immediately following final incubation, tissues were mounted on glass slides with DAKO fluorescent mounting medium for imaging under a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan).

Immunohistochemistry

Collected tissues (distal and proximal colon) were placed in oxygenated PBS (pH 7.2) containing nifedipine (3 µM) (Sigma-Aldrich, Australia) for 20 min to inhibit smooth muscle contraction. Samples were cut open along the mesenteric border, cleared of their contents, maximally stretched and dissected mucosa down to expose either the submucosal plexus (distal colon) or myenteric plexus (distal and proximal colon). Tissues were then fixed with Zamboni's fixative (2% formaldehyde, 0.2% picric acid) overnight at 4°C. Preparations were cleared of fixative by washing three times for 10 min with DMSO (Sigma-Aldrich, Australia) followed by 3 × 10 min washes with PBS. Fixed tissues were stored at 4°C in PBS for a maximum of 5 days.

Whole-mount preparations were incubated with 10% normal donkey serum (Chemicon, USA) for 1 h at room temperature. Tissues were then washed (2 × 5 min) with PBS and incubated with primary antibodies against β-tubulin class III (TuJ1) (chicken, 1:1000, Abcam, MA, USA), nitrotyrosine (rabbit, 1:1000, Millipore, CA, USA), nNOS (goat, 1:500, Abcam, MA, USA) and cleaved caspase-3 (rabbit, 1:500, Cell

Signalling Technologies, MA, USA) overnight at 4°C. Tissues were then washed in PBS (3 × 10 min) before incubation with species-specific secondary antibodies labelled with different fluorophores: donkey anti-chicken Alexa 594 (1:200, Jackson Immuno research Laboratories, PA, USA), donkey anti-goat Alexa 488 (1:200, Jackson Immuno research Laboratories, PA, USA) and donkey anti-rabbit Alexa 488 and 647 (1:200, Jackson Immuno research Laboratories, PA, USA) for 2 h at room temperature. Double immunohistochemical labelling was performed using mixtures of antibodies raised in different species and using species specific secondary antibodies labelled with different fluorophores. Tissues were given a further 3 × 10 min washes with PBS, followed by a 2 min incubation with the fluorescent nucleic acid stain, 4'-6-diamidino-2-phenylindole (DAPI; 14 nM) (Invitrogen, Australia). Whole-mount preparations were given three final 10 min washes in PBS and then mounted on glass slides using fluorescent mounting medium (DAKO, Australia). Whole-mount preparations were observed under a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan); eight randomly chosen images from each preparation were captured with a 20× objective and processed using NIS Elements software (Nikon, Japan). The numbers of β -tubulin-immunoreactive (IR) neurons, nNOS-IR neurons and neurons displaying translocation of nitrated proteins to the nuclei were quantified in both submucosal and myenteric ganglia within a 2 mm² area of each distal colon preparation. Proximal colon preparations were used to quantify the numbers of β -tubulin-IR and nNOS-IR neurons. Images were then calibrated to standardized minimum baseline fluorescence, converted to binary, and changes in caspase-3 fluorescence were detected using Image J software (NIH, MD, USA). Quantitative analyses were conducted blindly.

Histology

The distal colon was harvested and placed in a 10% formalin solution overnight and then transferred into 70% ethanol the following day. Paraffin embedded colon sections were cut 5 μ m thick and de-waxed in a 60°C oven for 30 min. To examine the morphological changes to the colon, a standard haematoxylin and eosin staining protocol was followed (26, 27). Ten sections per preparation were analysed. All images were analysed blindly.

Imaging

Three dimensional (z-series) images of wholemount preparations were taken using a Nikon Eclipse Ti laser scanning microscope (Nikon, Japan). Fluorophores were visualized using excitation filters for Alexa 594 Red (excitation wavelength 559 nm), Alexa 488 (excitation wavelength 473 nm) and Alexa 405 (excitation wavelength 405 nm). Z-series images were taken at step size of 1.75 μ m (1600 × 1200 pixels).

Western blotting

The whole colon was harvested, cut along the mesenteric border and pinned mucosa side up to a silicone-based petri dish containing physiological saline. The colon was flushed of its contents, and the mucosa, submucosa and the circular muscle layers were dissected and discarded. The remaining longitudinal muscle-myenteric plexus (LMMP) whole-mount preparations were then snap frozen using liquid nitrogen.

Frozen tissues were homogenized with a Polytron homogeniser (Kinematica AG, Lucerne, Switzerland) for 20 s in ice-cold WB buffer (40 mM Tris, pH 7.5; 1 mM EDTA; 5 mM EGTA; 0.5% Triton X-100; 25 mM β -glycerophosphate; 25 mM NaF; 1 mM Na₃VO₄; 10 μ g·mL⁻¹ leupeptin; and 1 mM PMSF), and the whole homogenate was used for Western blot analysis. Sample protein concentrations were determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and equivalent amounts of protein from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on SDS-PAGE acrylamide gels. Following electrophoretic separation, proteins were transferred to a PVDF membrane, blocked with 5% powdered milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h followed by an overnight incubation at 4°C with rabbit anti-iNOS primary antibody (D6B6S, Cell Signalling Technology, Danvers, MA, USA) dissolved in TBST containing 1% BSA. The following day membranes were washed for 30 min in TBST and then probed with a peroxidase-conjugated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Following 30 min of washing in TBST, the blots were developed with a DARG CCD camera mounted to a Fusion FX imaging system (Vilber Lourmat, Germany) using ECL Prime reagent (Amersham, Piscataway, NJ, USA). Densitometric measurements of the protein of interest were carried out using Fusion CAPT Advance software (Vilber Lourmat, Germany). Membranes were then stained with Coomassie Blue, scanned and total protein loaded quantified using Image J software. The signal intensity of the protein of interest was normalized to the signal for total protein loaded.

Intracellular recordings

Segments of the distal colon proximal to the pelvic brim were collected from day 14 sham and oxaliplatin-treated mice and placed in physiological saline bubbled with 95% O₂ and 5% CO₂ at room temperature. The physiological saline contained the L-type Ca²⁺ channel blocker nifedipine (3 μ M) (Sigma-Aldrich, Australia) to limit contractions. The distal colon was opened along the mesenteric border and pinned in a Sylgard-lined (Dow Corning, USA) Petri dish. The mucosa and submucosa were removed revealing the circular muscle layer. A 20 mm long, full circumference segment was transferred into a Sylgard-lined recording chamber and pinned with 50 μ m gold-plated tungsten pins. The recording chamber was placed on the stage of an inverted microscope fitted with fluorescent optics (Zeiss Axiovert 200), and was continuously superfused with physiological saline (3 mL·min⁻¹) pre-heated to yield a bath temperature of 35°C. Following 2 h recovery from dissection (Carbone *et al.*, 2012), circular smooth muscle cells were impaled with conventional intracellular borosilicate glass capillary electrodes filled with 5% 5,6-carboxyfluorescein in 20 mM Tris buffer (pH 7.0) in 1 M KCl. Electrode resistances ranged from 60–120 M Ω . Recordings were made using an Axoclamp 2B amplifier (Axon Instruments, USA), digitised at 1–10 kHz via a Digidata 1440A interface (Molecular Devices, USA) and stored using PClamp 10.0 (Molecular Devices) on a PC. Carboxyfluorescein-labelled cells were identified as circular muscle cells *in situ* from their morphology (Carbone *et al.*, 2012). Intracellular hyperpolarising current pulses (duration 500 ms, intensity

100–500 pA) were used to determine input resistance (R_{in}). A tungsten electrode (10–50 mm tip diameter, placed 1 mm circumferential to the recording microelectrode) was connected to an ISO-Flex stimulator controlled by a Master-8 pulse generator (AMPI, Israel). Single pulse stimuli (20 V, 0.4 ms duration) and short trains of high frequency pulses (20 V, 3 pulses, 40 ms interval, 0.4 ms duration) were used to activate nerve fibres. Junction potential responses were recorded in the impaled smooth muscle cells. Responses in 3–4 cells were averaged per test condition, in each animal. Data were analysed using AxoGraph 10 software.

Contraction force

Freshly excised distal colon was cut into 3 mm rings, cleaned of connective tissue and fat and then placed in a custom built organ-bath chamber containing physiological saline oxygenated with 95% O₂ and 5% CO₂ and maintained at a constant temperature of 37°C and pH of 7.4. Colonic rings were mounted between two small metal hooks attached to force displacement transducers (Zultek Engineering, Australia), and stretched to a resting tension of 0.2 g. After 30 min, rings were returned to resting tension and allowed to stabilize for 2 h with physiological saline changed every 20 min (Habiyyakare *et al.*, 2014). Following stabilization, 10 μ M sodium nitroprusside (SNP) was added to organ bath. Baseline values were obtained by averaging 60 s of data 5 min prior to drug application; maximum relaxation was calculated as absolute change from the baseline values.

Colonic motility experiments

The entire colon was removed from day 14 sham and oxaliplatin-treated mice and set up in organ-bath chambers to record motor patterns *in vitro* (Wafai *et al.*, 2013). Briefly, the colon was placed into warmed (35°C), oxygenated physiological saline until the faecal pellets were expelled. The empty colon was cannulated at both ends and arranged horizontally in organ-bath chambers. The proximal end of the colon was connected to a reservoir containing oxygenated physiological saline to maintain intraluminal pressure. The distal end was attached to an outflow tube that provided a maximum of 2 cm H₂O back-pressure. Organ baths were continuously superfused with oxygenated physiological saline solution, and preparations were left to equilibrate for 30 min. Contractile activity of each segment was recorded with a Logitech Quickcam Pro camera positioned 7–8 cm above the preparation. Videos (2 \times 20 min) of each test condition were captured and saved in *avi* format using VirtualDub software (version 1.9.11).

Colonic migrating motor complexes (CMMCs) were defined as propagating contractions directed from the proximal to the distal end of the colon, which travelled more than 50% of the colon length (Roberts *et al.*, 2007; Roberts *et al.*, 2008). Contractions that propagated less than 50% of the length of the colon were considered to be short contractions. Another form of incomplete contraction was identified as fragmented contractions occurring simultaneously at different parts of the colon rather than propagating over the length of the colon. Recordings were used to construct spatiotemporal maps using in-house edge detection software (Gwynne *et al.*, 2004). Spatiotemporal maps plot the diameter of the colon

at all points during the recording allowing contractile motor patterns to be analysed with Matlab software (version 12).

Faecal water content and colonic faecal content

Wet weight of faecal pellets was measured immediately upon pellet expulsion. Pellets were then dehydrated for 72 h at room temperature prior to measurement of the dry weight. Water content was calculated as the difference between the wet weight and dry weight. Total number of faecal pellets along the entire length of the colon was counted in freshly excised intact colons from day 14 sham and oxaliplatin-treated mice.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data are presented as mean \pm SEM. Sample size was calculated based on our previous studies on the enteric neuropathy and intestinal dysmotility associated with chemotherapy (Wafai *et al.*, 2013; McQuade *et al.*, 2016). To detect a 30% change at a power 0.8 and α = 0.05 with 10% SD, the effect size should be minimum n = 5 animals per group as calculated by the GPOWER program. Data were assessed using two-way ANOVA, Welch's two-tailed *t*-test and Student's two-tailed *t*-test. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., CA, USA). Differences between group means were considered statistically significant at P < 0.05.

Materials

MRS2500 [(1R,2S,4S,5S)-4-[2-iodo-6-(methylamino)purin-9-yl]-2-phosphonoxy-1-bicyclo [3.1.0]hexanyl]methyl dihydrogen phosphate) was from Tocris, UK; atropine, carbachol, N^o-nitro-L-arginine (L-NNA), nicardipine and SNP were all from Sigma-Aldrich, Australia. These compounds were prepared as stock solutions and diluted in physiological saline daily before addition to preparations.

Results

Loss of enteric neurons and increase in subpopulations of nNOS-immunoreactive neurons following oxaliplatin treatment

To investigate changes to the total number of myenteric neurons, whole-mount preparations of the distal and proximal colon were labelled with β -tubulin antibody to count neurons within a 2 mm² area (Figure 1). Repeated *in vivo* administration of oxaliplatin caused myenteric neuronal loss in both the proximal and distal colon when compared to sham (Figure 2A). Significant neuronal loss was also observed in the submucosal plexus in the distal colon from oxaliplatin-treated mice, compared to sham (Figure 2A').

To determine if oxaliplatin administration was associated with changes in subpopulations of myenteric neurons, inhibitory muscle motor and interneurons IR for nNOS were analysed. Fewer nNOS-IR neurons were observed in the myenteric plexus of the both the proximal and distal colon following oxaliplatin administration when compared with sham (Figure 2B), but not in the submucosal plexus. The

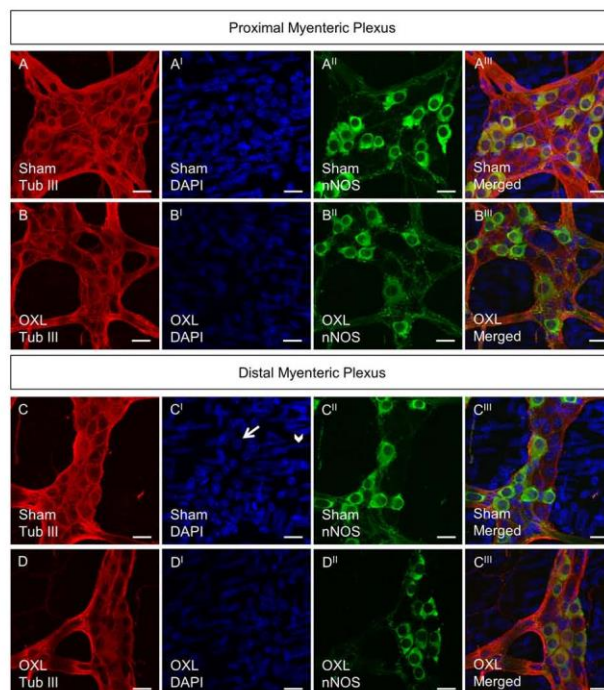


Figure 1

Whole-mount preparations of myenteric neurons in the proximal and distal colon following 14 days of *in vivo* oxaliplatin treatment. Myenteric neurons labelled with anti- β -tubulin III antibody (Tub III, red) counterstained with DAPI (blue) that labels neuronal nuclei within the ganglion (arrow) and smooth muscle cell nuclei outside the ganglion (arrowhead) (C'). nNOS-IR neurons (green). Scale bar = 20 μ m.

proportion of nNOS-IR neurons was, however, significantly increased in the myenteric and submucosal plexus of the distal colon, but not the myenteric plexus of the proximal colon from oxaliplatin-treated mice, when compared to sham-treated mice (Figure 2C,C').

Reactive oxygen species, iNOS expression and protein nitration in the submucosal and myenteric ganglia of the colon following in vivo oxaliplatin treatment

To evaluate production of ROS following long-term oxaliplatin treatment, distal colon samples were probed with a fluorescent mitochondrial superoxide marker MitoSOX[™] Red M36008. Increased MitoSOX fluorescence was found in both the submucosal (Figure 3A,A' and B,B') and myenteric (Figure 3C,C' and D,D') plexuses of the distal colon from oxaliplatin-treated mice compared to sham-treated animals (Figure 3E).

Western blot analysis revealed a 40% increase in the expression of iNOS in LMMP preparations from the colon of oxaliplatin-treated mice ($n = 5$ mice/group, Figure 3F).

An antibody against nitrotyrosine was used to label nitrated proteins within the submucosal (Figure 4A–A'' and B–B'') and myenteric (Figure 4C–C'' and D–D'') plexuses. The number of neurons per 2 mm² displaying translocation of nitrated proteins to the nuclei was higher in both the submucosal and myenteric plexuses of the colon from oxaliplatin-treated, compared with sham-treated (Figure 4E).

Apoptosis of submucosal and myenteric neurons in the colon

Diffusion of cytochrome c out of the mitochondria as a result of mitochondrial membrane depolarisation and increased permeability was measured via fluorescence (green) of monomeric JC-10. Increased monomeric JC-10 fluorescence was

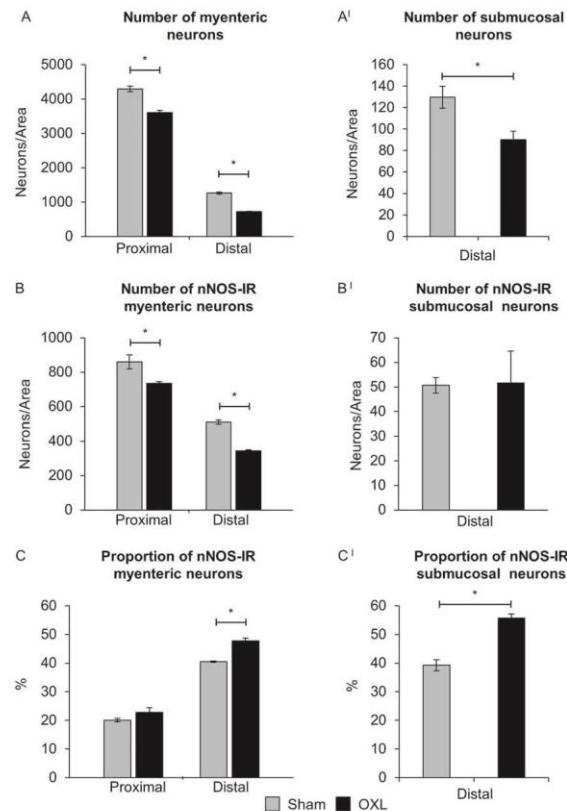


Figure 2

Effect of *in vivo* oxaliplatin treatment on total number of neurons and average number and proportion of nNOS-IR enteric neurons. Average number of myenteric neurons in the proximal and distal colon and submucosal neurons was counted per 2 mm² in the distal colon from day 14 sham and oxaliplatin-treated mice (A,A'). Average number of nNOS-IR neurons (B,B') in myenteric and submucosal ganglia counted within 2 mm² area. Proportion of nNOS-IR neurons to the total number of myenteric and submucosal neurons (C,C'). Data presented as mean \pm SEM. **P* < 0.05, significantly different as indicated; *n* = 6 mice per group per time point.

found in both submucosal (Figure 5A,A' and B,B') and myenteric (Figure 5C,C' and D,D') plexuses of the distal colon from oxaliplatin-treated mice compared with sham-treated animals (Figure 5E). Both tissues from both sham and oxaliplatin-treated mice experienced the same dissection and therefore the same level of cellular stress associated with dissection. Cellular stress associated with dissection can induce short-term mitochondrial depolarisation, resulting in modest increases in JC-10 fluorescence in tissues from both sham and oxaliplatin-treated mice. However, the level of JC-10 fluoresce in tissue from oxaliplatin-treated mice was

significantly higher (Figure 5) due to long-term mitochondrial depolarisation associated with neurotoxicity.

Caspase-3 immunoreactivity was absent in both the submucosal and myenteric ganglia in the colon preparations from sham-treated mice, but was observed in both the submucosal and myenteric ganglia in the colon preparations from oxaliplatin-treated mice indicating neuronal apoptosis (not shown, *n* = 6 mice/group). Caspase-3 labelling appeared both within the neuronal cell body as well as smaller, irregular patches of apoptotic debris that did not colocalise with β -tubulin III immunoreactivity.

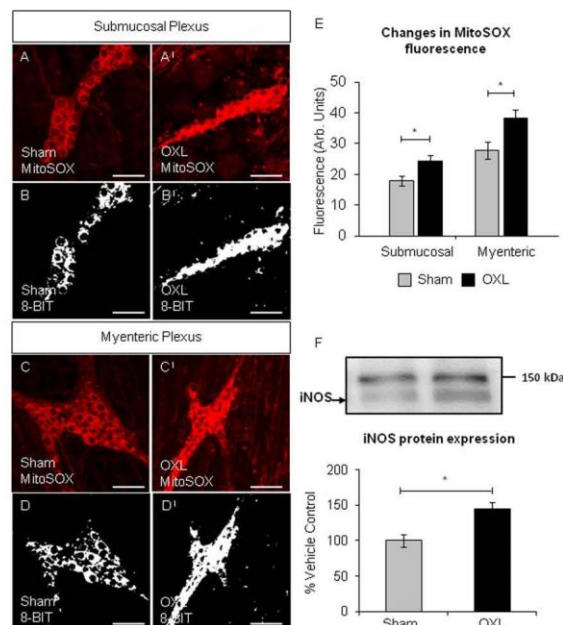


Figure 3

Mitochondrial superoxide in the colonic submucosal and myenteric ganglia and iNOS protein expression. Fluorescent and binary images of wholemount preparations of submucosal (A,A' and B,B') and myenteric (C,C' and D,D') ganglia labelled with MitoSOX™ Red in the colons from day 14 sham and oxaliplatin-treated mice. Scale bar = 50 μ m. (E) Quantification of the levels of mitochondrial superoxide production visualized by fluorescent probe in submucosal and myenteric ganglia in colonic preparations from day 14 sham and oxaliplatin-treated animals. * $P < 0.05$, significantly different as indicated; $n = 6$ mice per group. (F) Representative images and quantification of the Western blot analysis for iNOS in LMMP tissue from day 14 sham and oxaliplatin-treated mice. iNOS protein was normalized to total protein values obtained from the Coomassie Blue membrane staining (see Methods section). All values are expressed as a percentage of the values obtained from sham-treated mice. Data presented as mean \pm SEM. * $P < 0.05$, significantly different as indicated; $n = 5$ mice per group.

Effects of oxaliplatin treatment on neuromuscular transmission in the colon

Neuromuscular transmission in the distal colon was investigated using intracellular electrophysiology. Electrical stimulation of nerve fibres innervating the circular muscle in distal colon segments evoked junction potentials in single smooth muscle cells that were defined as fast inhibitory junction potentials (fIJP), slow inhibitory junction potentials (sIJP) and excitatory junction potentials (EJP) using pharmacological blockers.

In control physiological saline, the resting membrane potential (RMP) of smooth muscle cells from sham-treated mice averaged -38 ± 1.0 mV ($n = 6$ mice) and did not differ from that of smooth muscle cells from oxaliplatin-treated mice (-39 ± 1.0 mV, $n = 6$ mice). RMPs in both groups were unaffected by the selective P2Y₁ receptor antagonist MRS2500 (1 μ M) (sham: -39 ± 1.4 mV, oxaliplatin: -37 ± 1.3 mV), L-NNA (1 mM) (sham: -39 ± 1.3 mV, oxaliplatin: -38 ± 0.9 mV) or the muscarinic antagonist,

atropine (1 μ M) (sham: -38 ± 1.4 mV, oxaliplatin: -41 ± 3.3 mV). The input resistance of smooth muscle cells from sham-treated mice averaged 8.3 ± 0.9 M Ω ($n = 6$ mice) in control physiological saline; this did not differ significantly from the input resistance of smooth muscle cells from oxaliplatin-treated mice (8.7 ± 1.0 M Ω , $n = 6$ mice).

Single pulse and compound stimuli (trains of 20 V, 40 ms interval, 0.4 ms pulse duration) evoked fIJP in colonic smooth muscle cells (Figure 6A, A'). The amplitudes of fIJP recorded in smooth muscle cells from the distal colon of sham-treated mice following a 20 V compound stimulus did not differ significantly from those recorded from the distal colon of oxaliplatin-treated mice (Figure 6B). The duration of fIJP (width at half amplitude) was not different between (sham: 397 ± 17 ms) and oxaliplatin-treated (422 ± 27 ms) groups. The amplitudes of fIJP increased with increasing stimulus strength, but no significant differences were found in responses between sham

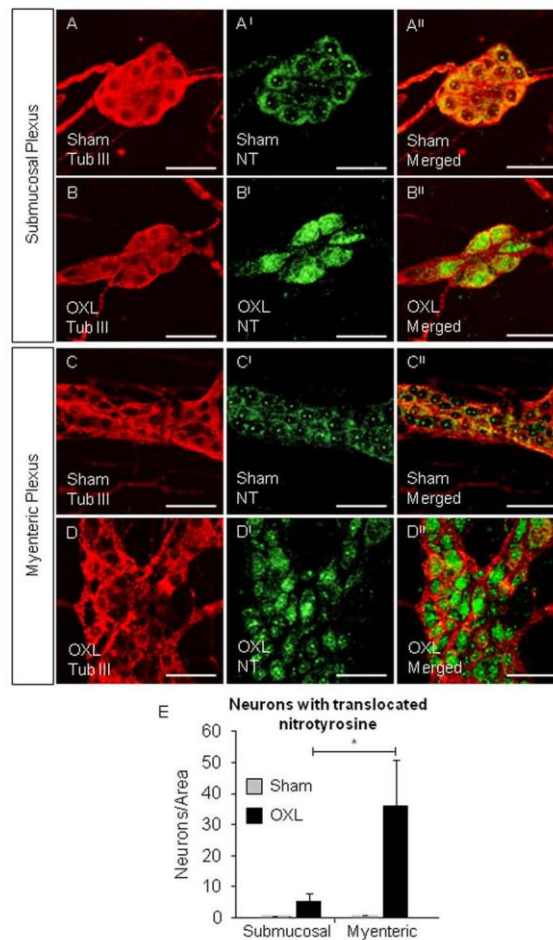


Figure 4

Translocation of nitrotyrosine to the nuclei of submucosal and myenteric neurons. Whole-mount preparations of colonic submucosal ganglia from day 14 sham (A–A'') and oxaliplatin-treated (B–B'') mice and myenteric ganglia from sham (C–C'') and oxaliplatin-treated (D–D'') mice. Scale bar = 50 μ m. Neurons and ganglia were labelled with anti- β -tubulin III antibody (Tub III, red). Nitrotyrosine within the ganglia was labelled with anti-nitrotyrosine antibody (NT, green). * $P < 0.05$, significantly different as indicated; $n = 6$ mice per group.

and oxaliplatin-treated mice (Figure 6C). Fast IJPs were inhibited by MRS2500 (1 μ M).

Short compound stimuli (trains of 20 V, 3 pulses, 40 ms interval, 0.4 ms pulse duration) applied in the presence of MRS2500 evoked sIJPs (Figure 6D), whose amplitudes were

greater in smooth muscle cells from the distal colon of mice treated with oxaliplatin than from sham-treated mice (Figure 6E). The sIJPs were abolished by addition of L-NNA (1 mM).

Short compound stimuli (trains of 20 V, 3 pulses, 40 ms interval, 0.4 ms pulse duration) of nerve fibres in the presence

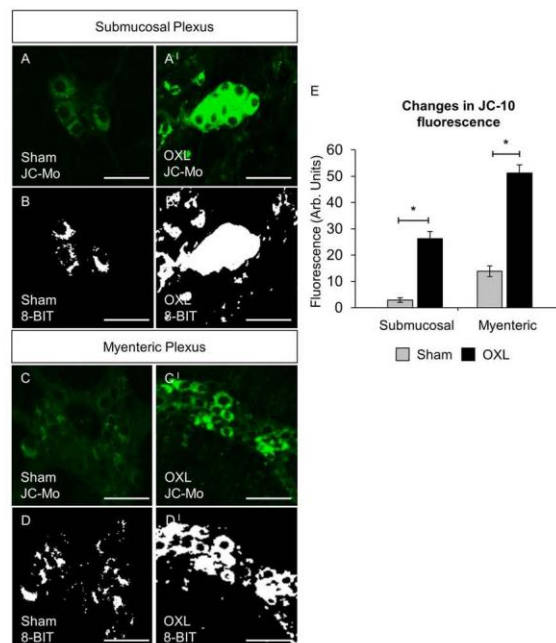


Figure 5

Changes in neuronal mitochondrial membrane potential indicative of cytochrome *c* release in submucosal and myenteric plexuses. Fluorescent and binary wholemount preparations of submucosal (A, A' and B, B') and myenteric (C, C' and D, D') ganglia labelled with JC-10 dye in the colons from day 14 sham and oxaliplatin-treated mice. Green fluorescent colour (JC monomeric form) is due to the JC-10-labelled release of cytochrome *c* diffusing out of the mitochondria as a result of mitochondrial depolarisation and increased permeability. Scale bar = 50 μ m. (E) Quantification of the levels of monomeric JC-10 production visualized by fluorescent probe in submucosal and myenteric ganglia in colonic preparations from sham and oxaliplatin-treated animals. * $P < 0.05$, significantly different as indicated; $n = 6$ mice per group.

of both MRS2500 and L-NNA, inhibiting fast and slow IJPs respectively, evoked EJPs (data not shown). EJPs were inhibited by atropine (1 μ M). The EJPs recorded in smooth muscle cells from the distal colon of sham-treated mice (6 ± 0.4 mV) did not differ from those of oxaliplatin-treated mice (6 ± 0.7 mV). Atropine reduced the amplitude of responses in sham-treated mice from 6 ± 0.4 to 0.8 ± 0.3 mV ($P < 0.05$) and from 6 ± 0.7 to 0.8 ± 0.2 mV in oxaliplatin-treated mice ($P < 0.05$, $n = 6$ animals per group).

Effects of oxaliplatin treatment on colonic smooth muscles

Smooth muscle tone of the distal colon was studied in organ bath experiments using force transducers. We measured the force produced by 3 mm wide circular muscle rings. Application of SNP (10 μ M) to organ baths containing distal colon segments resulted in a decrease in circular muscle tone, that is relaxation, in tissues from both sham (Figure 7A) and

oxaliplatin-treated (Figure 7A') mice. The reduction in tension produced by SNP was greater in the colon segments from sham-treated mice than in segments from oxaliplatin-treated mice (Figure 7B). Thus, the relaxation force in response to SNP was reduced in colonic circular muscles from oxaliplatin-treated mice.

The resting colon diameter measured in organ-bath experiments between contractions was larger in preparations from mice treated with oxaliplatin (2.4 ± 0.1 mm) compared with sham-treated mice (2.1 ± 0.1 mm) ($n = 7$ animals per group) (Figure 7C). Histological analysis of colon sections revealed thinning of the colonic muscle layer in oxaliplatin-treated mice when compared with sham (Figure 7D). Quantitative analysis confirmed that this reduction was statistically significant (Figure 7D').

Colonic motility

Colonic motor patterns. Analysis of the total number of contractions included all types of motor patterns in the colon

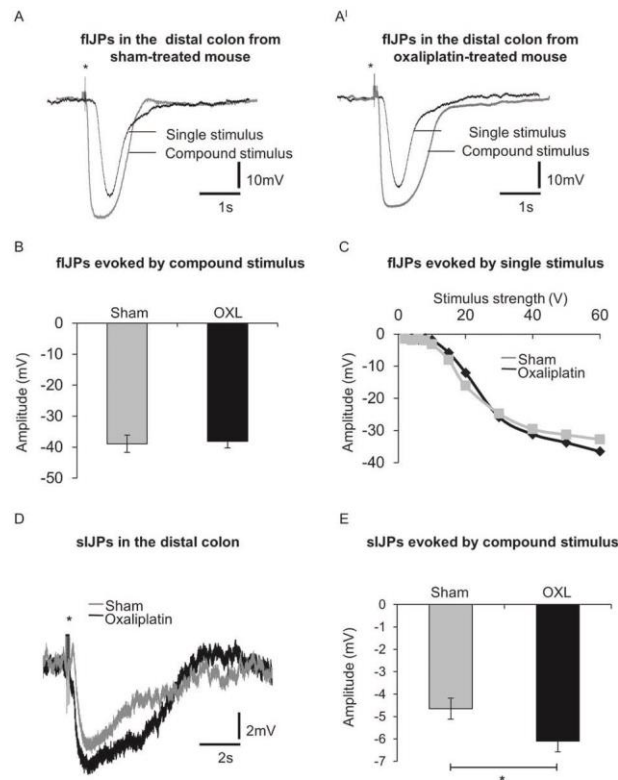


Figure 6

Intracellular recordings of fast and sIPs from colonic smooth muscle cells. Single pulse electrical stimulus (40 V, 0.4 ms duration) and high frequency compound stimulus (20 V, 0.04 ms interval, 0.4 ms duration) evoked fIPs in smooth muscle cells from day 14 sham (A) and oxaliplatin-treated (A') mice (- stimulus artefact). (B) The mean amplitude of fIPs in response to compound stimuli from smooth muscle cells of sham and oxaliplatin-treated mice ($n = 4$ mice/group). (C) Amplitudes of fIPs to increasing strength of the electrical stimuli (2–60 V) in smooth muscle cells of sham and oxaliplatin-treated mice. (D) Blocking fIPs with a selective antagonist of P2Y₁ receptors, MRS2500 (1 μ M) revealed sIPs in smooth muscle cells from both sham and oxaliplatin-treated mice. (E) Comparison of the amplitude of sIPs in smooth muscle cells from sham and oxaliplatin-treated mice. Data presented as mean \pm SEM. * $P < 0.05$, significantly different as indicated; $n = 6$ mice per group.

(CMMCs, short and fragmented contractions) (Figure 8A). The frequency, speed of propagation and length of contractions were measured for each specific type of motor activity. The total number and rate of rise of contractions were reduced in the colons from oxaliplatin-treated animals compared to sham-treated mice ($P < 0.05$ for both, Table 1). The addition of L-NNA did not alter the overall number of contractions in either group (Figure 8B, Table 1). Further analysis revealed differences in occurrence of

various types of colonic motor patterns in the oxaliplatin-treated group with a significant decrease in the proportion of CMMCs ($P < 0.05$, Figure 8C) and increase in the proportion of fragmented contractions ($P < 0.05$, Figure 8E). Application of L-NNA restored the proportion of CMMCs in oxaliplatin-treated colons to the levels comparable with sham-treated colons (Figure 8C), but had no significant effect on the proportion of either short or fragmented contractions (Figure 8D,E).

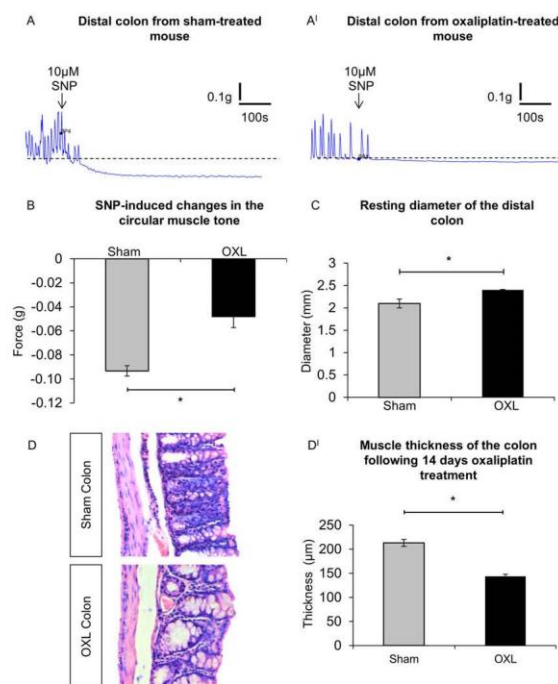


Figure 7

Effects of oxaliplatin treatment on colonic smooth muscles. (A, A') Smooth muscle relaxation following application of the NO donor, SNP (10 μ M) to the colon from day 14 sham (A) and oxaliplatin-treated (A') mice. (B) Comparison of the maximum relaxation produced by circular muscles in response to SNP in colonic preparations from sham and oxaliplatin-treated mice quantified as an absolute change in the force transduction from the basal values. * P < 0.05, significantly different as indicated; n = 6 mice per group. (C) Resting diameter of the distal colon from sham and oxaliplatin-treated mice. * P = 0.05, significantly different as indicated; n = 7 mice per group. (D) Gross morphological changes in the colon following repeated *in vivo* oxaliplatin administration. Colonic crypt length was shorter in oxaliplatin-treated mice and muscle thickness was reduced in comparison to the sham-treated animals. (D') Statistical analysis of the muscle layer thickness in the colon preparations from sham and oxaliplatin-treated mice. Data presented as mean \pm SEM. * P < 0.05, significantly different as indicated; n = 6 mice per group, 10 sections per preparation from each animal.

Colonic migrating motor complexes. *In vivo* treatment with oxaliplatin was associated with a lower CMMC frequency than in sham-treated preparations (P < 0.05). Application of L-NNA significantly increased the frequency of CMMCs in colons from oxaliplatin-treated (P < 0.05), but not sham-treated mice (Figure 9A, Table 1). The rate of rise of CMMCs was comparable between sham-treated and oxaliplatin-treated mice, but increased CMMC rate of rise following addition of L-NNA was noted in the colons from sham (P < 0.05), but not oxaliplatin-treated animals (Figure 9B, Table 1).

Short contractions. Treatment with oxaliplatin resulted in a reduced frequency of short contractions in the colon

(Figure 9C, Table 1). L-NNA had no effect on the frequency of short contractions in either group. The rate of rise of short contractions was twofold slower in colons from oxaliplatin-treated mice (Table 1). L-NNA significantly enhanced the propagation speed of short contractions in the sham (P < 0.05), but not the oxaliplatin-treated group. The frequency of short antegrade contractions in the distal region of the colon was significantly reduced in oxaliplatin-treated animals compared with sham-treated mice (Figure 9E, Table 1). Short contractions initiated in the distal colon were also found to travel in a retrograde direction. These retrograde short contractions occurred at a significantly reduced frequency in colons from oxaliplatin-treated mice compared to sham-treated mice (Figure 9F,

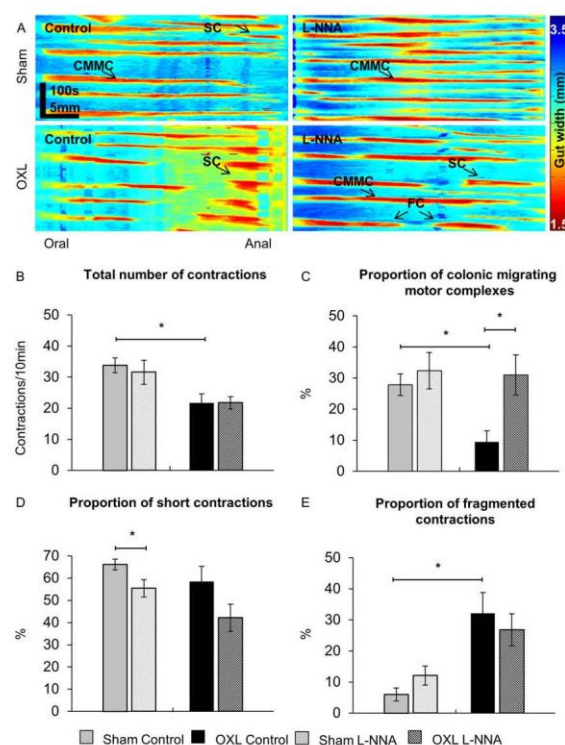


Figure 8

Total number of contractions and proportion of different types of contractile activity in the colon before and after application of L-NNA. (A) Examples of spatiotemporal maps generated from digital video recordings of colonic motility from day 14 sham and oxaliplatin-treated mice before (control) and after addition of L-NNA. Each contraction can be seen as a reduction in the gut width (red), while relaxation as an increase in the gut width (blue). CMMCs propagate >50% of the colon length, short contractions (SCs) propagate <50% of the colon length and fragmented contractions (FCs) are interrupted by period(s) of relaxation during contraction. (B) The total number of contractions including all types of contractile activity in the colons from sham and oxaliplatin-treated mice. The proportion of CMMCs (C), short contractions (D) and fragmented contractions (E) to the total number of contractions was calculated in maps from sham-treated and oxaliplatin-treated mice before and after addition of L-NNA. Data are presented as an average of contractions per 10 min from a total of 40 min of video recording at baseline intraluminal pressure prior to and after the addition of L-NNA. Data presented as mean \pm SEM. * P < 0.05, significantly different as indicated; n = 10 mice per group.

Table 1). L-NNA had no effects on anterograde or retrograde short distal contractions in either group.

Fragmented contractions. These were defined as incomplete contractions occurring simultaneously rather than propagating over the length of the colon (Figure 10A). The frequency of fragmented contractions was significantly higher in oxaliplatin-treated than in sham-treated mice (Figure 10B, Table 1). Application of L-NNA did not affect the frequency of fragmented contractions in either oxaliplatin-treated or sham-treated mice.

Colonic faecal content

To define the clinical symptoms resulting from the altered patterns of colonic motor activity, fresh faecal pellets were collected from both sham and oxaliplatin-treated mice and faecal water content was calculated as the difference between wet and dry pellet weight (Figure 10C,D). The fresh pellets (wet weight) from oxaliplatin-treated mice weighed significantly less than those of sham-treated mice (Figure 10C). After dehydration, the dry weight of pellets from sham-treated mice and from oxaliplatin-treated mice were not different (Figure 10C). Thus, the water content was significantly lower

Table 1

Parameters of different types of colonic contractions

| | | Sham-treated (n = 10 mice) | Oxaliplatin-treated (n = 10 mice) |
|---|---------|-------------------------------|--------------------------------------|
| All contractions | | | |
| Frequency (contractions/10 min) | Control | 33.8 ± 2.4 | 21.7 ± 2.9 ^a |
| | L-NNA | 31.6 ± 3.9 | 21.8 ± 3.9 |
| Speed (mm/s) | Control | 1.8 ± 0.1 | 1.3 ± 0.2 |
| | L-NNA | 3.4 ± 0.5 | 2.1 ± 0.4 |
| CMMCs | | | |
| Proportion (%) | Control | 27.8 ± 3.5 | 9.4 ± 3.7 ^a |
| | L-NNA | 32.4 ± 5.9 | 31.0 ± 6.4 ^b |
| Frequency (contractions/10 min) | Control | 9.0 ± 0.9 | 2.5 ± 1.0 ^a |
| | L-NNA | 9.8 ± 2.2 | 6.0 ± 1.2 ^b |
| Speed (mm/s) | Control | 2.2 ± 0.3 | 2.0 ± 0.5 |
| | L-NNA | 3.5 ± 0.8 ^a | 1.9 ± 0.5 |
| Short contractions | | | |
| Proportion (%) | Control | 66.2 ± 2.4 | 58.5 ± 6.9 |
| | L-NNA | 55.5 ± 3.9 ^a | 42.2 ± 6.2 |
| Frequency (contractions/10 min) | Control | 22.7 ± 2.4 | 13.0 ± 2.0 ^a |
| | L-NNA | 17.6 ± 2.3 | 9.8 ± 1.9 |
| Speed (mm·s ⁻¹) | Control | 1.6 ± 0.1 | 0.8 ± 0.1 ^a |
| | L-NNA | 2.8 ± 0.6 ^a | 1.2 ± 0.2 |
| Distal anterograde short contraction frequency (per 10 min) | Control | 14.5 ± 1.5 | 6.4 ± 1.5 ^a |
| | L-NNA | 10.3 ± 1.5 | 4.8 ± 1.4 |
| Distal retrograde short contraction frequency (per 10 min) | Control | 2.0 ± 0.6 | 0.1 ± 0.1 ^a |
| | L-NNA | 1.0 ± 0.3 | 0.3 ± 0.1 |
| Fragmented contractions | | | |
| Proportion (%) | Control | 6.0 ± 2.1 | 32.1 ± 6.1 ^a |
| | L-NNA | 12.1 ± 3.1 | 26.8 ± 5.2 |
| Frequency (contractions/10 min) | Control | 2.1 ± 0.7 | 6.2 ± 1.2 ^a |
| | L-NNA | 4.2 ± 1.2 | 6.0 ± 1.1 |

^a*P* < 0.05 compared with sham-control.^b*P* < 0.05 compared with oxaliplatin-control.

in pellets collected from oxaliplatin-treated mice than in those collected from sham-treated mice (Figure 10D). Total number of pellets within the entire length of excised colons from oxaliplatin-treated mice was significantly higher than the number in sham-treated mice (Figure 10E).

Discussion

This study is the first to examine the role of oxidative stress *in vivo* on enteric neurons of the colon that may be involved in mechanisms underlying colonic dysmotility associated with oxaliplatin treatment. The results show that mitochondrial superoxide and nitrated protein levels are significantly increased in the myenteric and submucosal ganglia after oxaliplatin treatment. Mitochondrial depolarisation and increased mitochondrial permeability leading to release of cytochrome *c* from mitochondria were increased in

myenteric ganglia in the colon from oxaliplatin-treated mice. This presumably led to increased expression of an 'executioner' caspase-3 in both the submucosal and myenteric ganglia in the colon preparations from oxaliplatin-treated mice. Oxidative stress and neuronal apoptosis resulted in functional changes in the colon (increased nitrgenic neuromuscular transmission, decreased smooth muscle tone, changes in the patterns and parameters of colonic motility) leading to symptoms of constipation in mice after long-term treatment with oxaliplatin.

Oxaliplatin-induced oxidative stress and neuronal apoptosis

Oxidative stress in enteric neurons following oxaliplatin treatment was shown by increased mitochondrial superoxide and translocation of nitrated proteins in submucosal and myenteric plexuses as well as the increase in expression of

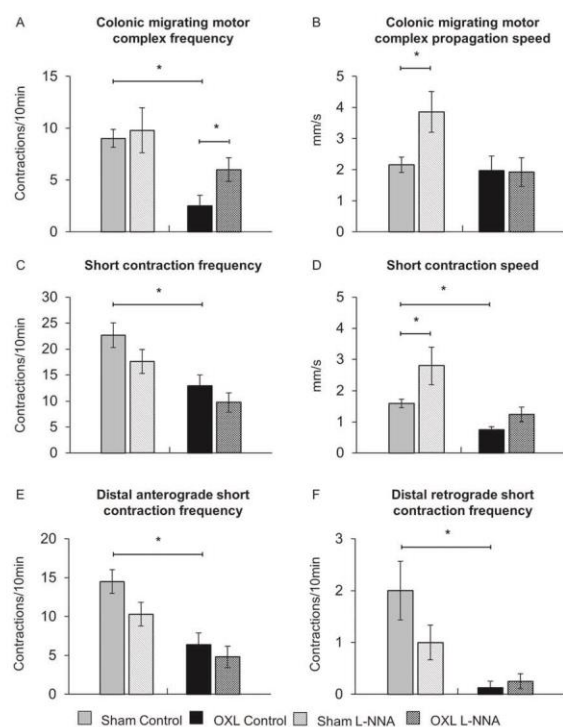


Figure 9

Effects of L-NNA on CMMCs and short contractions. (A) The frequencies of CMMCs quantified in spatiotemporal maps from sham-treated and oxaliplatin-treated mice before and after L-NNA application. (B) Speed of CMMCs in the colons from sham and oxaliplatin-treated mice in both test conditions. * $P < 0.05$, significantly different as indicated; $n = 10$ mice per group. (C) The frequencies of short contractions were quantified in spatiotemporal maps from sham and oxaliplatin-treated mice before and after L-NNA application. (D) Speed of all short contractions analysed before and after L-NNA application. Changes in the frequency of distal anterograde (E) and retrograde (F) short contractions in the colons from sham and oxaliplatin-treated mice in both test conditions. Data presented as mean \pm SEM. * $P < 0.05$, significantly different as indicated; $n = 10$ mice per group).

iNOS observed in our study. Mitochondria are responsible for most of the ROS burden under both normal and pathological conditions. The increased level of mitochondrial superoxide observed in enteric neurons is consistent with findings showing increased mitochondrial ROS in neuronal cultures from dorsal root ganglia of oxaliplatin-treated rats (Kelley *et al.*, 2014). Inhibition of mitochondrial electron transport chain complexes I and III involved in mitochondrial ROS formation improves sensory hyperalgesia in a rat model of peripheral neuropathy induced by another platinum-based chemotherapeutic drug, cisplatin (Joseph and Levine, 2009). Mitotoxicity affecting mitochondrial respiration and ATP production were found in sciatic nerve from oxaliplatin-treated rats and linked to oxaliplatin-induced peripheral

neuropathy (Zheng *et al.*, 2011), which could be prevented by peroxynitrite decomposition catalyst (Janes *et al.*, 2013). Thus, reduction of oxidative stress-related biomarkers in peripheral neurons may be a promising avenue for alleviation of chemotherapy-induced neuropathies.

Oxaliplatin-induced mitochondrial damage has also been proposed as a potential mechanism leading to activation of NOS (Areti *et al.*, 2014). The direct toxicity of NO can be greatly enhanced by reacting with superoxide to form peroxynitrite, which modifies tyrosines in proteins to create nitrotyrosines. Nitration of structural proteins can have major pathological consequences, but can also be an indicator of oxidative stress (Beckman and Koppenol, 1996). We identified translocation of nitrated proteins in both submucosal

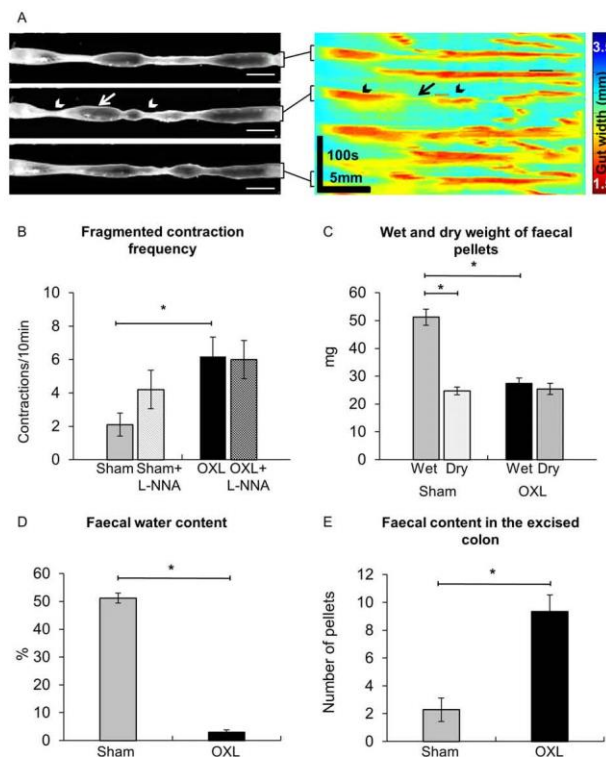


Figure 10

Effects of L-NNA on fragmented contractions and colonic faecal content. (A) Fragmented contractions were defined as interrupted contractions consisting of period(s) of relaxation (arrow) and simultaneously occurring contractions (arrowheads). The frequency (B) of fragmented contractions in the colon from sham and oxaliplatin-treated mice before and after L-NNA application. (C) Wet weight of faecal pellets measured immediately upon pellet expulsion; dry weight of faecal pellets measured after 72 h of dehydration at room temperature. (D) Faecal water content calculated as the difference between the wet weight and dry weight. (E) Total number of faecal pellets along the entire length of the colon counted in freshly excised intact colons. Data presented as mean \pm SEM. * $P < 0.05$, significantly different as indicated; $n = 10$ mice per group.

and myenteric plexuses of the colon from oxaliplatin-treated mice. Translocation of nitrotyrosine has been previously found in myenteric neurons following ischaemia and reperfusion in mice (Rivera *et al.*, 2011b) and induction of colitis in rats (Zingarelli *et al.*, 2003); both of which were correlated with increases in NO and ROS. The increased level of iNOS observed in the LMMP preparations in our study probably leads to increased production of NO contributing to the accumulation of nitrotyrosine in enteric neurons. Although nitration can be used as an indirect measure of peroxynitrite, accumulation of nitrotyrosine itself is correlated with increased susceptibility to NO-induced apoptosis (Savidge, 2011). Accumulation of nitrotyrosine has been

linked to protein misfolding, mitochondrial dysfunction and neuronal degeneration (Nakamura and Lipton, 2008). Increased ROS and concurrent oxidative stress are linked to damage in various cellular components, and neuronal death (Wei *et al.*, 2000).

One of many by-products of oxaliplatin metabolism is oxalate and increases in oxalate levels trigger intracellular Ca^{2+} influx (Webster *et al.*, 2005). Increased cytoplasmic Ca^{2+} activates nNOS, potentially leading to excessive NO production as well as activating the release of cytochrome *c* (Yagihashi *et al.*, 2000). Release of JC-10-labelled cytochrome *c* diffusing out of the mitochondria as a result of mitochondrial depolarisation and increased permeability was observed in

both submucosal and myenteric neurons in our study. The increase in superoxide and NO production may be a direct consequence of the loss of mitochondrial cytochrome *c* (Düssmann *et al.*, 2003). The rate of superoxide production is linked to the magnitude of mitochondrial membrane potential and mitochondrial permeability (Brand *et al.*, 2004). The magnitude of mitochondrial depolarisation dictates cytochrome *c* release and hence the activation of apoptosis. Modest mitochondrial depolarisation is transient and reversible, and has been found to be protective against moderate tissue injury (Schweizer and Richter, 1994; Gottlieb *et al.*, 2003). Apoptotic cells exhibit distinctive biochemical features, including the presence of specific cleaved proteins (Hengartner, 2000). Caspase-3 is a key effector or 'executioner' caspase, cleaving various substrates to cause the morphological and biochemical changes seen in apoptotic cells (Slee *et al.*, 2001; Elmore, 2007; McIlwain *et al.*, 2013). Our results are consistent with previous studies using caspase-3 antibody, which found both intracellular immunoreactivity and detected extracellular aggregates of apoptotic debris outside neuronal cells (Pasinelli *et al.*, 2000). Due to the fact that the half-life of activated caspase-3 is 8 h, it is difficult to detect significant amounts of activated caspase 3 within enteric ganglia. It is possible that the activation of caspase-3 might have occurred at the earlier stages of oxaliplatin treatment resulting into neuronal apoptosis; this would explain both neuronal loss and presence of apoptotic debris observed in our study at day 14. Increased expression of caspase-3 observed in our study is indicative of oxaliplatin-induced apoptosis which resulted in 43% loss of myenteric neurons in the distal colon, 16% of myenteric neurons in the proximal colon and 30% of submucosal neurons in the distal colon. Similarly, oxaliplatin treatment induced 12% loss of myenteric and 21% submucosal neurons in the mouse ileum at day 14 (Robinson *et al.*, 2016). Although oxaliplatin causes neuronal death in all studied intestinal regions, it seems that neurons in the distal colon are more susceptible to oxaliplatin-induced damage than neurons in the proximal colon and ileum. There may be many reasons for these regional differences, but it should be noted that the proportion of the nNOS-IR neurons was significantly increased in the distal, but not proximal colon, consistent with a role for NO in oxaliplatin-induced enteric neuronal loss. Moreover, repeated *in vivo* oxaliplatin administration induces decreases in glial fibrillary acidic protein-IR enteric glia, contrasting with increases in s100 β -IR enteric glial cells in both the myenteric and submucosal ganglia at day 14 after commencement of treatment (Robinson *et al.*, 2016).

Changes in neuromuscular transmission and smooth muscle tone

NO released from nNOS expressing inhibitory motor neurons supplying the intestinal smooth muscles is an integral inhibitory neurotransmitter in the gastrointestinal tract (Lecci *et al.*, 2002; Takahashi, 2003). nNOS produces basal levels of NO to maintain the hyperpolarised state of the circular muscle cells leading to physiological tonic inhibition of the intestine (Daniel *et al.*, 1994). Excessive NO production and consequent nitrosylation has been linked to inhibition

of dynamin-related protein 1, resulting in synaptic impairment and disruption to synaptic transmission (Savidge, 2011). In our study, intracellular recordings from colonic circular muscles revealed changes in neuromuscular transmission with an increase in amplitude of NO-mediated sIJP potentials in the oxaliplatin-treated group. No changes in ATP-mediated fIJP or ACh-mediated EJPs were observed. These results differ from findings in inflamed guinea pig colon where oxidative stress leads to reduced purinergic neuromuscular transmission (Roberts *et al.*, 2013). This may be due to differences in mechanisms of oxaliplatin-induced neuronal damage from inflammation-induced enteric neuropathy. Oxaliplatin exerts its cytotoxic effects via direct binding to nuclear and mitochondrial DNA and formation of platinum-DNA adducts (Graham *et al.*, 2000; Goodman *et al.*, 2006). Direct mitochondrial damage leads to excessive NO production and activation of apoptotic cascades as discussed above.

Direct oxaliplatin toxicity and increased NO signalling alter the functions of colonic circular muscles. The relaxation evoked by the NO donor, SNP, was reduced by about 50% in the distal colon from oxaliplatin-treated mice. The baseline diameter of the distal colon was greater in oxaliplatin-treated animals than in sham-treated mice suggesting a reduction in the colonic muscle tone after oxaliplatin treatment. This was reversed by application of L-NNA inhibiting all NOS isoforms including iNOS, which was increased in longitudinal muscle-myenteric neuron preparations after oxaliplatin treatment. The reduced smooth muscle tone as well the reduced response to the NO donor could be a result of the relatively higher inhibitory activity compared with the excitatory due to the increased proportion of nNOS neurons observed in oxaliplatin-treated mice. The reduction in muscle thickness that we observed after oxaliplatin treatment may also contribute to a reduction in muscle tone. Decreased basal tone and, thus, a larger resting diameter of the distal colon compromised the ability of smooth muscles to further relax. Similar findings have been observed following intestinal inflammation where smooth muscle relaxation in response to a NO donor was decreased (Van Bergeijk *et al.*, 1998; Rajagopal *et al.*, 2015). This compromised ability to relax was in part attributed to iNOS-mediated protein nitrosylation (Rajagopal *et al.*, 2015).

Oxaliplatin-induced changes in colonic motility

To investigate the role of NO in colonic dysmotility resulting from oxaliplatin treatment, we performed experiments with and without application of the NOS inhibitor, L-NNA, and analysed phases of contraction in various parts of the colon, overall colonic motor patterns and parameters of specific types of motor activity.

Alterations in the basal tone of the colon and augmented nitrgic transmission may be attributed to increased NO release providing enhanced inhibitory input to the smooth muscle and altering colonic contractile patterns. Analysis of spatiotemporal maps revealed reductions in the proportion and frequency of CMMCs as well as in frequency and speed of short contractions in the distal colon from oxaliplatin-treated mice. Anterograde and retrograde short contractions

were observed in the distal colon of both sham and oxaliplatin-treated mice. Both types of short contractions were significantly reduced in the distal colon after oxaliplatin treatment. In contrast, the proportion and frequency of fragmented contractions were greater in colons from oxaliplatin-treated mice. These findings are consistent with previous studies demonstrating inhibition of CMMCs in the colon of oxaliplatin-treated mice (Wafai *et al.*, 2013) and inhibition of intestinal transit in cisplatin-treated rats (Vera *et al.*, 2011).

Previous studies using NOS inhibitors have established a role of NO in the pathophysiology of gastrointestinal functional disorders (Rivera *et al.*, 2011a). NO is a primary regulator of complex intestinal motor patterns and increased NO release can disrupt CMMCs (Sarna *et al.*, 1993; Rodriguez-Membrilla *et al.*, 1995; Castro *et al.*, 2012). In our study, application of the NOS inhibitor, L-NNA, restored the proportion and frequency of CMMCs in oxaliplatin-treated mice. NOS inhibition increased the rate of rise of all types of contractions (CMMCs, short and fragmented) in the colons from sham-treated, but not oxaliplatin-treated mice. This is consistent with previous studies reporting increased speed of contraction induced by L-NNA in control mice (Rodriguez-Membrilla *et al.*, 1995). Inhibition of NOS by L-NNA did not affect short and fragmented contractions in colons from oxaliplatin-treated mice. This suggests that the augmented proportion and frequency of fragmented contractions and reduced frequency of short contractions observed in oxaliplatin-treated mice are not due to increased NO release, but can be attributed to either disruption of enteric neural pathways or a direct effect on colonic smooth muscle function. Colonic dysmotility was associated with reduction in faecal water content and increase in number of pellets in the colon providing evidence that oxaliplatin-treated mice had constipation.

In conclusion, oxaliplatin treatment induces severe gastrointestinal side-effects such as nausea, vomiting, constipation and diarrhoea, which account for dose limitations and/or cessation of treatment. These symptoms can persist up to 10 years after the treatment has been ceased (Denlinger and Barsevick, 2009). Oxidative stress has been suggested as a potential mechanism underlying platinum neurotoxicity (Di Cesare Mannelli *et al.*, 2012). Our study is the first providing evidence that oxidative stress is a key player in enteric neuronal death, changes in intestinal smooth muscle tone and neuromuscular transmission underlying colonic dysmotility and leading to chronic constipation associated with oxaliplatin treatment. Further studies elucidating mechanisms of oxaliplatin-induced oxidative stress are warranted in order to develop strategies to alleviate gastrointestinal side-effects of chemotherapy and achieve more effective anti-cancer treatment.

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Author contributions

R.M.M. conception and design, collection, analysis and interpretation of data, manuscript writing; S.E.C. collection and interpretation of data, manuscript revision; V.S. collection and interpretation of data, manuscript revision; A.R. analysis and interpretation of data, manuscript revision; R.M.G. collection and analysis of data; A.M.R. collection and analysis of data; C.A.G. collection and analysis of data, manuscript revision; J.C.B. interpretation of data, manuscript revision; K.N. conception and design, interpretation of data, manuscript revision. All authors approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Gastrointestinal dysfunction and enteric neurotoxicity following treatment with anticancer chemotherapeutic agent 5-fluorouracil

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Key Points

- 5-FU is the first-line chemotherapy for colorectal cancer; its use is associated with severe long-term gastrointestinal side-effects. Mechanisms underlying 5-FU-induced gastrointestinal dysfunction have not been investigated in depth.
- This is the first study in a mouse model demonstrating that short-term 5-FU treatment induces increased gastrointestinal transit associated with acute intestinal inflammation, which may lead to persistent changes in the ENS contributing to delayed gastrointestinal transit and colonic dysmotility.
- These findings provide insight into the mechanisms underlying chemotherapy-induced gastrointestinal dysfunction.

Abstract

Background The use of the anticancer chemotherapeutic agent 5-fluorouracil (5-FU) is often limited by nausea, vomiting, constipation, and diarrhea; these side-effects persist long after treatment. The effects of 5-FU on enteric neurons have not been studied and may provide insight into the mechanisms underlying 5-FU-induced gastrointestinal dysfunction. **Methods** Balb/c mice received intraperitoneal injections of 5-FU (23 mg/kg) 3 times/week for 14 days. Gastrointestinal transit was analysed in vivo prior to and

following 3, 7, and 14 days of 5-FU treatment via serial x-ray imaging. Following 14 days of 5-FU administration, colons were collected for assessment of ex vivo colonic motility, gross morphological structure, and immunohistochemical analysis of myenteric neurons. Fecal lipocalin-2 and CD45⁺ leukocytes in the colon were analysed as markers of intestinal inflammation. **Key Results** Short-term administration of 5-FU (3 days) increased gastrointestinal transit, induced acute intestinal inflammation and reduced the proportion of neuronal nitric oxide synthase-immunoreactive neurons. Long-term treatment (7, 14 days) resulted in delayed gastrointestinal transit, inhibition of colonic migrating motor complexes, increased short and fragmented contractions, myenteric neuronal loss and a reduction in the number of ChAT-immunoreactive neurons after the inflammation was resolved. Gross morphological damage to the colon was observed following both short- and long-term 5-FU treatment. **Conclusions & Inferences** Our

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results indicate that 5-FU induces accelerated gastrointestinal transit associated with acute intestinal inflammation at day 3 after the start of treatment, which may have led to persistent changes in the ENS observed after days 7 and 14 of treatment contributing to delayed gastrointestinal transit and colonic dysmotility.

Keywords 5-fluorouracil, 5-FU, colonic motility, enteric neuropathy, gastrointestinal transit, myenteric neurons.

Abbreviations: 5-FU, 5-fluorouracil; CMMC, colonic migrating motor complex; CRC, colorectal cancer; ENS, enteric nervous system; FC, fragmented contraction; IR, immunoreactive; SC, short contraction.

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer related mortality,^{1–3} leading to over 1 million deaths annually⁴; it is one of the most frequently diagnosed cancers worldwide. Due to late stage diagnosis approximately 50% of patients have metastasis to surrounding tissues, most frequently to the liver.⁵ Therefore, dual therapy consisting of chemotherapy and surgical resection is common.⁶

Since the discovery of fluoropyrimidines in the 1950's, 5-fluorouracil (5-FU) has been the backbone of therapy for many solid tumors and is considered as the standard first line therapy for metastatic CRC.⁷ 5-fluorouracil is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen.⁸ Upon entering a cell, 5-FU is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate, and fluorouridine triphosphate. These active metabolites exert their cytotoxic effects via misincorporation of fluoronucleotides into RNA and inhibition of nucleotide synthetic enzyme thymidylate synthase.⁹

Combination therapies including 5-FU are reported to improve response rates for advanced CRC by 40–50%,^{10,11} however, severe gastrointestinal side-effects such as nausea, vomiting, constipation, and diarrhea remain significant hurdles in the clinical application of 5-FU. It has been suggested that 5-FU-induced gastrointestinal dysfunction results from inflammation, epithelial degradation, and intestinal ulceration triggering intestinal mucositis.¹² However, recent evidence suggests that 5-FU induced gastrointestinal dysmotility outlasts intestinal mucositis.¹³ One system that has been overlooked in 5-FU-induced gastrointestinal dysfunction is the enteric nervous system

(ENS), embedded within the wall of the gastrointestinal tract and controlling its functions.¹⁴ Neuronal loss and phenotypic changes within the ENS following chemotherapeutic administration of other agents, cisplatin and oxaliplatin, have been found to result in downstream effects on gastrointestinal muscle tone and transit.^{15,16}

The aims of this study were to investigate the effects of short- and long-term 5-FU treatment on: (i) gastrointestinal transit time and gastric emptying; (ii) *ex vivo* colonic motility functions; (iii) histological architecture of the colonic mucosa; (iv) level of inflammation in the colon; and (v) the total number of myenteric neurons and subpopulations of inhibitory and excitatory neurons controlling colonic motility.

MATERIALS AND METHODS

All procedures were approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Animals

Male Balb/c mice aged 6–8 weeks (18–25 g) supplied from the Animal Resources Centre (Perth, Australia) were used for the experiments. Mice had free access to food and water and were kept under a 12 h light/dark cycle in a well-ventilated room at an approximate temperature of 22 °C. Mice acclimatized for up to 7 days prior to the commencement of *in vivo* intraperitoneal injections. A total of 42 mice were used for this study.

In vivo 5-FU injections

Mice received intraperitoneal injections of 5-FU (23 mg/kg; Sigma-Aldrich, Castle Hill, NSW, Australia), 3 times a week via a 26 gauge needle. 5-FU was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) to make 1 M/L stock solution refrigerated at –20 °C. The stock was then defrosted and diluted with sterile water to make 0.1 M/L (10% DMSO) solutions for intraperitoneal injections. The dose of 5-FU was calculated to be equivalent to standard human dose per body surface area.¹⁷ The low doses of 5-FU (10–40 mg/kg) have been shown to have antitumor efficacy in mouse models of cancer.¹⁸ Sham-treated mice received 10% DMSO in sterile water via intraperitoneal injection three times a week via a 26 gauge needle. The injected volumes were calculated to the body weight; the maximum volume did not exceed 200 µL per injection. Mice were euthanized via cervical dislocation at 3 (2 treatments), 7 (3 treatments), and 14 (6 treatments) days after the first injection and colon was collected for *in vitro* experiments.

Gastrointestinal transit

Prior to performing x-ray imaging, animals were trained/conditioned for oral gavage using a non-irritating substance such as 0.9% w/v saline (volume 0.1–0.4 mL); this was repeated at least three times with each animal with at least 24 h between each training.

The training/conditioning with restraint were done by placing the restrainer into the mouse cages at least 24 h prior to the x-ray procedure. Gastrointestinal transit was studied by x-ray prior to first treatment (day 0) and following 3, 7, and 14 days of 5-FU treatment. The contrast agent, 0.4 mL of suspended barium sulfate (X-OPAQUE-HD, 2.5 g/mL), was administered via oral gavage. Radiographs of the gastrointestinal tract were performed using a HiRay Plus Porta610HF x-ray apparatus (JOC Corp, Kanagawa, Japan; 50 kV, 0.3 mAs, exposure time 60 ms). Mice were immobilized in the prone position by placing them inside a transparent plastic restraint tube with partly open front side for breathing which comfortably restrains animal movement essential for maximum of 1–2 min for successful x-ray imaging. X-rays were captured using FujiFilm cassettes (24 × 30 cm) immediately after administration of barium sulfate (T0) every 5 min for the first hour, every 10 min for the second hour, then every 20 min through to 480 min (T480). Animals were closely monitored during and after all procedures. Images were developed via a FujiFilm FCR Capsula XLII and analysed using eFilm 4.0.2 software. Speed of gastrointestinal transit was calculated as time in minutes taken to reach each region of the gastrointestinal tract (stomach, small intestines, cecum, and large intestines). Organ emptying was calculated as the time taken for complete barium emptying from specific gastrointestinal regions (stomach, small intestines).^{19–21}

Colonic motility experiments

The entire colon was removed from day 14 sham and 5-FU-treated mice and set up in organ-bath chambers to record motor patterns *in vitro*.¹⁵ Briefly, the colon was placed into warmed (35 °C), oxygenated physiological saline until the fecal pellets were expelled. The empty colon was cannulated at both ends and arranged horizontally in an organ-bath chamber. The proximal end of the colon was connected to a reservoir containing oxygenated physiological saline to maintain intraluminal pressure. The distal end was attached to an outflow tube that provided a maximum of 2 cm H₂O back-pressure. Organ baths were continuously superfused with oxygenated physiological saline solution and preparations were left to equilibrate for 30 min. Contractile activity of each segment was recorded with a Logitech Quickcam Pro camera positioned 7–8 cm above the preparation. Videos (2 × 20 min) of each test condition were captured and saved in *avi* format using VirtualDub software (Avery Lee, Cambridge, MA, USA) (version 1.9.11).

Colonic migrating motor complexes (CMMCs) were defined as propagating contractions directed from the proximal to the distal end of the colon which travelled more than 50% of the colon length.^{22–24} Contractions that propagated less than 50% colon length were considered to be short contractions (SCs). Incomplete non-propagating phasic contractions occurring concurrently at different parts of the colon rather than propagating over the length of the colon were defined as fragmented contractions (FCs). Recordings were used to construct spatiotemporal maps using in-house edge detection software.²⁵ Spatiotemporal maps plot the diameter of the colon at all points during the recording allowing contractile motor patterns to be analysed with Matlab software (Mathworks, Natick, MA, USA) (version 12).

Drugs used

Hexamethonium Bromide (HEX; Sigma-Aldrich) and tetrodotoxin (TTX) (Abcam, Cambridge, MA, USA) were prepared as stock solutions and diluted in physiological saline daily before addition to preparations.

Histology

The colon was harvested and placed in a 10% formalin solution overnight and then transferred into 70% ethanol the following day. Paraffin embedded colon sections were cut 5 µm thick and de-waxed in a 60 °C oven for 30 min. To examine the morphological changes to the colon, a standard Hematoxylin and Eosin staining protocol was followed.^{26,27}

Immunohistochemistry in wholemount preparations

Colon sections (2–3 cm) were placed in oxygenated phosphate-buffered saline (PBS; pH 7.2) containing nicardipine (3 µM; Sigma-Aldrich) for 20 min to inhibit smooth muscle contractions. Samples were cut open along the mesenteric border, cleared of their contents, maximally stretched and dissected mucosa up to expose the myenteric plexus attached to the longitudinal muscle layer. Tissues were fixed with Zamboni's fixative (2% formaldehyde, 0.2% picric acid) overnight at 4 °C. Preparations were cleared of fixative by washing 3 × 10 min with DMSO followed by 3 × 10 min washes with PBS. Fixed tissues were stored at 4 °C in PBS for a maximum of 5 days.

Wholemount preparations were incubated with 10% normal donkey serum (Chemicon, EMD Millipore Corporation, Billerica, MA, USA) for 1 h at room temperature. Tissues were then washed (2 × 5 min) with PBS and incubated with primary antibodies against Protein Gene Product 9.5 (PGP9.5) (chicken, 1 : 500; Abcam), neuronal nitric oxide synthase (nNOS; goat, 1 : 500; Abcam), and choline acetyl transferase (ChAT; goat, 1 : 200; Abcam) overnight at 4 °C. Tissues were then washed in PBS (3 × 10 min) before incubation with species-specific secondary antibodies labeled with different fluorophores: donkey anti-chicken Alexa 594 (1 : 200; Jackson Immuno Research Laboratories, West Grove, PA, USA) and donkey anti-goat Alexa 488 (1 : 200; Jackson Immuno Research Laboratories) for 2 h at room temperature. Wholemount preparations were given 3 × 10 min final washes in PBS and then mounted on glass slides using fluorescent mounting medium (DAKO, Glostrup Municipality, Hovedstaden, Denmark). Wholemount preparations were observed under a Nikon Eclipse Ti laser scanning microscope (Nikon, Tokyo, Japan), eight randomly chosen images from each preparation were captured with a 20× objective and processed using NIS Elements software (Nikon). The number of PGP9.5, nNOS, and ChAT immunoreactive neurons was quantified in the myenteric ganglia within a 2 mm² area of each preparation.

Immunohistochemistry in cross-sections

Colon sections (1–2 cm) were placed in oxygenated PBS containing nicardipine (3 µM; Sigma-Aldrich) for 20 min to inhibit smooth muscle contractions. Samples were cut open along the mesenteric border, cleared of their contents, and pinned mucosa up without stretching. Tissues were fixed with Zamboni's fixative overnight at 4 °C. Preparations were cleared of fixative by washing 3 × 10 min with DMSO (Sigma-Aldrich) followed by 3 × 10 min washes with PBS. After washing, tissues were embedded in 100% OCT and frozen using liquid nitrogen (LN₂) and isopentane (2-methyl butane) and stored in –80 °C freezer. Tissues were cut at 20 µm section thickness using Leica CM1950 cryostat (Leica Biosystems, Wetzlar, Hesse, Germany), adhered to slides and allowed to rest for 30 min at room temperature before processing.

Cross-section preparations were incubated with 10% normal donkey serum (Chemicon) for 1 h at room temperature. Tissues

were then washed (2×5 min) with PBS and incubated with primary antibodies against CD45 (Rat, 1 : 500; BioLegend, San Diego, CA, USA), overnight at 4°C . Sections were then washed in PBS (3×10 min) before incubation with secondary antibodies labeled with fluorophore donkey anti-rat Alexa488 (1 : 200; Jackson Immuno Research Laboratories) for 2 h at room temperature. The sections were given 3×10 min final washes in PBS and then cover slipped using fluorescence mounting medium (DAKO). Sections were viewed under a Nikon Eclipse Ti laser scanning microscope (Nikon), eight randomly chosen images from each preparation were captured with a $20\times$ objective and processed using NIS Elements software (Nikon). The number of CD45⁺ immunoreactive cells was quantified within a 2 mm^2 area in every colonic section.

Imaging

Three dimensional (z-series) images of wholemount preparations were taken using a Nikon Eclipse Ti laser scanning microscope (Nikon). Fluorophores were visualized using excitation filters for Alexa 594 Red (excitation wavelength 559 nm), Alexa 488 (excitation wavelength 473 nm), and Alexa 405 (excitation wavelength 405 nm). Z-series images were taken at step size of $1.75\text{ }\mu\text{m}$ (1600×1200 pixels).

Quantification of fecal lipocalin-2

Fresh fecal pellets were collected from mice after 3, 7, and 14 days of 5-FU administration and stored immediately at -80°C . Fecal samples were thawed and suspended in PBS with 0.1% Tween (100 mg/mL) overnight prior to processing. The next day samples were centrifuged at 13.8 g at 4°C for 10 min. Samples were then agitated and centrifuged for another 5 min at $12\text{ }000\text{ rpm}$ at 4°C . Supernatant was collected and lipocalin-2 levels were quantified using DuoSET ELISA Mouse Lipocalin-2 kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Data were assessed using two-way ANOVA, Welch's two-tailed *t*-test, and Student's two-tailed *t*-test. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA). Data are presented as mean \pm SEM. Value differences were considered statistically significant at $p < 0.05$.

RESULTS

Altered gastrointestinal transit following 5-FU administration

To determine the effects of 5-FU administration on gastrointestinal transit, series of radiographic images were used to track barium sulfate throughout the gastrointestinal tract before the first injection (day 0), after two injections (day 3), three injections (day 7), and six injections (day 14; Figs 1 and 2, Table 1). The speed of barium movement was calculated by tracing barium entry from one part of the gastrointestinal tract to the next. After 3 days of 5-FU administration, movement of barium in both the cecum and colon was faster than before treatment (Fig. 2A), however, after 7 and

14 days of 5-FU administration barium movement was significantly delayed compared to day 0 in the cecum and colon (Fig. 2A, Table 1).

Although tracing barium movement allowed analysis of real time transit speed, gastrointestinal organ filling and emptying does not happen simultaneously, thus we further analysed the time taken for complete barium emptying from specific regions.

No changes in gastric emptying time were observed at day 3 of 5-FU administration, but significant delays in gastric emptying were seen after 7 and 14 days of 5-FU administration (Fig. 2B, Table 1). Intestinal emptying was faster after 3 days of 5-FU administration, but significantly delayed after both 7 and 14 days of 5-FU administration (Fig. 2C, Table 1).

Pellet formation time was decreased after 3 days of 5-FU administration (Fig. 2D), but significantly increased after 7 and 14 days of 5-FU administration (Fig. 2D). Thus, increased intestinal transit was observed after short-term 5-FU treatment, while prolonged treatment induced delays in gastrointestinal transit.

Changes in colonic motility following 5-FU treatment

To investigate effects of 5-FU treatment on colonic motility, excised colons were studied in organ bath experiments at day 14 of 5-FU treatment. The total number of contractions (including all types of motor patterns in the colon: CMMCs, short and FCs, Fig. 3A) was increased in colons from 5-FU-treated animals compared to sham-treated mice (sham: 27.7 ± 1.4 , 5-FU: 36.1 ± 2.1 , $p < 0.01$, Fig. 3B). To determine if this increase was due to changes in a specific type of motor activity, the frequency, proportion, and propagation speed were analysed for each type of motor pattern.

Colonic migrating motor complexes were defined as sustained anally directed contractions propagating more than 50% of the colon length. Colonic migrating motor complexes are mediated mainly by myenteric neurons, although inputs from the mucosa may modulate their activity.²⁸ *In vivo* treatment with 5-FU was associated with a decrease in the frequency of CMMCs in comparison with sham-treated mice (sham: 11.2 ± 0.6 , 5-FU: 3.5 ± 1.0 , $p < 0.0001$, Fig. 3B). Similarly, the proportion of CMMCs following 5-FU treatment was significantly decreased compared to sham-treated mice (sham: $41.1 \pm 3.0\%$, 5-FU: $12.4 \pm 3.8\%$, $p < 0.0001$, Fig. 3C). No significant difference in the speed of CMMCs was found between sham-treated and 5-FU-treated mice (sham: $2.9 \pm 0.2\text{ mm/s}$, 5-FU: $2.3 \pm 0.3\text{ mm/s}$, Fig. 3D).

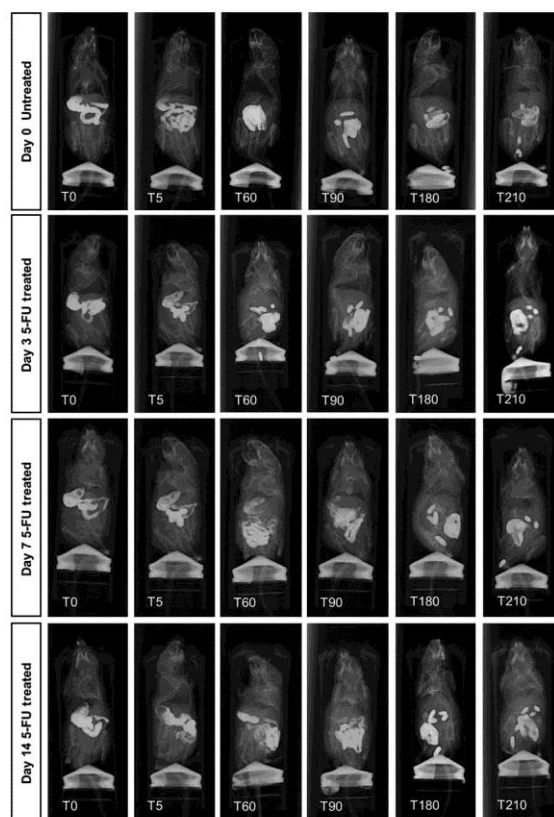


Figure 1 X-ray images following repeated *in vivo* 5-FU (2.5 mg/kg, 3 times/week) administration. Representative x-ray images obtained from mice 0–210 min after intragastric barium sulfate (0.4 mL, 2.5 mg/mL) administration at day 0 (prior to 1st injection) and following 3, 7, and 14 days of 5-FU administration.

Contractions that propagated less than 50% of the colon length were termed SCs [Fig. 3A]. Treatment with 5-FU resulted in both increased frequency (sham: 10.1 ± 1.1 , 5-FU: 20.5 ± 3.0 , $p < 0.01$, Fig. 3B) and increased proportion (sham: $36.0 \pm 2.2\%$, 5-FU: $54.2 \pm 4.1\%$, $p < 0.01$, Fig. 3C) of SCs in the colon. A significant increase in the speed of SCs was found following 5-FU administration when compared to sham-treated mice (sham: 0.7 ± 0.0 mm/s, 5-FU: 1.9 ± 0.3 mm/s, $p < 0.01$, Fig. 3D). Incomplete contractions that did not propagate, but rather occurred concurrently over the length of the colon were defined as FCs. Both the frequency (sham: 6.4 ± 0.8 , 5-FU: 11.8 ± 1.2 , $p < 0.01$, Fig. 3B) and the proportion (sham:

$22.9 \pm 2.3\%$, 5-FU: $32.5 \pm 1.8\%$, $p < 0.01$, Fig. 3C) of FCs were significantly higher in 5-FU-treated compared to sham-treated mice. No significant difference in the speed of FCs was found between sham-treated and 5-FU-treated mice (sham: 1.5 ± 0.1 mm/s, 5-FU: 2.1 ± 0.3 mm/s, Fig. 3D).

To investigate whether neurogenic components are involved in the recorded colonic motor patterns, a voltage-gated sodium channel blocker TTX and a nicotinic cholinergic antagonist HEX were added into the organ baths. TTX [$1 \mu\text{M}$, 20 min] abolished CMMCs, SCs, and FCs in colons from both sham and 5-FU-treated mice (Fig. 3E), indicating that these motility patterns are neurogenic. However, a persistent TTX-resistant

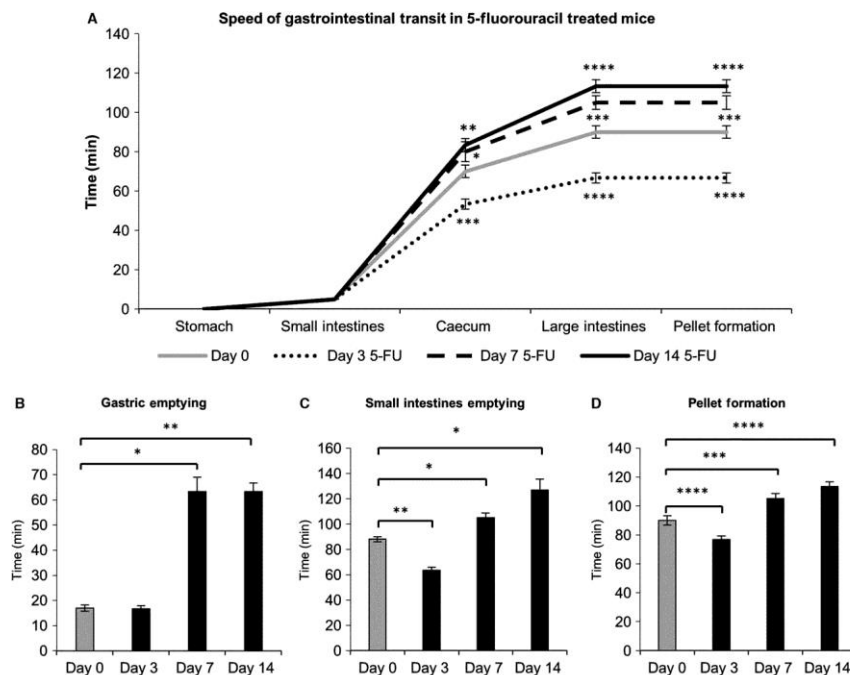


Figure 2 Gastrointestinal transit time, gastric and intestinal emptying following repeated *in vivo* 5-FU administration. (A) Time (min) taken for barium sulfate to reach the stomach, small intestines, caecum, and large intestines before (day 0) and at 3, 7, and 14 days following 5-FU administration. (B) Time (min) taken for complete emptying of barium from the stomach. (C) Time (min) taken for complete emptying of barium from the small intestines. (D) Time (min) taken to form first pellet before (day 0) and at 3, 7, and 14 days following 5-FU administration. Data represented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 6$ per group/time point.

tonic constriction in the distal part of the colon blocked by HEX ($100 \mu\text{M}$, 20 min) was observed in sham-treated mice. In 5-FU-treated mice, TTX-resistant tonic constrictions were prominent in both proximal and distal parts of the colon; the proximal tonic constriction was not blocked by application of HEX (Fig. 3E).

Morphological damage to the colon following 5-FU administration

Colonic architecture from sham-treated animals at days 3, 7, and 14 appeared healthy with a visible brush border and uniform crypts (Fig. 4A C). Histological examination of the colon at day 3 from the 5-FU-treated group demonstrated no obvious changes to the epithelial brush border; however, there was a severe

loss of colonic crypts and goblet cells and cellular infiltration within the lamina propria when compared to the sham-treated group (Fig. 4A and A'). By days 7 and 14, there was thickening of the epithelial brush border indicative of regeneration of the colonic crypts in the 5-FU-treated groups, but they still appeared short, distended, and disorganized with an increased number of goblet cells (Fig. 4B' and C') when compared to colon from the sham-treated group (Fig. 4B and C).

Intestinal inflammation following 5-FU treatment

To investigate if 5-FU treatment causes inflammation, immune cell infiltration in the colon and the concentration of a neutrophil gelatinase-associated protein,

Table 1 Speed of transit and emptying following repeated *in vivo* 5-fluorouracil administration

| Parameters measured | Day 0 | Day 3 | Day 7 | Day 14 |
|---|--------|------------|-------------|-------------|
| Speed of transit (time to reach each region, min) | | | | |
| Stomach | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Small intestines | 5 ± 0 | 5 ± 0 | 5 ± 0 | 5 ± 0 |
| Cecum | 70 ± 3 | 53 ± 3*** | 80 ± 5* | 83 ± 4** |
| Large intestines | 90 ± 3 | 66 ± 3**** | 105 ± 4**** | 113 ± 4**** |
| Time for complete barium emptying (min) | | | | |
| Gastric emptying | 17 ± 1 | 16 ± 1 | 63 ± 6* | 63 ± 3** |
| Intestinal emptying | 88 ± 2 | 63 ± 3** | 105 ± 4* | 126 ± 8* |
| Pellet formation | 90 ± 3 | 66 ± 3**** | 105 ± 4**** | 113 ± 4**** |

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, significantly different to Day 0 ($n = 5$ per group).

lipocalin-2, a highly sensitive biomarker for intestinal inflammation^{29,30} in fecal samples were analysed.

Immune cells in colonic-cross sections were labeled with a pan leukocyte marker anti-CD45 antibody following 3 (Fig. 5A and A'), 7 (Fig. 5B and B'), and 14 (Fig. 5C and C') days of sham and 5-FU treatment. Total numbers of CD45 positive cells were counted within a 2 mm² area. A significant increase in the number of CD45 positive cells was found in the colon following 3 days of 5-FU administration [99 ± 2, $p < 0.0001$] when compared to sham [48 ± 1, Fig. 5D]. No significant changes in the number of CD45 positive leukocytes were found after 7 [sham: 49 ± 2, 5-FU: 53 ± 3] and 14 [sham: 51 ± 4, 5-FU: 54 ± 0.1] days of treatment (Fig. 5D). A significant increase in the concentration of lipocalin-2 was observed at day 3 after 5-FU administration (1592 ± 160 ng, $p < 0.05$) compared to sham-treated mice (883 ± 100 ng), but not at days 7 [sham: 946 ± 230 ng, 5-FU: 867 ± 210 ng] and 14 [sham: 1122 ± 160 ng, 5-FU: 881 ± 120 ng] (Fig. 5E).

Reduction in the total number of myenteric neurons and changes in neuronal subpopulations following administration of 5-FU

To investigate any changes to the total number of myenteric neurons, wholemount preparations of the colon were labeled with anti-PGP9.5 antibody to count neurons within a 2 mm² area. Repeated *in vivo* administration of 5-FU induced myenteric neuronal loss at days 7 [sham: 1225 ± 6; 5-FU: 1150 ± 5, $p < 0.001$] and 14 [sham: 1229 ± 3; 5-FU: 1091 ± 5, $p < 0.001$], but not at day 3 [sham: 1234 ± 4; 5-FU: 1231 ± 10, Fig. 6].

To determine if 5-FU administration was associated with changes in subpopulations of myenteric neurons controlling intestinal muscle activity, inhibitory muscle motor, and interneurons IR for nNOS (Fig. 7) and neurons IR for ChAT (Fig. 8) were analysed.

Fewer nNOS-IR neurons were observed at days 3 [sham: 357 ± 7; 5-FU: 286 ± 3, $p < 0.0001$] and 14 [sham: 380 ± 11; 5-FU: 310 ± 3, $p < 0.0001$] following 5-FU administration (Fig. 7A' and C) when compared to sham (Fig. 7A and C). However, no reduction in the number of nNOS-IR neurons from the 5-FU-treated group was observed at day 7 [sham: 363 ± 5; 5-FU: 341 ± 4; Fig. 7B, B' and D]. The proportion of nNOS-IR neurons was reduced on day 3 [sham: 29.5 ± 0.3%; 5-FU: 23.8 ± 0.3%] when compared to sham [$p < 0.01$, Fig. 7E], but not on days 7 [sham: 29.3 ± 0.6; 5-FU: 29.7 ± 0.3] and 14 [sham: 30.9 ± 0.9; 5-FU: 28.5 ± 0.4, Fig. 7E].

The total number of ChAT-IR neurons increased on day 3 [sham: 294 ± 4; 5-FU: 319 ± 4], but decreased on days 7 [sham: 322 ± 2; 5-FU: 300 ± 4] and 14 [sham: 320 ± 3; 5-FU: 282 ± 5] following 5-FU administration (Fig. 8A', B', C', and D) when compared to the sham-treated group (Fig. 8A D). However, no significant difference in the proportion of ChAT-IR neurons was observed at any time point after 5-FU administration began (Fig. 8E).

DISCUSSION

This study is the first to examine 5-FU-induced enteric neuropathy and its effect on gastrointestinal function. Our results show that 5-FU administration causes a significant increase in gastrointestinal transit time at day 3, but delays in intestinal and gastric emptying at days 7 and 14. Significant reductions in the frequency and proportion of CMMCs, but increased frequency and proportions of short and FCs were prominent after 14 days of 5-FU treatment. All types of contractions were abolished by TTX, a residual tonic constriction in the distal colon was blocked by HEX in both sham and 5-FU-treated mice. A TTX-resistant tonic constriction of the proximal colon seen in 5-FU treated mice was not blocked by HEX. Severe crypt ablation and mucosal destruction occurred in the colon at day 3. Consistent crypt disorganization and hypoplasia persisted throughout the experimental period; however, mucosal barrier regeneration was evident after both 7 and 14 days. Acute inflammation, confirmed by increased levels of fecal lipocalin-2 and CD45 positive leukocytes in the colon, was seen on day 3 of 5-FU treatment but subsided by days 7 and 14. Loss of myenteric neurons was seen on days 7 and 14, accompanied by

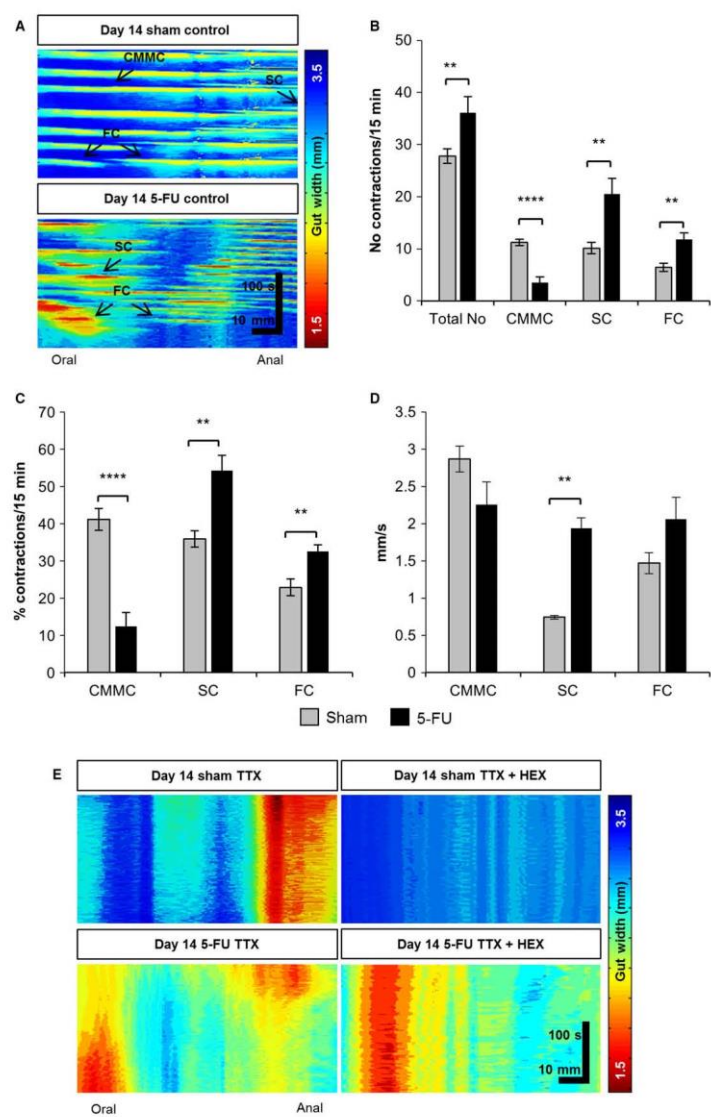


Figure 3 Total number, proportion, and speed of different contractions following repeated *in vivo* 5-FU administration. (A) Representative spatiotemporal maps generated from digital video recordings of colonic motility from sham and 5-FU-treated mice. Each contraction can be seen as a reduction in the gut width (red), while relaxation as an increase in the gut width (blue). Colonic migrating motor complexes (CMMCs) propagate >50% of the colon length, short contractions (SCs) propagate <50% of the colon length and fragmented contractions (FCs) are interrupted by period(s) of relaxation during contraction. (B) Total number of contractions including all types of contractile activity in the colons from sham and 5-FU-treated mice. (C) The proportion of CMMCs, SCs, and FCs to the total number of contractions. (D) The speed of CMMCs, SCs, and FCs. Data represented as mean \pm SEM. ** $p < 0.01$, **** $p < 0.0001$, $n = 6$ per group/time point. (E) Spatiotemporal maps from day 14 sham and 5-FU-treated mice after addition of tetrodotoxin (TTX, 1 μ M, 20 min) and hexamethonium (HEX, 100 μ M, 20 min).

reduced numbers of ChAT-IR neurons at these time points.

Although 5-FU is routinely used in the treatment of CRC and is known to cause gastrointestinal side-effects, the neurological mechanisms underlying these side-effects have not been studied.^{31,32} Our study demonstrates that, while short-term 5-FU treatment accelerates overall gastrointestinal transit without a change in gastric emptying, more prolonged treatment results in significant delays to overall gastrointestinal transit, gastric emptying and intestinal emptying. Our findings are consistent with previous studies showing that short- and long-term administration of the chemotherapeutic agent cisplatin differentially alters gastrointestinal transit.^{19,20} Our findings suggest that mechanisms underlying these differential changes in transit are different. Accelerated gastrointestinal transit observed after 3 days of treatment was associated with acute intestinal inflammation and reduced proportions of nNOS-IR neurons. Intestinal inflammation was

resolved after days 7 and 14 when delays to overall gastrointestinal transit, gastric, and intestinal emptying, and colonic dysmotility were observed. The loss of myenteric neurons observed at these time points might contribute to the functional changes in the gastrointestinal tract. Gastric and intestinal transit has previously been investigated using radioactivity retention technique in rats where it was found that delayed gastric emptying and increased fundus and duodenum muscle contractions occurred 3 and 15 days after a single high dose of 5-FU (150 mg/kg) injection.¹³ However, our study is the first to use radiographic analysis to evaluate overall gastrointestinal transit alongside ENS damage following 5-FU administration in mice. Postinflammatory delays to gastric and intestinal emptying have been reported in a plethora of gastrointestinal disorders.³³⁻³⁵ Gastric emptying of solids is significantly delayed in patients with postinfectious functional dyspepsia, irritable bowel syndrome, non-obstructive Crohn's disease and colitis.³⁴⁻³⁸

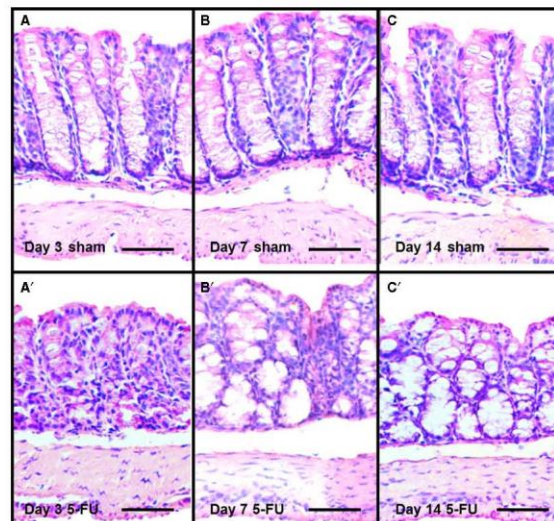


Figure 4 Gross morphological changes in the colon following repeated *in vivo* 5-FU administration. H&E staining in the colon from sham and 5-FU-treated mice at 3 (A, A'), 7 (B, B'), and 14 (C, C') days. Scale bar = 100 μ m, $n = 4$ per group/time point.

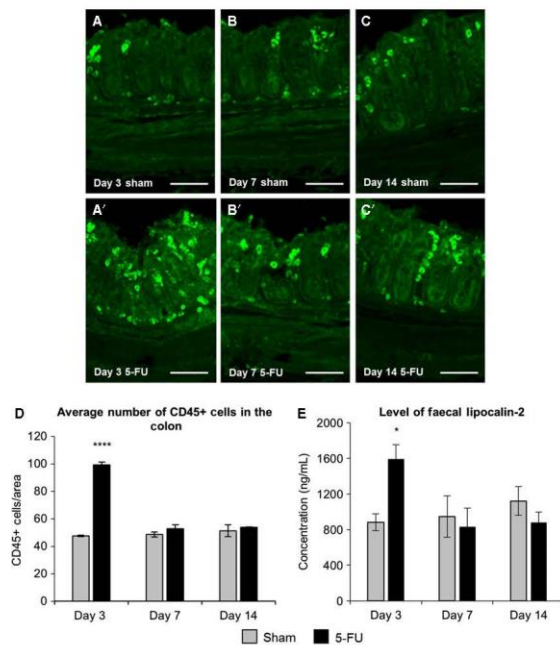


Figure 5 Inflammatory markers in the colon and fecal pellets. Cross-sections of the colon labeled with antibody against CD45⁺ leukocytes (green) following 3 (A, A'), 7 (B, B'), and 14 (C, C') days of sham or 5-FU treatment, scale bar = 50 μ m. Average number of CD45⁺ cells in the colon was counted per 2 mm² at 3, 7 and 14 days in both sham and 5-FU treated mice. (D) Concentration of lipocalin-2 (ng/mL) in fecal pellets collected following 3, 7, and 14 days of 5-FU administration (E). Data represented as mean \pm SEM. * p < 0.05, **** p < 0.0001, n = 4 per group/time point.

Mucosal inflammation, epithelial degradation and intestinal ulceration, manifesting as mucositis, following 5-FU administration have been suggested as causes

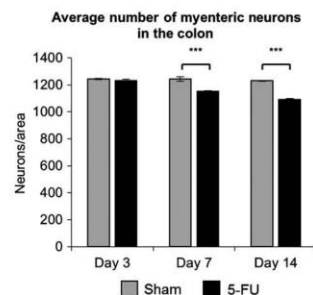


Figure 6 Effect of repeated *in vivo* 5-FU administration on the total number of myenteric neurons. Average number of PGP9.5-IR neurons in the colon was counted per 2 mm² at 3, 7, and 14 days in both sham and 5-FU-treated mice. Data represented as mean \pm SEM. *** p < 0.001, n = 6 per group/time point.

of gastrointestinal dysfunction.¹² Our results show that, while short-term 5-FU treatment was associated with destruction of the epithelial brush border and severe loss of colonic crypts and goblet cells, chronic treatment did not worsen epithelial damage. In fact regeneration of mucosa was evident during chronic 5-FU treatment, although crypts still appeared shorter, distended, and disorganized. These results are consistent with previous findings in the rat duodenum following 3 days of 5-FU treatment, where loss of crypt architecture, shortening of villi and inflammatory infiltration in the lamina propria was reported.¹³ This was followed by the restoration of intestinal villi and crypts following 15 days of 5-FU treatment, but with sparse neutrophil infiltration in the muscular layer.¹³ Such evidence indicates that duodenal mucosal inflammation resolves during 15 days of 5-FU treatment.

Both *in vitro* and *in vivo* treatment with 5-FU promote the attraction of inflammatory cells.³⁹ Intestinal inflammation is associated with gastrointestinal dysmotility both at the site of inflammation and at distant non-inflamed sites.^{40–42} Further to this a

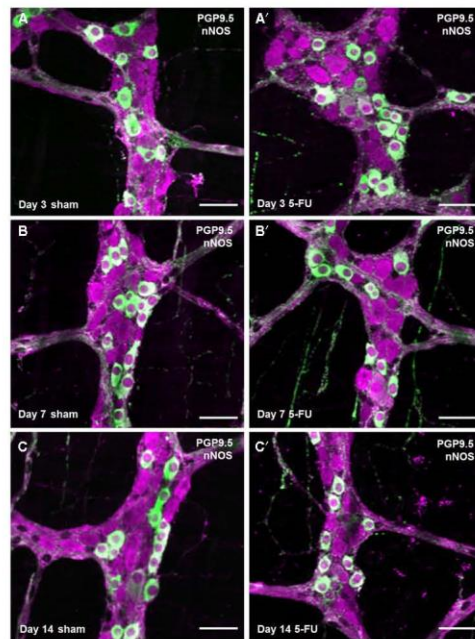
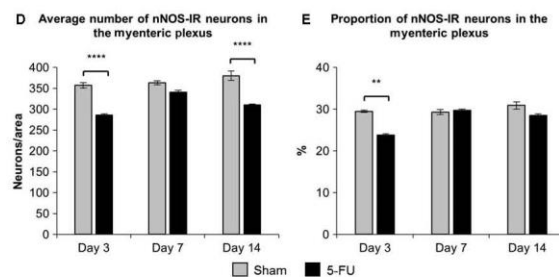


Figure 7 Effect of repeated *in vivo* 5-FU administration on average number and proportion of nNOS-IR myenteric neurons. Wholemount preparations of myenteric neurons in colon following 3 (A, A'), 7 (B, B'), and 14 (C, C') days of sham or 5-FU treatment, scale bar = 50 μ m. (D) Average number of nNOS-IR neurons (green in A–C) in the colon was counted per 2 mm² at 3, 7 and 14 days in both sham and 5-FU treated mice. (E) Proportion of nNOS-IR neurons (green in A–C) to the total number of PGP9.5-IR myenteric neurons (magenta in A–C) in the colon was counted at 3, 7, and 14 days in both sham and 5-FU-treated mice. Gray column: sham-treated, black column: 5-FU-treated. Data represented as mean \pm SEM. ** p < 0.01, **** p < 0.0001, n = 6 per group/time point.



number of inflammatory mediators have been implicated in both short- and long-term smooth muscle contractility changes.⁴³ Accordingly, many gastrointestinal side-effects experienced during and after 5-FU administration have been attributed to inflammation-induced mucosal damage which we observed in the rat model of acute toxicity induced by a high dose (150 or 300 mg/kg) of 5-FU [R. Abalo, unpublished data] and in

this study after a short-term treatment of mice with a low dose (23 mg/kg) of 5-FU. However, 5-FU-induced gastrointestinal dysmotility appears to outlast intestinal mucositis,¹³ indicating that a more complex and multifaceted pathophysiology is at play.

Our results show that chronic 5-FU administration results in significant neuronal loss in the myenteric plexus of the colon at days 7 and 14 when intestinal

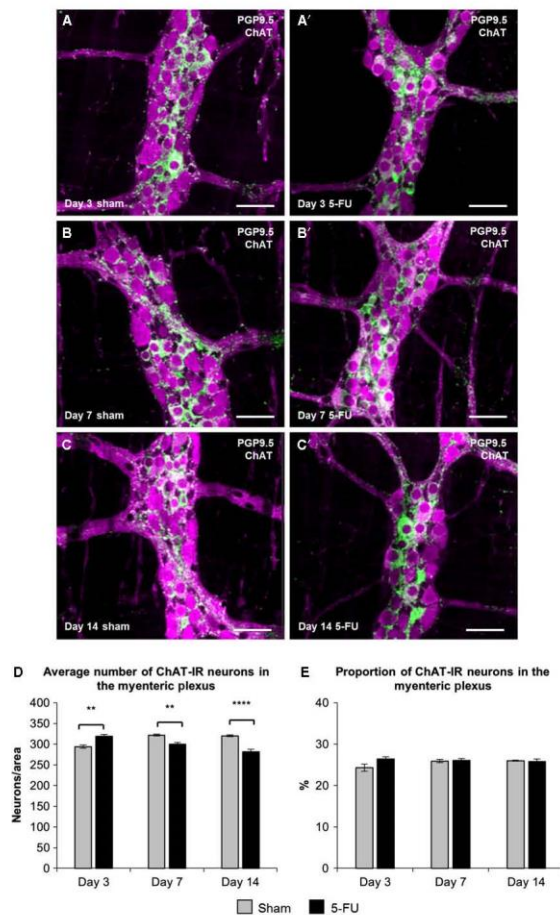


Figure 8 Effect of repeated *in vivo* 5-FU administration on the average number and proportion of ChAT-IR myenteric neurons. Wholemount preparations of myenteric neurons in the colon following 3 (A, A'), 7 (B, B'), and 14 (C, C') days of sham or 5-FU, scale bar = 50 μ m. (D) Average number of ChAT-IR neurons (green in A–C) in the colon was counted per 2 mm² at 3, 7, and 14 days in both sham and 5-FU-treated mice. (E) Proportion of ChAT-IR neurons (green in A–C) was counted to the total number of PGP9.5-IR myenteric neurons (magenta in A–C) in the colon was counted per 2 mm² at 3, 7, and 14 days in both sham and 5-FU-treated mice. Gray column: sham-treated, black column: 5-FU-treated. Data represented as mean \pm SEM. ** p < 0.01, **** p < 0.0001, n = 6 per group/time point.

inflammation subsided. Our findings are consistent with previous studies investigating effects of platinum-based anticancer chemotherapeutics, which also demonstrated that long-term treatments induce loss of myenteric neurons.^{15,16} However, 5-FU treatment induced less severe damage to myenteric neurons with 12% loss at day 14 compared to 25% of myenteric neuronal loss at day 14 of oxaliplatin treatment.¹⁵ Although previous studies demonstrated that the loss of enteric neurons does not

necessarily lead to changes in CMMCs^{44,45} our results demonstrated that the loss of myenteric neurons correlated with colonic dysmotility in 5-FU-treated mice. The loss of myenteric neurons leads to long-term alterations in gastrointestinal functions as was shown in rats after cisplatin treatment¹⁶ and mice after oxaliplatin treatment.¹⁵ 5-FU treatment inhibited CMMCs, but the total number of contractions of the isolated colon was significantly increased due to an increase in the number and

proportion of short and FCs. Short and FCs play a vital role in the construction of productive motor patterns in the healthy intestine.²⁵ Short distance contractions result in a segmenting motor pattern essential for mixing and absorption of colonic contents.⁴⁶ Alteration in short segmenting contractions induces intestinal dysmotility.⁴⁷

Our results demonstrated that all types of contractions were neurogenic; residual tonic constriction in the distal colon was blocked by HEX in both sham and 5-FU-treated mice. Tetrodotoxin-resistant tonic constriction in the proximal colon observed in 5-FU treated mice was not blocked by HEX. These results suggest that there is TTX-insensitive tonic excitatory drive to the smooth muscle that presumably involves at least one nicotinic synapse and hence must involve neurons with TTX-resistant action potentials like those described by Foong *et al.* in the mouse submucosal plexus.⁴⁸ This activity seems to be differentially sensitive to 5-FU. Thus, initial acute inflammation caused by 5-FU treatment may have led to persistent changes in the ENS and gut dysfunction. Similarly, in animal models of intestinal inflammation, long-term gut dysfunction persists long after resolution of acute inflammation due to neuronal loss, axonal damage, and neuronal dysfunction in the myenteric plexus leading to changes in synaptic transmission between neurons and to the smooth muscles.^{26,49–54}

We found that long-term treatment with 5-FU induced loss of both excitatory (ChAT-IR) and inhibitory (nNOS-IR) neurons in the myenteric plexus. However, only nNOS-IR neuron proportions were reduced at day 3 post 5-FU treatment, which is consistent with increased intestinal transit at this time point. Loss of nNOS-IR neurons was also observed in cisplatin and oxaliplatin-treated animals^{15,16} however, ChAT-IR neurons were not investigated in these studies. Thus, our results suggest that the loss of neurons after 5-FU treatment is not restricted to a specific neurochemically defined subpopulation. Differential effects of various chemotherapeutic compounds on enteric neurons might be attributed to the differences in their mechanisms of action with platinum-based compounds having direct

neurotoxicity due to platinum adducts to DNA⁵⁵ compared to inflammation-induced neuropathy associated with 5-FU treatment.¹³

Damage to the intestinal smooth muscles, alterations in neuromuscular transmission, smooth muscle sensitivity to neurotransmitters and morphological and functional changes of interstitial cells of Cajal might be also involved in 5-FU-induced dysmotility and these require further investigation.

In conclusion, this study is the first to demonstrate that 5-FU administration induces accelerated gastrointestinal transit associated with acute intestinal inflammation at day 3 post-treatment, which may have led to persistent changes in the ENS and gastrointestinal dysfunctions: delayed gastrointestinal transit, gastric and intestinal emptying, and colonic dysmotility. The loss of myenteric neurons observed at days 7 and 14 of treatment may contribute to the functional changes in the gastrointestinal tract. Further research investigating the effects of 5-FU on the electrophysiological properties of myenteric neurons is warranted.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

RMM conception and design, collection, analysis and interpretation of data, manuscript writing, VS collection, analysis and interpretation of data, manuscript revision; ED interpretation of data, manuscript revision; RA interpretation of data, manuscript revision; JCB interpretation of data, manuscript revision; KN conception and design, interpretation of data, manuscript revision. All authors approved final version of the manuscript.

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