THE UNIVERSITY of ADELAIDE

Exploring the Effects of Toll-Like Receptor 4 Antagonism on Gastrointestinal Mucositis and Tumour Activity

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Thesis Abstract

Gastrointestinal mucositis (GIM) is a hallmark of chemotherapy-induced gastrointestinal toxicity. It affects up to 80% of patients with cancer depending on their treatment regimen. Symptoms of GIM include weight loss, diarrhoea and bleeding. These symptoms can be so severe and debilitating that it often necessitates a reduction of treatment doses or discontinuation of the treatment which compromises patient survival. Unfortunately, there are no effective treatment strategies for these patients and more studies are required to develop potential intervention strategies.

TLR4 is an intra- and extra-cellular receptor expressed on endosomes and cytoplasmic membranes. TLR4 recognises pathogen-associated molecular patterns (PAMPs) (flagellin and LPS) and damage-associated molecular patterns (DAMPs) (calprotectin, S100A8/9 HMGB1 and HSP70) through its co-receptors MD-2 and CD-14. The activation of TLR4 has been proposed to have a major influence on inflammatory signalling pathways and the pathogenesis of GIM. Inhibition of TLR4 has been postulated as an effective way to treat intestinal inflammation. However, there is a limited number of studies looking into the potential of TLR4 antagonism as a therapeutic approach for gastrointestinal (GI) inflammation.

The work described in this thesis focussed primarily on the influence of TLR4 antagonism on GI toxicity stemming from irinotecan/CPT-11, a DNA topoisomerase I inhibitor used in the treatment of advanced colorectal cancer. The TLR4 antagonists studied were TAK-242 and IAXO-102, due to their potential to serve as alternative treatment options for GIM.

Firstly, I modelled binding sites and affinity of IAXO-102, TAK-242 and SN-38 (the active metabolite of CPT-11) to the human TLR4/MD-2 complex, identifying specific amino acid residues of interaction and performed 3D structural analysis through *in*

silico docking analysis. Computational techniques provide the possibility to explore drug development opportunities in order to rapidly provide structural, chemical, and biological data to improve understanding of potential drugs and their targets. The results from this study could contribute to rational development of therapeutic anti-inflammatory drugs targeting TLR4 in the GI tract.

Secondly, I assessed the potential of the TAK-242 and IAXO-102 to attenuate GI inflammation in 2 different models; 1) an *in vitro* model using intestinal epithelial cell lines (T84, HT-29) and monocyte-like cells (U937), and 2) an *ex vivo* model using segments of mouse colon. Both models were induced with inflammation using TLR4 agonists and inflammatory mediators. Results from this study did not show significant protection with TAK-242 or IAXO-102 which, highlighted the limitation of *in vitro* and *ex vivo* models to accurately simulate GIM.

Finally, from the *in vitro* and *ex vivo* studies, the TLR4 antagonist with the greatest potential for clinical development, IAXO-102 was evaluated for effectiveness to attenuate GI inflammation as well as supress tumour activity in a colorectal-tumour bearing mouse model of CPT-11-induced GIM. Results showed that IAXO-102 was able to prevent diarrhoea in mice treated with CPT-11 as well as reduce tumour volume. However, it had no effect in protecting the colon from tissue damage or changing proliferation and apoptosis rates in both the colon and tumour. As such, it was concluded TLR4 activation plays a partial role in GIM development but further research is required to understand the specific inflammatory signals underpinning tissue-level changes.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Shu Yie Janine Tam

06/04/2022

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Publications arising from this thesis

1. **Tam JSY**, Coller JK, Hughes PA, Prestidge CA, and Bowen JM (2021) Toll-like receptor 4 (TLR4) antagonists as potential therapeutics for intestinal inflammation. Indian Journal of Gastroenterology 40:5-21. 10.1007/s12664-020-01114-y

2. **Tam JSY**, Coller JK, Prestidge CA, and Bowen JM (2022) Investigation of TLR4 Antagonists for Prevention of Intestinal Inflammation. Inflammation 10.1007/s10753-022-01714-0

3. **Tam JSY**, Crame EE, Elz AS, Coller JK, Wignall A, Prestidge CA, and Bowen JM (2022) Effects of a novel toll-like receptor 4 antagonist IAXO-102 in a murine model of chemotherapy-induced gastrointestinal toxicity. Cancer Chemotherapy and Pharmacology 10.1007/s00280-022-04463-x

Other publications completed during candidature

1. Secombe KR, Crame EE, **Tam JSY**, Wardill HR, Gibson RJ, Coller JK, and Bowen JM (2021) Intestinal toll-like receptor 4 knockout alters the functional capacity of the gut microbiome following irinotecan treatment. Cancer Chemotherapy Pharmacology 10.1007/s00280-021-04382-3

Conference presentations

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Investigation of TLR4 Antagonists for Prevention of Intestinal Inflammation

Multinational Association of Supportive Care in Cancer Annual Meeting (2021)

TLR4 Antagonists, TAK-242 and IAXO-102, Do Not Provide Significant Protection in Models of Colonic Inflammation.

Annual Australian Society for Medical Research (ASMR) SA Scientific Meeting (2021)

TLR4 Antagonists, TAK-242 and IAXO-102, Do Not Provide Significant Protection in Models of Colonic Inflammation.

Florey Postgraduate Research Conference (2021)

TLR4 Antagonists, TAK-242 and IAXO-102, Do Not Provide Significant Protection in Models of Colonic Inflammation.

Florey Postgraduate Research Conference (2019)

Using Silica Nanoparticles to Target Inflammation in Pre-Clinical Models of Intestinal Disease.

List of abbreviations

For ease of reading, these abbreviations are also introduced within each chapter.

µg: Microgram

5-FU: 5-fluorouracil

AA: Asiatic acid

ALF: Acute liver failure

ANOVA: Analysis of variance

AO-I/ AT-I: Atractylenolide I

APC: Adenomatous polyposis coli

AS: Anti-sense

AβO: Amyloid-β oligomers

BALB/c: Albino, immunodeficient laboratory-bred strain of the house mouse

BDF1: Bromodomain-containing factor 1

BqLOS: Lipooligosaccharide from Bartonella quintana

C57Bl/6: C57 black 6

CBZ: Carbamazepine

CD: Crohn's disease

CD-14: Cluster of differentiation 14

cDNA: Complementary DNA

CFTR: Cystic fibrosis transmembrane conductance regulator

CIN: Chromosomal instability

CLP: Caecal ligation and puncture

C_n: Conformation number

COX-2: Cyclooxygenase-2

CPT-11: Camptothecin-11

CRC: Colorectal cancer

Cyp: LPS from *Oscillatoria Planktothrix FP1*

DAI: Disease activity index

DAMP: Damage-associated molecular pattern

DCs: Dendritic cells

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DSS: Dextran sulphate sodium

E5564/E5531: Eritoran

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epithelial growth factor receptor

ELISA: Enzyme-linked immunosorbent assays

ERK: Extracellular-signal-regulated kinase

EtOH: Ethanol

FBS: Foetal Bovine Serum

FP7: Synthetic glycolipid active as a TLR4 antagonist

g: Grams

GalN: D-galactosamine

GI: Gastrointestinal

GIM: Gastrointestinal Mucositis

H&E: Haematoxylin and eosin

h: Hour

HBE: Human bronchial epithelial cell line

HEK: Human embryonic kidney

HMGB1: High mobility group box chromosomal protein 1

HPLC/MS: High performance liquid chromatography/mass spectrometry

HSP: Heat shock protein

HUVEC: Human umbilical cord vein endothelial cells

IBD: Inflammatory bowel disease

ICV: Intracerebroventricular

IEC: Intestinal epithelial cells

IFN-γ: Interferon-gamma

IHC: Immunohistochemistry

IKK: Inhibitor of NF-κB kinase complex stimulation

IL: Interleukin

iNOS: Nitric oxide synthase 2

IP/i.p.: Intraperitoneal

IRAK: Interleukin-1 receptor-associated kinase

IRF3: Inteferon regulating transcription factor 3

IV/i.v.: Intravenous

JNK: c-Jun N-terminal kinase

kg: Kilograms

KO: Knockout

KRAS: Kirsten rat sarcoma virus

L: Litre

LBP: Lipopolysaccharide-binding protein

LOS: Lipooligosaccharide

LPS: Lipopolysaccharide

LRR: Leucine-rich repeat

m: Minutes

MAMPs: Microbial-associated molecular patterns

MAP: Mitogen-activated protein

MAPK: Mitogen-activated protein kinase

MCP-1: Monocyte chemoattractant protein-1

MD-2: Myeloid differentiation factor 2/ Protein lymphocyte antigen 96

mg: Milligrams

MIP-2: Macrophage inflammatory protein-2

mL: Millilitre

MPO: Myeloperoxidase

mRNA: Messenger ribonucleic acid

mRNA: Messenger RNA

MyD88: Myeloid differentiation primary response 88

NCI/CTC: National Cancer Institute Common Toxicity Criteria

NF-κB: Nuclear factor-kappa B

nm: Nanometers

NO: Nitric oxide

PAMP: Pathogen-associated molecular pattern

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PI3K: Phosphatidylinositol 3-kinase

PMN: Polymorphonuclear leukocytes

PRR: Pattern recognition receptor

PTL: Parthenolide

RAW264.7: Macrophage cell line

RIP: Receptor-interacting serine-threonine kinase 1 protein

Rmsd/lb: RMSD lower bound

Rmsd/ub: RMSD upper bound

RMSD: Root mean square deviation

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute

RsDPLA: Rhodobacter sphaeroides lipid A

RT-PCR: Real-time polymerase chain reaction

SEM: Standard error of the mean

SN-38: Active metabolite of irinotecan/ 7-Ethyl-10-hydroxycamptothecin

SN-38G: SN-38 glucuronide

SsnB: Sparstolonin B

TAB 1/2/3: TAK1-binding proteins

TAK1: Transforming growth factor-β (TGF-β)-activated kinase 1

TAK-242: Resatorvid

TBI: Traumatic brain injury

TBK1: TANK-binding kinase 1

TGF-β: Transforming growth factor-β

THP-1: Human acute monocytic leukemia cell line

TIR: Toll-interleukin-1 receptor

TIRAP: TIR-domain-containing adaptor protein

TLR: Toll-like receptor

TNF: Tumour necrosis factor

TNFR1/2: TNF-α receptors

TP53: Tumour protein P53

TRAF: Tumour necrosis factor receptor-associated factor

TRAM: TRIF-related adaptor molecule

TRIF: TIR-domain-containing adaptor protein inducing interferon-β

UC: Ulcerative colitis

UGT1A1: UDP Glucuronosyltransferase Family 1 Member A1

WT: Wild-type

XN: Xanthohumol

XTT: Sodium 3´-[1- (phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate

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Chapter 1: General introduction

This thesis investigates the roll of toll-like receptor 4 (TLR4) antagonists as potential treatment options for gastrointestinal mucositis (GIM). GIM is a common side-effect of the chemotherapeutic agent, irinotecan (CPT-11, Camptosar®), and is characterised by inflammation of the GI tract leading to high levels of diarrhoea. Irinotecan is a mainline treatment for advanced colorectal cancer (CRC), one of the most common causes of cancer death worldwide. Cancer research has been progressing steadily through the years with new and more effective treatments for cancer being discovered. However, there has been a lack of research into the side-effects these treatments actually cause in patients. TLR4 has recently been recognised as a key player in the development of bowel cancer and GIM. Therefore, inhibiting TLR4 has emerged as an exciting target for mitigating side-effects and ultimately improving cancer treatment. The purpose of this general introduction is to provide the relevant background information regarding the current state of knowledge of TLR4 antagonists with a specific focus on their uses in the treatment of GIM, as well as details on how *in silico* docking and protein binding assays help determine the specific binding and activation sites of these novel TLR4 antagonists.

Colorectal Cancer (CRC)

Bowel cancer, also known as colorectal cancer (CRC), develops from the inner lining of the bowel and is usually preceded by growths called polyps, which may become invasive cancer if undetected [1]. CRC can be divided into 4 stages as seen in Table 1.1 [2] and is responsible for 5000 Australian deaths each year, with more than 10,000 new cases diagnosed annually (https://www.aihw.gov.au/getmedia/0ea708eb-dd6e-4499-9080-1cc7b5990e64/aihw-can-144.pdf.aspx?inline=true) [3]. CRC symptoms include blood in the stools, abdominal pain, bloating or cramping and anal or rectal

pain [4]. Several genetic and environmental factors are implicated in the aetiology of CRC, with the risk of CRC increasing with certain ethnic groups such as Asian, African and Israeli, as well as patients with a family history of CRC and colorectal adenomas [5]. A diet low in folate and vegetables and high in fat and red meat and alcohol, together with a sedentary lifestyle and smoking are all associated with an increased relative risk of developing CRC [4].

Table 1.1: The 4 stages of CRC created and updated by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) [2].

Stage 0	Carcinoma *in situ*: there are abnormal cells in the bowel lining that may become cancerous
Stage 1	The cancer has spread from the bowel lining to deeper layers of the bowel wall/submucosa/muscle
Stage 2	The cancer has spread through the muscle of the bowel wall and may have spread to nearby organs
Stage 3	The cancer has spread to nearby lymph nodes and organs
Stage 4	The cancer has spread through the lymph nodes or blood to other parts of the body such as the lungs or liver

Genomic instability is also an important feature in CRC development. The chromosomal instability (CIN) pathway is considered the main cause of 80-85% of CRC cases [6]. CIN causes an imbalance in the number of chromosomes, which leads to loss of heterozygosity, affecting mechanisms such as chromosome segregation, telomere dysfunction and DNA damage response [6]. The adverse changes in these mechanisms also affect genes such as adenomatous polyposis coli (APC), kirsten rat sarcoma virus (KRAS), phosphatidylinositol 3-kinase (PI3K) and tumour protein P53 (TP53) which are important for normal cell function [6]. Mutations in APC causes the translocation of β-catenin into the nucleus, which promotes the transcription of genes that are associated with tumorigenesis and invasion [6]. KRAS and PI3K mutations continually activate the mitogen-activated protein (MAP) kinase, increasing cellular proliferation [6]. Lastly, TP53 mutations encode for p53 which regulates cell division and prevents the uncontrolled growth and division of cells [6]. A review detailing the prevalence of these CRC mutations was conducted by Alharbi *et al*, however, this was only in a specific ethnic population and may not reflect the prevalence of CRC mutations in the wider population [7].

Chemotherapy and radiotherapy have established roles in the treatment of invasive CRC and can contribute positively to cure rate, prolongation of survival, reduction of local rates of recurrence and enhanced quality of life in people with advanced disease [8]. The previous standard treatment of advanced CRC was 5-fluorouracil (5-FU) in combination with methotrexate and leucovorin. However, key developments introduced new therapies such as oxaliplatin and irinotecan (CPT-11). Clinical studies treating patients with a combination of 5-FU/leucovorin, oxaliplatin, and CPT-11 had significant anti-tumour response rates but also increased toxicities [9]. It was revealed that treatment regimens that included CPT-11 had a higher overall survival rate and

improved quality of life in patients with CRC, which makes it one of the main current treatment options for CRC [10].

Over the past few years, there have been major advances in our understanding of the molecular basis of CRC and its progression from adenoma to carcinoma that hold potential for translation into novel strategies for treatment [11]. Epidermal growth factor receptor (EGFR) is one such strategy for cancer therapy. EGFRs are frequently overexpressed in epithelial tumours and have been and have been associated with the pathogenesis and progression of tumours [11]. Anti-EGFR monoclonal antibodies such as cetuximab and panitumumab were developed and they were the first therapeutic agents that specifically targeted a molecular pathology [12].

Irinotecan hydrochloride (CPT-11)

Irinotecan hydrochloride (CPT-11) is an analogue of camptothecin, an extract from the Chinese tree Camptotheca acuminata [13]. It was approved by the United States Food and Drug Administration in 1996 for the treatment of metastatic carcinoma. CPT-11 is available under various brand names (Camptosar®, Campto® and Camptothecin-11) and was licensed to Pfizer. CPT-11 is currently combined with 5-FU, oxaliplatin and several molecularly-targeted drugs, resulting in the extension of overall CRC survival from 8-12 months to 18-24 months [14].

In early clinical development, CPT-11 was found to cause severe neutropenia and delayed diarrhoea (a feature of GIM). Diarrhoea is one of the main side-effects of people with cancer undergoing treatment and a major dose limiting factor. Chemotherapy-induced diarrhoea is graded using a number of clinical scales, with one example shown in Table 1.2. The incidence of diarrhoea (all grades) has been reported to be as high as 50–80% of treated patients, dependent on chemotherapy type and dose. The incidence of severe grade (3 and 4) diarrhoea during CRC treatment is

shown in Table 1.3 [15-19]. Diarrhoea is managed depending on its severity and presence of complications using loperamide, octreotide and/or removing exacerbating foods from the diet such as dairy [20]. Patients are hospitalised for severe and complicated diarrhoea. The current guidelines for managing chemotherapy-induced diarrhoea have been reviewed elsewhere [21].

Table 1.2 GIM diarrhoea grade as classified by the National Cancer Institute Common Toxicity Criteria (NCI/CTC) criteria [20].

Grade 1	Increase of less than four stools per day during pre-treatment
Grade 2	Increase of four to six stools per day or nocturnal stools
Grade 3	Increase of seven or more stools per day, or incontinence, or need for parenteral support for dehydration
Grade 4	Requiring intensive care for hemodynamic collapse
Grade 5	Death

Table 1.3: Diarrhoea occurrence in patients with CRC treated with chemotherapy [16-19].

Chemotherapy type	Grade 3-4 diarrhoea occurrence
5-FU (bolus)	32%
5-FU (continuous intravenous infusion)	6-13%
CPT-11	16-22%
anti-EGFR-antibodies	1-2%

As a prodrug, CPT-11 is converted into its active metabolite 7-ethyl-10-hydroxycampothecin (SN-38) by carboxylesterase in the liver and GI tract [22]. SN-38 is a topoisomerase 1 inhibitor, which prevents the replication of DNA causing apoptosis and is highly cytotoxic. However, the liver is also a major site of glucuronidation through UDP Glucuronosyltransferase Family 1 Member A1 (UGT1A1), which is an important enzyme for converting SN-38 to its inactive form SN-38 glucuronide (SN-38G) [23]. UGT1A1 is also found in the intestinal tract [23]. The conversion of SN-38 to SN-38G in the GI tract is important for detoxification through biliary secretion.

However, when SN-38G re-enters the GI tract through the excreted bile, it is converted back into SN-38 by β-glucuronidase produced by intestinal bacteria (*Escherichia coli.*, *Clostridium perfringens*) [24]. This re-conversion increases the GI toxicity and exacerbates GIM contributing to diarrhoea development [24]. A study by Brandi *et al* has shown that germ-free mice were protected against GIM after treatment with CPT-11 compared to wild-type (WT) mice [25]. Another study using antibiotics to inhibit β-glucuronidase activity by depleting the intestinal bacteria that produces it, has shown protective effects against GIM and diarrhoea after CPT-11 treatment [26]. These studies suggest an important role for intestinal bacteria in GIM pathobiology.

Gastrointestinal Mucositis (GIM)

Gastrointestinal mucositis (GIM) is the ulceration of the mucosa in the GI tract caused by chemotherapy and radiation and is an acute form of GI inflammation [27]. Chemotherapy, one of the most effective ways to treat cancer is known to also negatively affect healthy tissue. Unfortunately, the GI tract is particularly susceptible to the devastating effects of chemotherapy [27]. The severe ulceration caused can result in even more unfortunate effects as bacteria will be able to infiltrate from the lumen into the blood-stream lining the GI tract, thereby increasing an already

immunocompromised patient's susceptibility to infections [28]. In addition, the absorption of nutrients and water from the GI tract will be impaired leading to symptoms of diarrhoea and weight loss [29].

Moreover, GIM is known to have a significant impact on the quality of life of patients during cancer treatment. The total number of days a patient suffering from GIM needs to be hospitalised is 3 times more than the 4 days required on average by patients not suffering from GIM [30]. This increased length of stay will eventually increase the strain on hospital resources [31]. In certain cases, the symptoms of GIM will lead to a reduction in treatment doses, delay the next chemotherapy administration cycle and may even cause a discontinuation of their regimen which will affect the patient's survival [29]. Clinical representation of CPT-11-related GIM includes vomiting, abdominal pain and severe diarrhoea [32]. Unfortunately, the development of effective treatments for GIM have been slow due to the GI tract being largely inaccessible by non-invasive, standard diagnostic methods to monitor and detect GI disruption as a consequence of chemotherapy [33].

The pathobiology of GIM can be divided into a 5-phase model [28]:

1) Initiation; when chemotherapeutic agents have been administered causing direct DNA damage to both healthy and cancer cells. This develops into cell injury and generation of reactive-oxygen species (ROS) [34].

2) Upregulation and activation of messengers; when DNA damage caused by the chemotherapy and the presence of ROS promotes the activation of transcription factors involved in the regulation of cytokine expression and inflammatory protein complexes, such as nuclear factor kappa B (NF-κB) [34, 35]. These changes can lead to the activation and infiltration of immune cells (such as neutrophils, macrophages

and dendritic cells), as well as the production of inflammatory cytokines such as interleukin (IL)-6, and tumour necrosis factor-alpha (TNF-α) [36].

3) Signal amplification; The increased presence of pro-inflammatory cytokines induces more prominent tissue damage leading to a vicious cycle in which the signal amplification constantly increases the oxidative stress levels and pro-inflammatory cytokine release, which results in more intense tissue damage and apoptosis [37, 38]. The epithelium then begins to lose its integrity, which is exacerbated in the next phase.

4) Ulceration with inflammation; Characterised by loss of mucosal integrity and morphological changes in the tissue architecture. Due to the ulceration of the GI epithelium, the GI barrier becomes weak and “leaky” due to a loss of tight-junctions and further stimulates immune responses [39]. This increases the risk of bacterial translocation and sepsis occurring in patients.

5) Healing; Usually occurs after the discontinuation of chemotherapy [28]. The GI tract contains stem cells at the base of the crypts that are able to continuously divide and differentiate into the specialised cells of the GI epithelium to reform breaches in integrity [40]. Proliferation of these cells are promoted by various growth factors such as epidermal growth factor and fibroblast growth factor [40]. Cytokines such as IL-22 and IL-28 are also released to regulate immune homeostasis and mucosal wound healing [40]. In order to establish the mucosal barrier integrity, these proliferating cells need to differentiate and mature. Through Wnt/Notch signalling, the intestinal stem cells will differentiate into enterocytes [40]. Goblet cells and Paneth cells are differentiated through the expression of transcription factors such as Kruppel Like Factor and SRY-Box Transcription Factor 9, respectively [40].

Throughout all stages of GIM, the innate immune system plays multiple roles such as responding to danger signals, recruiting polymorphonuclear leukocytes to sites of

injury, and inducing inflammation. The innate immune system utilises various systems in the GI tract to maintain homeostasis such as pattern recognition receptors (PRR). One such family of PRRs is the TLRs, which are essential in identifying microbial molecular patterns. TLR4 is one such PRR which is located on GI epithelial cells as well as immune cells in the GI tract, including macrophages and dendritic cells [41]. TLR4 has been strongly implicated in the development of chemotherapy-induced GIM [42].

Toll-Like Receptor 4

TLRs are highly expressed within the GI tract and are implicated in inflammatory responses such as in GIM. They and are among the first receptors activated during any host–pathogen interaction [43]. TLRs are responsible for the induction of innate immunity responses upon the detection of pathogen-associated molecular patterns (PAMPs) [43]. TLR4 is the member of the TLR family specialised in the recognition of microbial components, lipopolysaccharides (LPSs) and lipooligosaccharides (LOSs) and their bioactive portion, the lipid A, commonly defined as endotoxin [44]. The induction of inflammatory responses by endotoxin is achieved by the co-ordinated and sequential action of four principal endotoxin-binding proteins: TLR4, myeloid differentiation protein (MD-2), lipopolysaccharide-binding protein (LBP), and the cluster of differentiation 14 (CD-14) antigen [44]. The TLR4 complex is arranged in a symmetrical fashion and adopts a horseshoe-like shape as seen in Fig. 1.1 [45]. MD-2 has a β-cup fold structure composed of two anti-parallel β-sheets forming a large hydrophobic pocket for ligand binding. Ligands such as LPS will bind to a LPS binding protein (LBP) and is delivered to the hydrophobic pocket of MD-2 via CD-14, directly mediating the dimerisation of the two TLR4/MD-2 complexes [45]. CD-14-dependent or independent TLR4 activation by endogenous factors (danger or damage-associated molecular patterns, DAMPs) such as heat-shock proteins, fibronectin, and oxidised

phospholipids has been recently related to a wide array of inflammatory disorders [46]. Consensus is growing that TLR-directed compounds have potential to provide new specific drugs against a wide array of diseases still lacking specific pharmacological treatment.

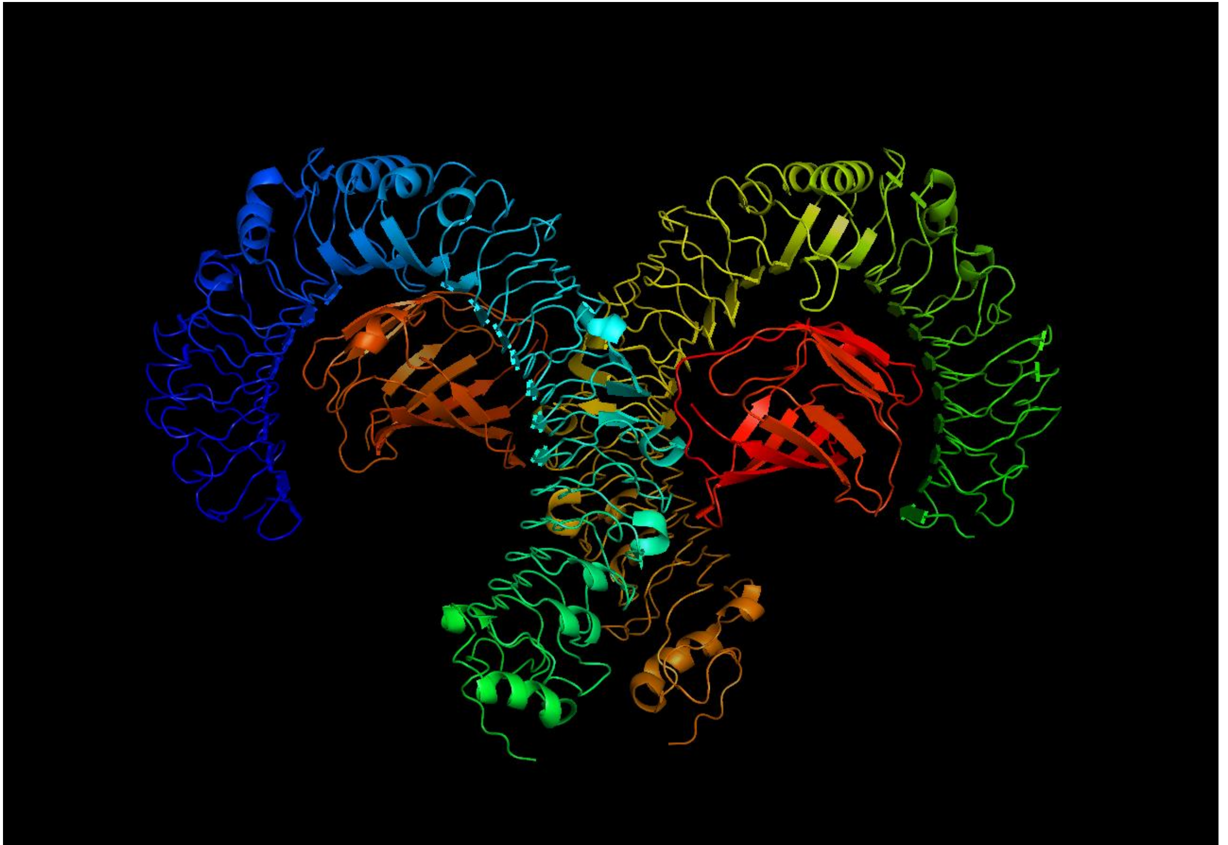

Figure 1.1: Crystal structure of TLR4/MD-2. The horse-shoe like shapes represent the TLR4 protein while the β-cup fold structure represents the co-receptor MD-2. Image was created using Auto Dock Tools (v.1.5.6).

The pathobiology of GIM has been linked to the activation of TLR4. To date, studies have shown a link between TLR4/MD-2 signalling and GIM development [42]. A study by Fort *et al* has shown that pharmacological inhibition of TLR4 was able to reduce disease activity and prevent morphological damage in an inflamed colon [47]. Another study demonstrated that TLR4 deficient mice were protected against CPT-11-induced GIM in the small intestine and that diarrhoea severity was also significantly less compared to the WT mice [42].

TLR4 has also been implicated in the development of CRC. It was observed that there is an increase in TLR4 expression in the mucosa of patients with CRC [48]. There is also an increase in TLR4 expression in CRC cell lines such as KM20, SW480 and HT-29 compared with normal intestinal epithelial cells (IECs) [48]. Additionally, studies have shown that TLR4 may be required for the formation of polyps and dysplasia [49, 50]. A study by Fukata *et al* has shown that TLR4 is overexpressed in human and mouse inflammation-associated colorectal neoplasia, and that TLR4 deficient mice were protected from colon carcinogenesis [50].

The use of a TLR4 antagonist has potential as a novel therapeutic for GIM patients whose disease pathogenesis relies heavily on TLR4 signalling. Previous studies have shown that inhibiting LPS-induced TLR4 stimulation with antagonists can reduce intestinal inflammation in animal models [47]. Studies have also shown that TLR4 signalling plays a major role in the adhesiveness and metastatic capacity of CRC cells [51].

Regardless of how promising TLR4 antagonists are in the treatment of intestinal inflammation and potentially even prevention of CRC, there are still challenges in their specificity and targeting capability. Nonetheless, anti-TLR4 therapies present a promising alternative for future innovative treatments for GIM. However, there is a need

for tissue-specific studies investigating these anti-TLR4 therapies in order to mimic the therapeutic setting of GIM.

In light of this knowledge, I designed a series of experiments to test the relationship between TLR4 antagonism and CPT-11-induced GIM. The work-flow included *in silico* docking experiments, *in vitro* and *ex vivo* models, and finally a CRC-bearing mouse model. These are now described.

In silico docking

While targeting TLR4 is a biologically supported approach to GI inflammatory conditions, there has been little progress in the field, which may be due to a lack of specific antagonists that selectively target the TLR4 protein and/or its associated co-receptors, MD-2, CD-14 and LBP. The integration between biological systems and computational techniques provides the possibility to advance drug development opportunities in order to rapidly provide structural, chemical, and biological data to improve understanding of potential drug targets. This analysis method also provides valuable information on the evidence on binding conformation, pattern and affinity of the bio-active peptides or chemical drug molecules when binding with specific receptors.

Hydrogen bonding plays a crucial role in the determination of protein structure and is equally central in many aspects of biological function [52]. For this reason, a crucial objective in *in silico* docking in biological systems is an accurate description of hydrogen bonding. An enormous variety of hydrogen bonds, both between various side-chain functional groups and involving the backbone peptide group, are possible.

The two antagonists investigated in this thesis are TAK-242 (resatorvid) and IAXO-102. TAK-242 is a cyclohexene derivative and a novel small-molecule compound [53], which selectively inhibits TLR4 signalling through the toll-interleukin-1 receptor (TIR)

domain of TLR4 via Cys747 [53]. IAXO-102 is another small-molecule compound classed as a cationic amphiphile [54]. IAXO-102 is able to bind with MD-2 and CD-14 in order to compete with LPS and displace it from TLR4 [55]. There is a lack of docking studies for these antagonists, especially in regards to SN-38. While some studies have looked into TLR4 antagonism and SN-38-induced GIM, they have not shown if SN-38 can act as a ligand for TLR4. Additionally, it is unknown if SN-38 would compete or interact with the TLR4 antagonists being investigated in this thesis.

The *in silico* docking study conducted in this thesis provides evidence for the ability of the antagonists investigated to bind to TLR4 with high binding energy values, docking scores, and protein-receptor interactions. The interactive association between various methods, such as *in silico* molecular docking and protein binding studies has been employed by researchers for the development of pharmacologically active drugs [56].

In vitro and *ex vivo* models of intestinal inflammation

Both TAK-242 and IAXO-102 have been used in recent research to determine their ability to negatively regulate TLR4 signalling in a model of abdominal aortic aneurysm and endotoxin shock, respectively [55, 57]. However, no studies have been conducted using these TLR4 antagonists in a model of GI inflammation. In this thesis the intestinal epithelial cells (IECs) T84 and HT-29 were used as they were derived from colon cancers, are well-characterised, express TLR4 and have been previously studied [58]. While the pro-monocytic, human myeloid leukaemia cell line U937 which are innate monocyte-like immune cells were used as a positive control due to their high expression levels of TLR4 [59]. Testing these TLR4 antagonists in an *in vitro* culture helped establish the ability of these antagonists to bind with TLR4/MD-2 in cell cultures and the subsequent downstream signalling effects, as well as identified the most effective antagonist to move forward with further investigations.

However, there are some limitations in using an *in vitro* model. Both T84 and HT-29 are immortalised cell lines which may not mimic a healthy human colon [60]. As such, TLR4 signalling in this system may not reflect the healthy GI tract response to challenge with chemotherapy. To overcome this limitation, an *ex vivo* model of GI inflammation was also developed. Previous studies have used these GI explant models with success and are a popular model for mimicking a normal GI tract [61, 62]. For example, a study by Guabiraba *et al* used mouse small intestinal explants treated with CPT-11 and SN-38 to determine if they were able to stimulate IL-33 production [61]. Another study also used mouse small intestinal explants to determine if IL-1β secretion was induced by CPT-11 [62]. However, these studies only determined the secretion of cytokines into the explant supernatant and did not investigate the histopathology of the explant tissue itself.

***In vivo* tumour bearing mouse model**

Due to the diverse clinical symptoms that present with GIM, animal models are required to improve our understanding of treatment toxicities and explore new ways to manage GIM. There are limited options to perform well-controlled clinical studies in chemotherapy patients, due to ethical restrictions and risks associated with invasive procedures [63]. Using animal models to study GIM will allow tissue sampling of the entire GI tract in response to the TLR4 antagonists in tumour-bearing animals.

It is well established that people with cancer, or animals that are tumour-bearing, will have changes in their immune function [64, 65]. Some examples of these changes include decreased lymphocyte proliferation response and function in response to suppressor T-cells and macrophages released by tumour cells impairing immune response [66]. Furthermore, tumours secrete a variety of different mediators, such as prostaglandin E2 and transforming growth factor beta, which act to decrease the

efficiency of immunological cells and promote tumour progression [67]. This is also why rodent xenograft models are not a viable option as the rodents used in these models are immunodeficient.

Tumours can also affect hormonal balance and metabolism [68]. Therefore, it is important to consider tumour-bearing animals. The MC-38 colon cell line was chosen to establish the model as it is a mouse syngeneic tumour, has a well characterised growth pattern, and is sensitive to irinotecan [69]. This model provided a clearer understanding of the damage that occurs to the GI tract after administration of CPT-11 and a targeted treatment against TLR4. This demonstrates the effect of targeted treatment on the tumour and the damage that chemotherapy causes.

Hypothesis and aims

Given the literature and experiments summarised above and the context of my research area, it was hypothesised that TLR4 signalling is the primary driver of upregulated pro-inflammatory cytokine expression involved in GI inflammation caused by chemotherapy. As such, TLR4 inhibition could modulate GI inflammation in patients treated by CPT-11.

Therefore, the fist aim was to model the potential binding mode of TAK-242, IAXO-102 and SN-38 to TLR4 using *in silico* docking methods.

The second aim was to identify the ability of TAK-242 and IAXO-102 to block TLR4 signalling in a cellular system using *in vitro* and *ex vivo* methods.

The third aim was to determine whether the TLR4 antagonist IAXO-102 could prevent GI toxicity in a pre-clinical model of CPT-11-induced GIM. In addition to GIM, this aim also explored the potential of IAXO-102 to decrease tumour burden.

Chapter 2: TLR4 Antagonists as Potential Therapeutics for Intestinal Inflammation

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Contribution to the Paper	Writing of the manuscript with input from all authors.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/04/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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Abstract

Gastrointestinal inflammation is a hallmark of highly prevalent disorders, including cancer treatment-induced mucositis and ulcerative colitis. These disorders cause debilitating symptoms, have a significant impact on quality of life, and are poorly managed. The activation of toll-like receptor 4 (TLR4) has been proposed as a major influence on the inflammatory signalling pathways of the intestinal tract. Inhibition of TLR4 has been postulated as an effective way to treat intestinal inflammation. However, there are a limited number of studies looking into the potential of TLR4 antagonism as a therapeutic approach for intestinal inflammation. This review surveyed available literature and reported on the *in vitro, ex vivo* and *in vivo* effects of TLR4 antagonism on different models of intestinal inflammation. Of the studies reviewed, evidence suggests that there is indeed potential for TLR4 antagonists to treat inflammation, although only a limited number of studies have investigated treating intestinal inflammation with TLR4 antagonists directly. These results warrant further research into the effect of TLR4 antagonists in the intestinal tract.

Introduction

Inflammation of the intestinal tract can result from acute or chronic manifestations of intestinal disease, which causes irritation, exposure to bacteria, and a dysregulation of the homeostatic balance. This leads to a range of debilitating symptoms that heavily affects patient quality of life. Current treatment modalities used for intestinal inflammation are associated with several disadvantages including poor efficacy and unwanted side effects. The incidence rates for intestinal inflammation have been steadily increasing around the world for the last 50 years with an increased prevalence most notable in newly industrialised nations [70, 71]. Factors such as cell types, immunological abnormalities, tissue specificity, and genetic/environmental factors are heavily involved in the pathogenesis of intestinal inflammation.

Toll-like receptors (TLRs) are type 1 transmembrane proteins belonging to the wider family of pattern recognition receptors (PRR) and are responsible for the recognition of a variety of molecular signals, including endogenous damage and pathogen associated signals (DAMPs and PAMPs, respectively). Both immune (dendritic cells, monocytes, mast cells, macrophages) and non-immune cells (fibroblasts, epithelial cells) express these PRRs [72]. The PRR-ligand binding between DAMPs and PAMPs prompt a downstream signalling cascade which results in the recruitment of leukocytes [72]. TLRs activate downstream signalling pathways, which originates from the toll-interleukin receptor-domain (TIR) containing adaptor proteins such as myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) [73]. These adaptor molecules are essential to produce inflammatory cytokines, type 1 interferons, chemokines and co-stimulatory molecules. At present, 13 human TLRs have been identified, and are

located on various cellular compartments (including extracellular membrane, endosome, and golgi apparatus) with each TLR responding to specific stimuli [73, 74].

In a healthy intestinal tract, enterocytes coexist with luminal and mucosal-associated commensal bacteria without the initiation of inflammatory responses. However, the exact mechanism behind bacterial tolerance within the intestinal tract is still largely unknown. TLRs play a pivotal role in immune tolerance to intestinal microbes [75]. This immune tolerance and responses are organised by Peyer's patches, mesenteric lymph nodes and the lamina propria [76]. These lymphoid organs are populated with dendritic cells which produce interleukin 10 (IL-10) in turn, transforming T-cells into growth factor-β (TGF-β) [76]. The production of these cytokines leads to immune tolerance and homeostasis, as well as unnecessary inflammation [76]. This suggests that TLR4 signalling has an effect not only on immune responses but also the balance of the intestinal microbial ecosystem [77, 78].

In contrast, during conditions of stress (such as disease) in the intestinal tract, inflammatory cytokines are released from enterocytes and mucosal immune cells responding to the stimulation of TLRs [79]. This leads to apoptosis and reduced proliferation of enterocytes, which in turn promotes translocation of bacteria into the lamina propria, exacerbating intestinal inflammation [79]. One of the most well characterised TLRs is Toll-like receptor 4 (TLR4) which has been shown to be involved in homeostasis, apoptosis, intestinal inflammation and inflammatory bowel disease [80]. The focus of this review of the literature will therefore be based on the role of TLR4 antagonism in inflammatory conditions with the purpose of generating a hypothesis to support the use of TLR4 antagonists in intestinal inflammation.

TLR4 activation and signalling

In 1997, toll proteins in Drosophila were discovered to mediate protection against fungal infections [81]. Toll proteins in Drosophila were activated by fungi and gram-positive bacteria which do not contain LPS. They do however, trigger a toxic shock response that is similarly induced by LPS [81]. This then led to research focusing on the now established TLR4-LPS signalling cascade. This early work also suggests a much broader role in homeostasis, tissue repair, and immune defence [82].

TLR4 is an intra- and extra-cellular receptor expressed on endosomes and cytoplasmic membranes, which recognises PAMPs (flagellin and LPS) and DAMPS (calprotectin, S100A8/9 HMGB1 and HSP70) through its co-receptors MD-2 and CD-14 [83, 84]. In addition, TLR4 has recently shown to be activated by certain pharmacological agents, including chemotherapeutic agents (paclitaxel). TLR4 is located on many different cell types (endothelial cells, lymphocytes, cardiac myocytes, glial cells) throughout the body [85-87]. In the intestine, TLR4 is expressed on antigen-presenting cells such as macrophages and dendritic cells, and on enterocytes and lymphocytes [88]. TLR4 consists of leucine-rich repeats (LRRs) with a horseshoe-like shape made up of 839 amino acids. The complex ligand specificity of the TLR4/MD-2 complex is composed of two antiparallel β sheets which form a large hydrophobic pocket in MD-2 [45]. LPS is able to bind to this hydrophobic pocket through its lipid chains which are completely buried in the MD-2 hydrophobic pocket [45]. However, one of these lipid chains is partially exposed to the outer surface which allows some interaction with TLR4 [45]. These hydrophilic and hydrophobic interactions between LPS and the TLR4/MD-2 complex mediate the dimerisation of extracellular domains in the TLR4. Thus, triggering a downstream signalling cascade leading to the release of pro-inflammatory cytokines [45]. A study by Abreu *et al* [58] discovered that increases in TLR4 expression alone would not result in a reaction from LPS without the accompanying

expression of MD-2. In the study, they challenged different intestinal epithelial cell lines (Caco-2, T84, HT-29) with LPS and found that a decreased expression of TLR4 and MD-2 correlated with intestinal epithelial protection against pro-inflammatory gene expression in response to bacterial LPS. It was concluded that careful regulation of both TLR4 and MD-2 is necessary to maintain homeostasis in the intestinal tract due to it being continuously exposed to high concentrations of bacteria.

Upon stimulation, TLR4 will activate two signalling pathways, the TRIF-dependent pathway (Figure 2.1) and the MyD88-dependent pathway (Figure 2.2). In the TRIF-dependent pathway, TLR4 heterodimers recruit TRAM, which is needed to activate TRIF, resulting in the binding of TRIF with TNF receptor-associated factor 3 (TRAF3) and TRAF6 for binding with a receptor-interacting serine-threonine kinase 1 protein (RIP). This subsequently leads to the activation of nuclear factor kappa B (NF-κB). The TRIF activated pathway leads to the activation of interferon regulatory transcription factor 3 (IRF3) by TANK-binding kinase 1 (TBK1) and inhibitor of NF-κB-kinase complex stimulation (IKK), which results in the production of type 1 interferons and anti-inflammatory cytokines (such as IL-10).

In the MyD88 signalling pathway, TLR4 heterodimers bind to MyD88, which results in the formation of IRAK (interleukin-1 receptor associated kinases) and TRAF6 complexes [83]. This formation of IRAK and TRAF6 complexes leads to a downstream signalling cascade. Various other complexes such as TAK1, TAB1/2/3, MAP kinases and IκB will be phosphorylated or activated to allow the translocation of NF-κB into the nucleus, ultimately driving the transcription of cytokine genes (such as TNFs, ILs, chemokines) to regulate pro-inflammatory responses [83, 89].

Dysregulation of TLR4 signalling has been linked to the development of a variety of diseases. Studies have investigated functional genetic variants of *TLR4* and their

impact on LPS signalling response. A study by Hold *et al* found that [cells carrying] *TLR4* D299G and T399I variants, when stimulated with LPS, had a 6-fold lower expression of NF-κB compared to wild-type *TLR4* [90]. Ferwerda *et al* demonstrated that patients carrying a variant at position 299 (Gly) but not at position 399 (Ile) had a stronger pro-inflammatory cytokine response with increased TNF-α levels in whole blood samples when stimulated with LPS compared to patients carrying wild-type *TLR4* alleles at both positions [91]. Weinstein *et al* showed these same *TLR4* variants in patients with acute ischemic stroke are associated with worse neurological outcomes and alterations in systemic markers of inflammation [92]. This dramatic difference in cytokine expression caused by dysregulation of TLR4 signalling due to genetic polymorphisms will affect a person's ability to respond to LPS leading to a dysregulated immune response to infection.

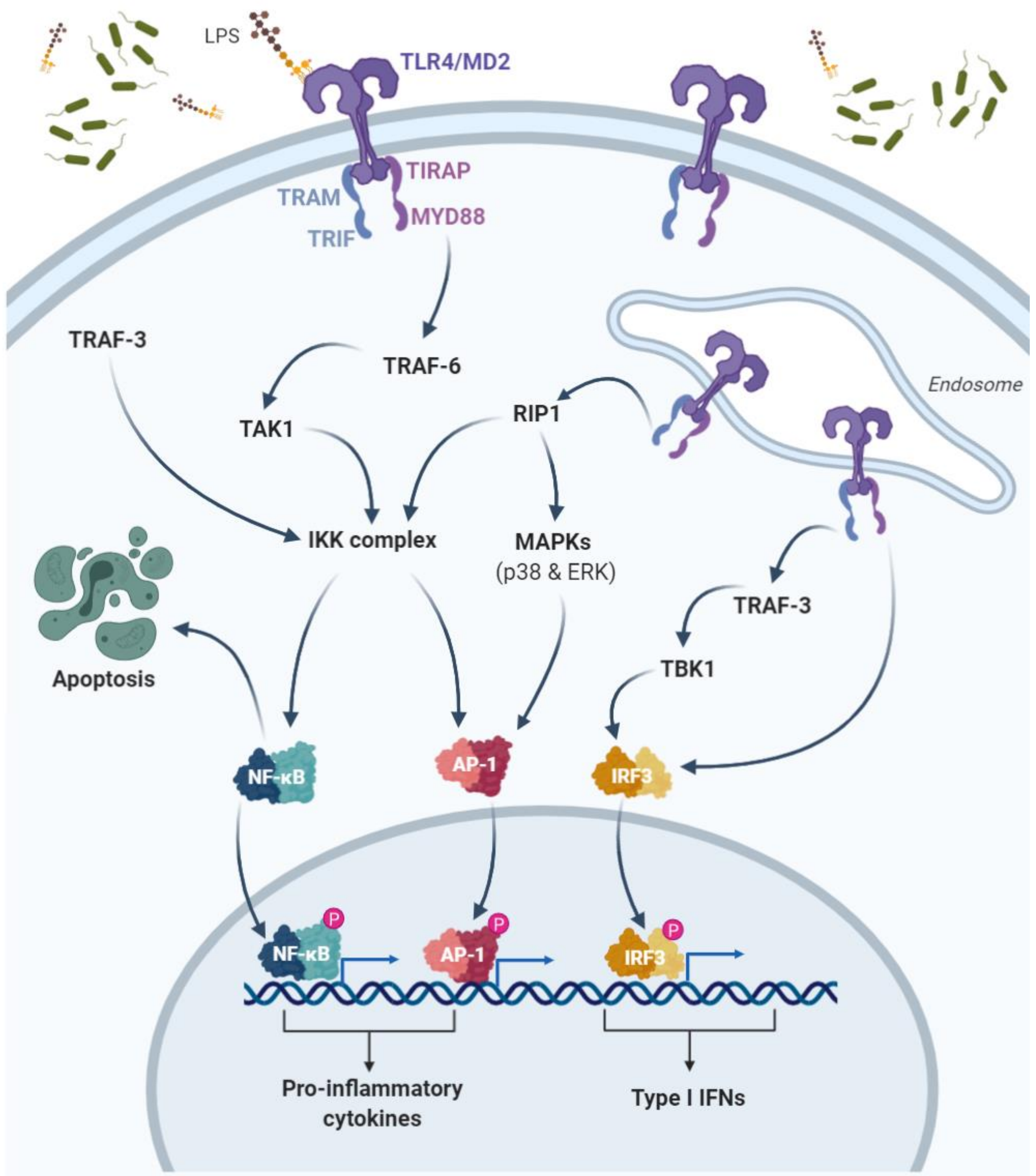

Figure 2.1: PAMP TLR4 signalling pathway in an enterocyte.

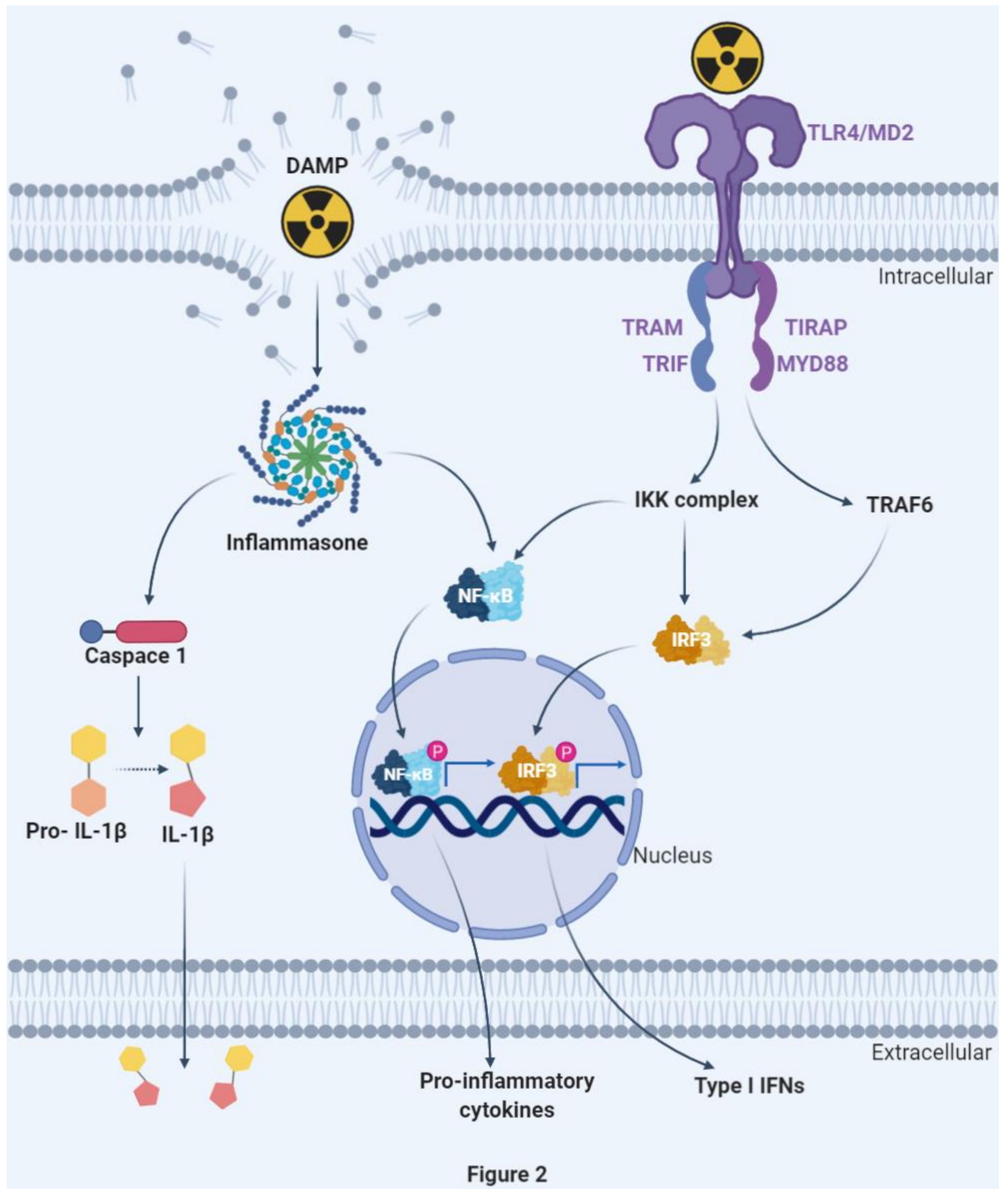

Figure 2.2: TLR4 activation by DAMPs from tissue damage. Leads to a downstream signalling pathway which induces inflammatory gene expression.

TLR4-mediated Intestinal inflammation

An important component of immunity and host-microbial interactions in the intestinal tract is the recognition of DAMPs, PAMPs and endogenous ligands by TLR4 expressed on the enterocytes and antigen-presenting cells. Any imbalance from this interaction may contribute to the pathogenesis of inflammation within the intestinal tract [93]. There has also been substantial evidence indicating the involvement of TLR4 in intestinal inflammatory diseases such as ulcerative colitis (UC), Crohn's disease (CD) and gastrointestinal mucositis (GIM). It was reported that in the colonic mucosa of patients with UC and CD, a significant increase in TLR4 mRNA and protein expression was observed compared to healthy controls [94, 95]. However, this may be due to the increased influx of TLR4 expressing innate immune cells. There is also mounting evidence that TLR4 polymorphism is associated with the development of UC and CD, whereby the allele frequencies of the *TLR4* Asp299Gly polymorphism was discovered to be significantly higher in UC and CD patients [96].

There have also been several studies using animal models of acute intestinal inflammation. TLR4 expression is strongly upregulated in animal models induced with colitis [50]. Animal models with TLR4 knocked-out were observed to be protected from colitis or colon tumorigenesis by preventing the downstream signalling pathways that induce colitis [50]. For example, TLR4 knockout (KO) mice induced with acute colitis had a decrease in COX-2 expression, prostaglandin production and NF-κB signalling, which lead to a significant reduction of acute inflammatory cells and therefore significantly reduced acute inflammation in the intestinal tract [97]. Other studies have observed an increase in pathogenic *E. coli* and a decrease in beneficial intestinal microbiomes (*Bifidobacterium* spp. and *Lactobacillus* spp.) in a DSS-induced mouse model, which may be associated with the increase in TLR4 expression observed in the colon of the animals [77]. Another study using TLR4 KO mice showed a reduction in

pathogenic *E coli.* compared to the DSS-induced wild-type mice, which showed a 10-fold increase in pathogenic *E coli* [78]. These TLR4 deficient mice also displayed reduced disease activity index and histopathological scoring.

It therefore stands to reason that by inhibiting TLR4, a protective effect from intestinal inflammation will be achieved. Recent research has shown that by inhibiting TLR4 using antagonists such as paeoniflorin, monoclonal-antibodies and CRX-526, DSS-induced intestinal inflammation was attenuated with a significant reduction in disease activity and histopathological scoring [47, 98, 99]. However, other studies have discovered that there was no protective effect observed in the clinical symptoms and histology scores when blocking TLR4 during chronic intestinal inflammation [100] despite the opposite results observed during acute intestinal inflammation [97]. This results is likely due to the low involvement of innate immune cells in chronic inflammation compared to acute [101].

TLR4 antagonists as a potential therapeutic alternative for treatment of intestinal inflammation

Inflammatory bowel disease (IBD) (consisting of UC and CD), are known as non-specific, chronic gastrointestinal inflammatory disorders [102]; with periods of disease activation and remission, and in some cases, progressive disease [103]. It is considered an autoimmune disease due to the combined effect of genetic factors and abnormal immune responses to the intestinal bacteria and other foreign substances [102]. In addition, one of the most serious complications that IBD patients encounter is colorectal cancer, which accounts for increased mortality rates associated with UC [104]. The severity of inflammation in the intestinal tract also correlates with the risk of colorectal cancer in patients with IBD [104].

Severe intestinal inflammation leads to a range of debilitating symptoms that significantly affect patient quality of life. Current treatment modalities for intestinal inflammation are associated with a range of limitations including poor efficacy and side effects. The TLR4 signalling cascade also plays an important role in intestinal inflammation; with its extracellular and intracellular components being attractive therapeutic targets for the treatment of both acute and chronic intestinal inflammation. The link between intestinal inflammation and colon cancer also offers the possibility of identifying and developing novel ways to prevent cancer.

The incidence rates for IBD have been steadily increasing around the world for the last 50 years with the majority of cases occurring in westernised and industrialised countries [70, 105]. IBD is a chronic lifelong condition that has no cure and requires a lifetime of care. It has a significant effect on patient quality of life and at times can leave patients bed-ridden or hospitalised [106, 107]. However, UC and CD are clinically distinct diseases and known to have different anatomical, clinical and histological features [108].

Presently, the aetiology and pathogenesis for IBD remains largely unknown [108]. A plausible hypothesis for the aetiology of IBD is the unregulated activation of both the body’s innate and adaptive immune system, potentially in response to resident gut microbes. This immune response may mediated by the innate immune receptor TLR4 in response to luminal antigens (fungi, bacteria, yeast) in the intestinal tract [108]. Treatment approaches includes aminosalicylates, corticosteroids and antibiotics [109, 110].

As previously stated, patients with IBD are at higher risk of developing colorectal cancer, and a common complication of cancer treatment is gastrointestinal mucositis (GIM); occurring in 40% of patients who receive a standard dose of chemotherapy and

100% of patients who are receiving high doses of chemotherapy [111, 112]. GIM is the ulceration and inflammation of the mucosa in the intestinal tract caused by chemotherapy and radiation for cancer, and is an acute form of intestinal inflammation [27].

Unfortunately, the gastrointestinal tract is particularly susceptible to the devastating effects of chemotherapy and radiation. TLR4 is the main receptor that detects DAMPs and responds to tissue damage in the intestinal tract [113]. The cytotoxic effects of chemotherapy and radiation on both normal and malignant cells causes the release of DAMPs. This produces a sustained innate immune activation which develops into the mucosal inflammation seen in patients with repeating cycles of chemotherapy and radiation treatments [113]. While GIM has been recognised as a major dose-limiting toxicity for decades, there is yet to be an effective treatment to manage intestinal inflammation. Pre-clinical studies have focused on inhibition of inflammation via multiple mechanisms, or accelerating healing with growth factors [114].

GIM has a significant impact on the quality of life of patients whereby the average number of days a patient suffering from GIM needs to be hospitalised is 3 times more than the 4 days required by patients not suffering from GIM [31]. This increased length of stay increases the strain on hospital resources [31]. Patients also suffer from symptoms such as vomiting, abdominal pain and severe diarrhoea. In certain cases, the symptoms of GIM will cause patients to require a dose reduction, delay, or even discontinuation of their regimen which will affect the patient’s survival [115]. To date, studies have shown a link between TLR4/MD-2 signalling and the development of IBD and GIM [42, 47]. TLR4 antagonists show potential as therapeutics in both settings. However, the majority of studies have focused on sepsis models as well as diseases and infections unrelated to the development of intestinal inflammation, leaving a significant gap in the literature.

TLR4 is overexpressed in both UC (fold increase: 2.33) and CD (fold increase: 1.71) [94, 116, 117]. In IBD, abnormal signal transmission mediated by the upregulation of TLR4 promotes the sustained release of inflammatory cytokines (IL-6, TNF-α). This in turn, develops and persists as intestinal inflammation. Only low levels of TLR4 and MD-2 are expressed on the intestinal epithelium and very little was known about their regulation in on intestinal epithelial cells. However, it was established that during inflammation, expression of both TLR4 and MD-2 is increased. It was later discovered by Abreu *et al* [44] that the expression of TLR4 and MD-2 in the intestinal tract is also regulated by immune-mediated signals. There was an increase in TLR4/MD-2 expression when the intestinal epithelial cell lines (T84, HT-29) were exposed with pro-inflammatory cytokines (IFN-γ, TNF-α), highlighting the potential link between the innate and adaptive immune systems in intestinal epithelial cells only in response to pathogenic organisms. Another study by Ungaro *et al* has also shown that the inflammation in IBD is decreased in TLR4 deficient mice [100]. However, this study also found that TLR4 deficient mice were unable to undergo mucosal healing and demonstrated decreased epithelial cell proliferation [100]. This shows that TLR4 serves as a mediator for both mucosal healing and inflammation in the intestinal tract.

A similar pattern can also be observed in GIM. A study by Wardill *et al* has shown that genetic deletion of TLR4 from mice was able to reduce chemotherapy-induced gut toxicity and pain [42]. TLR4 KO mice had reduced diarrhoea and weight loss compared to wild-type mice [42]. The TLR4 KO mice also exhibited a muted inflammatory response, with no significant increase in IL-1β, IL-6, or TNF-α, compared to their wild-type counterparts [42].

These studies highlight the critical role of TLR4 in regulating inflammation in the intestinal tract, and by targeting and inhibiting TLR4, the outcome of intestinal inflammation and its consequence may be prevented. However, careful selection of

TLR4 elimination vs selective or temporary inhibition as a therapeutic is needed since TLR4 has beneficial effects for mucosal healing and homeostasis.

TLR4 antagonists

Targeting TLR4 could represent a potential approach to regulate immune responses and treat inflammation. However, any potential therapeutic agent must be able to block the harmful effects of TLR4 activation without negatively affecting the host's defence functions. Currently, many different antagonists are being investigated for their potential in managing inflammatory-based diseases and settings, summarised in Table 2.1.

Naturally occurring antagonists

The first naturally occurring TLR4 antagonist discovered was from a photosynthetic gram-negative bacterium that was non-pathogenic known as *Rhodobacter sphaeroides* [118]. The LPS produced from this bacterium, known as *Rhodobacter sphaeroides* lipid A (RsDPLA), was non-toxic towards murine and human cells and was able to compete with toxic LPS for binding sites. RsDPLA was also able to interact with the TLR4/MD-2 complex found in rodents and humans with antagonistic effects [119]. Further *in vitro* and *in vivo* studies on the LPS produced by *Rhodobacter sphaeroides* and other bacteria/cyanobacteria have shown potent antagonistic activity of this type of LPS in murine and human cells as well as preventing endotoxic shock in mice.

Additionally, traditional Asian medicine produced from plants, including curcumin, turmeric and a variety of herbs provide a rich and natural source of molecules which are being investigated for bio-actives that act as TLR4 antagonists [120, 121]. The modulation of TLR4 using herbal extracts promoted a large area of research to determine their pharmacological potential. It was found that certain bio-actives from

bacteria or plants had a positive relationship against sepsis and septic shock [122-124]. These bio-actives were also discovered to have positive relationships against inflammatory diseases such as Alzheimer's, arthritis and inflammatory bowel diseases [100, 125, 126]. A summary of research conducted on some of these naturally sourced TLR4 antagonists can be found in Table 2.1. Although the main focus of this study is on the therapeutic potentials of TLR4 antagonists on intestinal inflammation, there are a limited number of studies which use intestinal inflammation as a disease model.

Table 2.1: Summary of the effect of natural TLR4 antagonists in previous pre-clinical *in vitro*, *in vivo* and *in silico* studies.

Study	Study model	TLR4 Antagonist	Outcome
Qureshi *et al* [122]	**Bacterial sepsis** *In vivo*: BDF1 mice injected with LPS (1 μg)	*Rhodobacter sphaeroides* lipid A (RsDPLA)	RsDPLA (100 μg, i.p.) pre-treatment was associated with 91% inhibition of LPS-induced response as measured by serum TNF-α concentration (246 +/- 95 pg/ml vs 2,653 +/- 286 pg/ml vehicle control).
Kirikae *et al* [127]	***In vitro* inflammation model** *In vitro*: mouse macrophage-like J774.1 cell line challenged with LPS		RsDPLA treatment decreased LPS response in a dose-dependent fashion as measured by TNF-α and IL-6 secretion (65% inhibition at 1:3 and 100% at 1:62 LPS:RsDPLA ratio). Mechanism proposed to be through binding of CD14 receptor.
Anwar *et al* [119]	***In silico***		Simulation predicted inhibitory behaviour of RsDPLA on the TLR4/MD-2 complex in rodents and humans.

	Molecular dynamics simulation		
Malgorzata-Miller *et al* *[123]*	**Septic shock** *In vitro*: Human PBMC challenged with LPS *In vivo*: C57Bl/6 mice injected with LPS	Lipooligosaccharide (LOS) from *Bartonella quintana* (BqLOS)	Human PBMCs pre-incubated with BqLOS (100 ng/mL) was associated with inhibition of LPS-induced response measured by supernatant concentration of IL-1β, TNF-α, IL-6, IL-8 ($P < 0.001$). Mice pre-treated with BqLOS (100 μg) had improved survival rates.
De Paola *et al* [128]	**Amyotrophic Lateral Sclerosis** *In vitro*: Motor neuron/glia co-cultures	LPS from *Oscillatoria Planktothrix FP1* (Cyp/VB3323)	*In vitro:* Cells exposed to LPS (1 μg/mL) reduced viability by 30.8 ± 11.9% ($P < 0.001$ vs control). This toxic effect was reduced by VB3323 (20 μg/mL) which almost completely restored

	In vivo: Wobbler mice		motor neuron viability in the cells (91.3 ± 9.9% with P < 0.001 vs LPS). *In vivo:* Wobbler mouse with spontaneous motor neuron degeneration chronically treated with VB3323 (5 mg/kg/d i.p., final concentration 0.5 mg/mL) displayed decreased microglial activation and morphological alterations of spinal cord neurons; and better performance in the paw abnormality and grip-strength tests.
Balducci *et al* [125]	**Alzheimer's disease** *In vivo*: C57Bl/6 mice		Amyloid-β oligomers (AβO) injection (7.5 μL at 1 μM) rapidly activated glial cells and induced a memory establishment deficit. When treated with CyP (10 μg, ICV) before AβO, the memory deficit was prevented (P = 0.0055).

Iori *et al* [129]	**Seizures** *In vivo*: C57Bl/6 mice		Carbamazepine (CBZ) is an anticonvulsant to treat neuropsychiatric disorders. Mice treated with CyP (1 mg/mouse, i.p.) + CBZ (20 mg/mouse, in food) during disease onset. CBZ-treated mice displayed a 3-fold higher seizure frequency compared to CyP-treated mice ($P < 0.01$). TLR4 antagonism by CyP was effective in delaying seizure onset and reduced recurrence in the established murine model of acquired epilepsy.
Yao *et al* [130]	**Inflammatory Bowel Disease** *In vitro*: Sprague-Dawley rats injected with 2,4,6-trinitro-benzene sulfonic acid	Probiotics, Golden bifid	Rats treated with the probiotics had a significantly lower disease activity ($P < 0.05$), histopathological score ($P < 0.05$) and inflammatory cytokine levels (TNF-α and IL-1β, $P < 0.05$) compared to control groups.

<table>
<tr>
<td rowspan="2">Chu et al
[131]</td>
<td>In silico

Docking analysis</td>
<td rowspan="2">Berberine, extracted from the herb huang lian (Rhizoma Coptidis)</td>
<td>Docking analysis suggested that 3 berberine molecules were able to bind to MD-2 and block TLR4/NF-κB downstream signalling. Binding free energies of the 3 berberine molecules was 7.70, -7.33 and -6.75 kcal/mol, respectively.</td>
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<tr>
<td>Bacterial infection

In vivo: BALB/c mice challenged with Salmonella typhimurium and bacterial endotoxin</td>
<td>Mice treated with 2 EU/ml endotoxin solution (i.p.) had a lethal rate of 80%. When treated with berberine at different doses (0.13, 0.16 and 0.20 g/kg) after endotoxin administration, mice had survival rate of 50, 50 and 60% respectively. Average time to death of each mouse group treated with berberine was significantly longer compared to mice only exposed to LPS ($P < 0.05$).</td>
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Liang *et al* [132]	***In vitro* inflammation model** *In vitro*: THP-1 human monocyte cells challenged with LPS **Sepsis** *In vivo*: C57Bl/6 mice administered with LPS	Sparstolonin B (SsnB) extracted from a Chinese herb (*Sparganium stoloniferum*)	*In vitro:* Ssnb (100 μM) inhibited LPS-induced (50 ng/mL) response as measured by an 18-fold decrease in TNF-α and 10-fold decrease in IL-6 expression levels vs LPS treated cells only. Mechanism proposed to be through binding to the CD-14/TLR4 receptor. *In vivo:* Mice co-treated with LPS (100 μg/mouse) and SsnB (100 μg/mouse) displayed lower expression of TNF-α ($P = 0.0075$), IL-6 ($P = 0.1077$) and IL-1β ($P < 0.0001$) vs LPS treated mice. SsnB was able to suppress inflammation induced by LPS by attenuating the TLR4-mediated activation of NF-κB.

Li *et al* [133]	**Leukaemia** *In vitro*: THP-1 cells treated with LPS	Parthenolide (PTL), extracted from the plant feverfew (*Tanacetum parthenium*)	3 and 12 µM PTL significantly decreased pro-inflammatory cytokine expression and diminished LPS-induced (1 µg/mL) TLR4 expression compared to LPS treated group ($P < 0.01$). PTL was able to inhibit the expression of these cytokines by blocking the TLR4 which in turn blocks the subsequent downstream signalling cascade.
Saadane *et al* [134]	**Cystic Fibrosis** *In vitro*: 16 HBE human bronchial epithelial cell line transfected with AS oligonucleotide that inhibits expression of CFTR. Stimulated using IL-1β/TNF (100 ng/ml each).		*In vitro*: At 3 h and 6 h, AS cells pre-treated with PTL (40 µM) had decreased IL-8 secretion vs non-treated cells ($P = 0.02$ and 0.03, respectively). *In vivo*: LPS (25 ng, intratracheally) treated mice had increased polymorphonuclear leukocytes (PMN) (9 ± 1.54% at 1 h, 38.8

	In vivo: Cystic fibrosis transmembrane conductance regulator (CFTR)-knockout mice challenged with LPS		± 7.23% at 3 h, and 63 ± 6.0% at 8 h). When co-treated with PTL (3 µg/gram) a decrease in PMN % at 8 h was observed (P = 0.006). Proposed mechanism of action for PTL was NF-κB dependent inhibition of cellular responses.
Gradišar *et al* [135]	***In vitro* inflammation model** *In vitro*: Human embryonic kidney (HEK)293 cells stimulated with LPS.	Curcumin, extracted from the Tumeric plant (*Curcuma longa*)	40% inhibition of TLR4/MD-2 complex observed at approximately equimolar concentration of curcumin and MD-2 in presence of LPS. Cells co-treated with LPS (100 ng/ml) and higher doses of curcumin (0-20 µM) showed no difference in NF-κB activity.

Zhu *et al* [136]	**Traumatic Brain Injury (TBI)** *In vivo*: Feeny weight-drop contusion model on C57Bl/6 mice		Injured brain tissue had a significant increase in TLR4 expression vs sham control brains 24 h post-trauma ($P < 0.01$). Curcumin (100 and 200 mg/kg) administered post-trauma reduced TLR4 expression and had lower neurological deficit scores and brain water content vs vehicle-treated mice with $P < 0.01$ and $P < 0.05$, respectively. A decrease in concentrations of inflammatory mediators (IL-1β, IL-6, TNF-α, MCP-1) vs vehicle-treated mice ($P < 0.01$) was also observed.
Zhang *et al* [137]	**Acute lung injury** *In vivo*: BALB/c mice with injury induced by LPS	Atractylenolide I (AO-I/ AT-I) extracted from the Chinese herb Cang zhu (*Rhizoma Atractylodis macrocephalae*)	LPS (10 μg) treated mice displayed pathological changes: inflammatory cell infiltration, interalveolar septal thickening, and oedema, which were attenuated in co-treated mice (LPS+AO-I at 5, 10 and 20 mg/kg). MPO activity and inflammatory cell infiltrate was reduced in co-treated mice (5

			mg/kg: P < 0.01, 10 mg/kg: P < 0.01 and 20 mg/kg: P < 0.01) and (5 mg/kg: P < 0.05, 10 mg/kg: P < 0.01 and 20 mg/kg: P < 0.01) vs LPS-treated mice, respectively.
Wang *et al* [124]	**Sepsis** *In vivo*: Caecal ligation and puncture (CLP) model of mice		Survival of mice increased with AT-I dose at 10, 20, and 40 mg/kg (i.p.) (P < 0.05, P < 0.01, P < 0.01, respectively) vs control, respectively. AT-I treated mice took a shorter time to return to normal temperature (P < 0.05) and displayed dose-dependent decrease in pro-inflammatory cytokines TNF-α and IL-6 (P < 0.05, P < 0.01, P < 0.01, respectively). Decrease in white blood cells (P < 0.05) and IL-1β (P < 0.05 and P < 0.01, respectively) was observed at 20 and 40 mg/kg doses.
Li *et al* [138]	**Acute respiratory distress syndrome**	Asiatic acid (AA) extracted from the plant Gotu	LPS treated mice displayed increased lung wet/dry weight ratio, inflammatory cell infiltrate and MPO activity. Co-treated mice (LPS + AA at 25, 50, and 100 mg/kg) displayed

	In vivo: BALB/c mice with LPS administered intranasally to induce lung injury	Kola/Pennywort (*Centella asiatica*)	decreased lung wet/dry weight ratio ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively) inflammatory cell infiltrate ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively) and MPO activity ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively) vs LPS group.
Lee *et al* [139]	***In vitro* inflammation model** *In vitro*: Bone marrow cells isolated from C57Bl/6 mice challenged with LPS	Celastrol extracted from the plant Thunder God Vine (*Tripterygium wilfordii*)	Celastrol (0.1, 0.5 and 1 μM) inhibited LPS-induced (10 ng/ml) responses measured by TNF-α, IL-6, IL-12, IL-1β at mRNA and protein levels ($P < 0.05$). Confocal imaging analysis of celastrol demonstrated decreased co-localisation of fluorescent LPS with MD-2.
Yuan *et al* [126]	**Arthritis** *In vivo*: C57Bl/6 mice with induced adjuvant arthritis		Celastrol (0.5 mg/kg) improved clinical outcome via clinical and histopathological scoring vs non-treated mice ($P < 0.01$). Decreased expression of TNF-α (1.9-fold) and IL-6 (3.1-fold) in celastrol treated mice vs non-treated mice.

Cho *et al* [140]	***In vitro* inflammation model** *In vitro*: RAW264.7 cells challenged with LPS	Xanthohumol (XN) extracted from the plant Hops (*Humulus lupulus*)	Cells co-treated with LPS (0.1 – 0.5 μg/ml) and XN (0.5, 1, 2.5, and 5 μg/ml) displayed a dose-dependent decrease in NO levels (2.5 & 5 μg/ml, P < 0.01 vs LPS group), TNF-α (2.5 & 5 μg/ml, P < 0.01 vs LPS group) and IL-1β (1, 2.5 & 5 μg/ml, P < 0.01 vs LPS group).
Ungaro *et al* [100]	**Inflammatory Bowel Disease** C57Bl/6J mice with dextran sulphate sodium (DSS) administered in drinking water.	IgG2b monoclonal antibody	Mice co-treated with DSS (2.5%) and IgG2b (20 mg/kg) displayed decrease in expression of TNF-α (141.5 ± 16.3 pg/ml vs. 336 ± 53.8 pg/ml, P < 0.01), IL-6 (4,816 ± 145.5 pg/ml vs. 5,850.4 ± 144.4 pg/ml, P < 0.01) and % dendritic cells in the lamina propria (3.4 ± 0.7 vs. 8.7 ± 0.4%, P < 0.05) vs control. No difference in DAI scoring vs control (1.28 ± 0.19 vs. 1.33 ± 0.23, P = 0.42, maximum score: 4).

Zhang *et al* [98]	**Inflammatory Bowel Disease** C57Bl/6 mice with dextran sulphate sodium (DSS) administered in drinking water.	Paeoniflorin extracted from peony root	Mice pre- or co-treated with paeoniflorin (50 mg/kg) and DSS (4%) suppressed weight loss: Pre-paeoniflorin at day 7 ($P < 0.05$), Co-paeoniflorin at day 5 – 6 ($P < 0.05$), day 7 ($P < 0.001$); Diarrhoea/ bloody diarrhoea: Pre-paeoniflorin at day 5 ($P < 0.05$), 6 ($P < 0.01$), 7 ($P < 0.001$), Co-paeoniflorin at day 6 – 7 ($P < 0.05$); Shortening of colon length: Pre- and co-paeoniflorin ($P < 0.05$); Histological score: Pre- and co-paeoniflorin ($P < 0.01$) vs vehicle control. Paeoniflorin treated mice had lower expression of TLR4 protein and mRNA vs DSS only mice ($P < 0.001$).

Synthetic antagonists

Although there are many plant-based products capable of targeting and inhibiting TLR4 *in vitro* and *in vivo* in both rodent and human models, they do not possess the necessary stability and target specificity to be considered a potential therapeutic option compared to products and molecules extracted from microorganisms [120]. Molecules produced from microorganisms such as *Rhodobacter sphaeroides* have therefore been used as a model to create synthetic antagonists. RsDPLA was used to design the synthetic TLR4 antagonists eritoran (E5564) and E5531 [141, 142]. E5531 was a first generation lipid A analogue synthesised as part of a program to develop therapeutic agents for septic shock [143]. Eritoran (E5564) is a second generation lipid A analogue designed for the same purpose but was found to be more potent in its anti-endotoxin effects, has a longer duration of action and is easier to manufacture compared to E5531 [144]. Studies on eritoran have shown a positive affect against sepsis [144] and other inflammatory effects [145, 146]. This led to the development of other synthetic analogues such as TAK-242 and FP7 with antagonistic effects on the TLR4/MD-2 complex to treat various inflammatory diseases such as neuroinflammation and influenza infections. Although the main focus of this study is on the therapeutic potential of TLR4 antagonists on intestinal inflammation, there are a limited number of studies which use intestinal inflammation as a disease model. Therefore, studies which encompass different inflammatory diseases have been included and summarised in Table 2.2 in order to show the potential broader anti-inflammatory effects of TLR4 antagonism.

Table 2.2: Summary of the effect of synthetic TLR4 antagonists in pre-clinical *in vitro* and *in vivo* research studies.

Study	Study model	TLR4 Antagonist	Outcome
Mullarkey *et al* [144]	**Sepsis** *In vivo*: C57Bl/6 mice, Hartley guinea pigs, Fischer rats challenged with IV LPS	Eritoran (E5564)	Mice: E5564 (100, 300, 1000 μg/kg) co-treatment was associated with 37, 81 and 93% inhibition of LPS-induced (100 μg/kg) response as measured by serum TNF-α levels, respectively ($P < 0.05$ vs control). Guinea Pigs: E5564 (30, 100, 300 μg/kg) co-treatment was associated with 29, 57 and 94% inhibition of LPS-induced (1000 μg/kg) response as measured by serum TNF-α levels, respectively ($P < 0.05$ vs control). Rats:

			E5564 (10, 100, 1000 µg/kg) co-treatment was associated with 84, 97 and 100% inhibition of LPS-induced (3 µg/kg) response as measured by serum TNF-α levels, respectively ($P < 0.05$ vs control).
Kitazawa *et al* [145]	**Acute liver failure (ALF)** *In vivo*: Wistar rats challenged with D-galactosamine (GalN) and LPS		Rats treated with E5564 after ALF (500 mg/kg GalN + 50 µg/kg LPS) displayed a decrease in serum TNF-α levels and had an improved survival rate of 42.9% compared to untreated rats ($P < 0.05$).
Liu *et al* [146]	**Inflammatory effects of ischemia-reperfusion in kidneys**		Rats treated with E5564 displayed a significant improvement in renal function as measure by serum creatinine levels ($P < 0.05$) and higher survival rates ($P < 0.05$) vs vehicle controls.

	In vivo: Fisher rats with kidney nephrectomy and ischemia performed.		
Sha *et al* [57]	**Endotoxin shock** *In vivo*: BALB/c mice treated with LPS i.p.	TAK-242 (resatorvid)	Pre-treatment of TAK-242 (0.1, 0.3, 1, and 3 mg/kg) was associated with a decrease in LPS-induced (10 mg/kg) responses as measured by IL-6, IL-10, MIP-2, IL-1β and NO serum levels vs vehicle control ($P < 0.025$). A 40% increase in survival rate of mice was also observed vs vehicle control ($P \leq 0.05$). Post-treatment of TAK-242 (1 mg/kg) was associated with a decrease in LPS-induced (5 mg/kg) response as measured by IL-6 and MIP-2 serum levels vs vehicle control ($P \leq 0.01$). A survival rate of 45% was also observed vs vehicle control ($P \leq 0.01$).

Kuno *et al* [147]	**Endotoximia** *In vivo*: Hartley guinea-pigs treated with LPS IV		TAK-242 (3 and 10 mg/kg) pre-treatment was associated with a dose-dependent decline in colonic muscle tension ($P = 0.001$ and $P < 0.001$, respectively) and mean arterial pressure ($P = 0.036$ and $P = 0.004$, respectively) caused by LPS (10 mg/kg, IV). A 50% survival rate was observed when pre-treated with TAK-242 at 10 mg/kg vs the 10% observed in the control group.
Garate *et al* [148]	**Neuroinflammation** *In vivo*: Wistar Hannover rats restrained to induce stress.		Pre-treatment of TAK-242 (0.5 mg/kg, IV) decreased expression of the pro-inflammatory enzymes: IL-1β, COX-2 and iNOS expression levels, $P < 0.05$ vs control, $P < 0.05$ vs stress only group.
Hua *et al* [149]	**Cerebral ischaemia**		Treatment with TAK-242 (3 mg/kg) was associated with reduce levels of serum TNF-receptor II, monocyte chemotactic protein-1,

	In vivo: C57Bl/6 mice induced with focal cerebral ischaemia/reperfusion		macrophage inflammatory protein-1γ, and tissue inhibitor of metalloproteinases-1 ($P < 0.05$ vs untreated mice). An 8.8% reduction in brain infarct size and improved neurologic function score (6.73) was also observed ($P < 0.05$ vs untreated mice).
Perrin-Cocon *et al* [150]	**Lethal influenza infection** *In vitro*: monocyte-derived dendritic cells (DCs) challenged with influenza virus, strain A/PR/8/34 *In vivo*: C57Bl/6 infected with mouse-adapted	FP7	*In vitro*: FP7 (1 & 10 µM) treatment was associated with decreased levels of LPS-induced (10 ng/ml) responses as measured by supernatant levels of IL-8, IL-6, MIP-1β, TNF-α, IL-12 and IL-10 in both monocytes and DCs ($P < 0.05$ vs LPS). *In vivo*: Mice treated with FP7 (200 µg/mouse, IV) after influenza infection displayed reduced gene production of TNF-α, IL-1β, IFN-β, murine IL-8 ($P < 0.01$) and IL-6 ($P < 0.05$) in the lungs. FP7-treated mice had

	influenza virus, strain A/PR/8/34		decreased viral load (log FP7-treated titre = 4.1 ± 0.39 vs vehicle-treated mice (log vehicle-treated titre = 5.27 ± 0.15) as measured by a virus titration assay ($P = 0.0225$).
Palmer *et al* [151]	**Cardiovascular inflammatory-based diseases** *In vitro*: Human umbilical vein endothelial cells (HUVEC), THP-1 and mouse RAW-264.7		*In vitro*: FP7 (0 – 10 μM) negatively regulated LPS-induced production (100 ng/ml) of pro-inflammatory cytokines in a dose-dependent manner: **THP-1**: IL-8 ($P < 0.001$), IL-6 ($P < 0.01$), MIP-1α ($P < 0.001$) at 5 μM and IL-1β ($P < 0.001$) at 0.1, 1, 5 μM vs LPS. **RAW-264.7**: p65 NF-κB at 1, 5, 10 μM ($P < 0.001$), IL-6 at 5 μM ($P < 0.05$), 10 μM ($P < 0.001$) and p38 MAPK at 0.1 ($P < 0.05$), 1 ($P < 0.01$), 5 and 10 μM ($P < 0.001$) vs LPS.

	macrophages challenged with LPS *In vivo*: Angiotensin II-infused apolipoprotein E-deficient mice		**HUVEC**: p38 MAPK and p65 NF-κB at 0.1, 0.5 and 1 μM ($P < 0.01$, $P < 0.05$, $P < 0.01$, respectively), MCP-1 at 1 μM ($P < 0.05$) vs LPS. *In vivo*: FP7 (3 mg/kg/d) inhibited angiotensin II-driven production of pro-inflammatory proteins, and MIP-1γ and JNK phosphorylation ($P < 0.05$ vs angiotensin II group).
Facchini *et al* [152]	**Inflammatory Bowel Disease**		*In vitro*: FP7 at 10 μM negatively regulated LPS-induced production (100 ng/ml) of pro-inflammatory cytokines:

	In vitro: Peripheral blood mononuclear cells and lamina propria mononuclear cells collected from patients with IBD *In vivo*: BALB/c mice with DSS administered in their water.		**mRNA relative expression:** TNF-α ($P < 0.001$); IL-1β ($P < 0.05$); IL-6 ($P < 0.05$). **ELISA**: TNF-α, IL-1β and IL-6 ($P < 0.05$). *In vivo*: FP7 (250 μg/kg) treatment was associated with a lower histological score ($P < 0.01$ vs DSS) and significantly reduced the release of inflammatory cytokines (TNF-α ($P < 0.05$), IL-1β ($P < 0.001$) and IL-6 ($P < 0.05$).
Huggins *et al* [55]	**Abdominal aortic aneurysm (AAA)**	IAXO-102	*In vitro*:

	In vitro: HUVEC challenged with LPS *In vivo*: C57Bl/6 mice induced with AAA		IAXO-102 (10 μM) blocked LPS-stimulated (100 ng/ml) production of JNK, ERK, p65 NF-κB ($P < 0.05$) and p38, MCP-1, IL-8 ($P < 0.01$) vs LPS. *In vivo*: IAXO-102 (3 mg/kg/day) blocked angiotensin II-induced response as measured by protein expression of JNK, ERK, p65, NF-κB ($P < 0.05$) vs angiotensin II only group. IAXO-102 also downregulated expression of MIP-1γ and TLR4 ($P < 0.05$ vs angiotensin II group) and reduced incidence of AAA (30% IAXO-102-treated vs 86% angiotensin II group).
Zhang *et al* [153]	**Acute lung injury (ALI)** *In vitro*: Mouse RAW 264.7 macrophages challenged with LPS	Chalcone derivatives - Compound 20	*In vitro*: Fluorescent probe determined compound 20 is a specific inhibitor of MD-2 (KD = 189 μM). Addition of compound 20 (10 μM) inhibited LPS-

	In vivo: Sprague Dawley rats with ALI induced by intratracheal LPS instillation		induced (0.5 μg/mL) secretion of TNF-α, IL-1β, COX-2 ($P < 0.01$) and IL-6 ($P < 0.05$) vs LPS. *In vivo*: Compound 20 (20 mg/kg) reduced LPS-induced (5 mg/kg) pulmonary oedema as measured by the decrease in lung wet/dry weight ratio ($P < 0.01$) vs LPS. Compound 20 also inhibited IL-1β secretion ($P < 0.01$) and MPO activity ($P < 0.05$) vs LPS.
Wang *et al* [154]	**Septic shock and lung injury** *In vitro*: Mouse primary peritoneal macrophages challenged with LPS	Curcumin Analogues - L48H37	*In vitro*: Fluorescent probe determined L48H37 is a specific inhibitor of MD-2 (KD = 11.3 μM). L48H37 (1, 2.5, 5, or 10 μM) inhibited LPS-induced (0.5 μg/ml) phosphorylation in a dose-dependent manner: ERK at 1, 2.5, 5, and 10 μM ($P < 0.01$), p38 at 2.5 μM ($P < 0.05$), 5 and 10 μM ($P < 0.01$), and JNK at 5 and 10 μM ($P < 0.01$) vs LPS. L48H37 (10 μM)

	In vivo: C57Bl/6 mice injected with LPS		inhibited secretion of TNF-α, IL-6, IL-1β and iNOS ($P < 0.01$ vs LPS-treated group); IL-10 and COX-2 ($P < 0.05$ vs LPS-treated group). *In vivo*: L48H37-treated (10 mg/kg) mice had higher survival rates vs LPS (20 mg/kg, i.v.) ($P < 0.01$). Pulmonary damage and LPS-injured tissue structure of lungs was amended.
Hodgkinson & Ye [155]	***In vitro* inflammation model** *In vitro*: Human embryonic kidney (HEK)293-CD-14-MD-2 cells challenged with LPS	Statins - Simvastatin - Pravastatin	Both simvastatin and pravastatin (2 μM) pre-treatment was associated with the inhibition of LPS-induced (5 ng/ml) response as measure by supernatant concentrations of NF-κB, IL-6 and TNF-α ($P < 0.05$ vs LPS).

Katsargyris *et al* [156]	**Carotid atherosclerotic plaques** *Ex vivo*: atherosclerotic plaques from patients		Patients who used statins had lower TLR4 expression in their endothelial cells and atherosclerotic plaques vs non-statin patients (P = 0.02 and P = 0.03, respectively). Prevalence cerebrovascular accident was 18.6% in statin group vs 61.4% of non-statin group (odds ratio (95% CI) 0.14 (0.07-0.31) P < 0.001).
Fort *et al* [47]	**Inflammatory Bowel Disease** BALB/c mice with DSS administered in their water.	lipid A-mimetic - CRX-526	CRX-526 (2, 10, 50 μg) treatment was associated with a lower DAI (P = 0.421, 0.056, 0.016, respectively) and histological score (P = 0.032, 0.008, 0.008, respectively) vs DSS in a dose-dependent manner.

The most well-known TLR4 antagonist to enter the clinical phase was eritoran, followed by TAK-242; and although many synthetic TLR4 antagonists have been developed and studied, very few have actually made it into clinical trials. Due to the limited evidence currently available. Table 2.3 summarises only the TLR4 antagonists that have been, or are, undergoing clinical trials in different inflammatory disease models. However, this will allow for a broader view of using TLR4 antagonists in inflammatory diseases to support its use in intestinal inflammation.

Table 2.3: Summary of TLR4 antagonists used in clinical trials.

TLR4 Antagonist	Condition/ Disease	Mechanism of Action	Clinical Trial Design & Aim	Trial Status & Outcome	Reference/Clinical Trial Number
Eritoran (E5564)	Sepsis/Severe sepsis/ Septic shock	Lipid A mimic, binds to MD2	Phase 2, A Safety and Efficacy Study of Intravenous E5564 in Patients With Severe Sepsis	Completed. Eritoran appeared well tolerated and showed a lower mortality rate (105 mg dose) in patients with severe sepsis and high predicted risk of mortality.	NCT00046072
			Phase 3, A Controlled Comparison of Eritoran	Completed.	NCT00334828

			Tetrasodium and Placebo in Patients With Severe Sepsis	Patients with severe sepsis did not have reduced 28-day mortality when administered with eritoran, compared with placebo.	
Resatorvid (TAK-242)	Severe sepsis	Binds covalently to Cys747 of TLR4-TIR domain and blocks TLR4/TIRAP and TLR4/TRAM interactions	Phase 3, A Pivotal, Multicenter, Multinational, Randomized, Double-Blind, Placebo-Controlled Study To Evaluate The Efficacy And Safety of TAK-242 in Adults With Severe Sepsis	Completed. TAK-242 did not suppress cytokine levels in patients with sepsis. TAK-242 was well tolerated but patients developed mild increases in serum methaemoglobin levels.	NCT00143611

			Phase 3, Randomised, Double-Blind, Placebo-Controlled Study of the Efficacy and Safety of TAK-242 Versus Placebo in Subjects With Sepsis-Induced Cardiovascular and Respiratory Failure	Terminated. Business Decision; No Safety Or Efficacy Concerns.	NCT00633477
NI-0101	Healthy volunteers	Monoclonal antibody blocking TLR4 signalling	Phase 1, Randomised double-blind study to determine the safety, tolerability and distribution and elimination of a novel therapeutic drug (NI-0101)	Completed. NI-0101 showed good tolerability, favourable safety and PK profile, and durable anti-inflammatory effect in healthy volunteers.	NCT01808469

			when administered to healthy volunteers.		
	Rheumatoid Arthritis		Phase 2, Randomised, placebo-controlled, double-blind study to explore the effect of a new antibody to treat patients with rheumatoid arthritis.	Completed. Results unavailable.	NCT03241108

Conclusions

Both IBD and GIM have significant effects on a patient's quality of life as well as economic and social burdens [31, 157, 158]. While the pathophysiology of chronic intestinal inflammation remains unknown, previous research has identified that TLR4 signalling in the intestinal tract is a critical regulator of intestinal immune homeostasis. The use of a TLR4 antagonists has potential as a novel therapeutic for IBD and GIM patients whose disease pathogenesis relies heavily on TLR4 signalling. Previous studies have shown that inhibiting LPS-induced TLR4 stimulation with antagonists can reduce intestinal inflammation in animal models [159]. Regardless of how promising TLR4 antagonists are in the treatment of intestinal inflammation, there are still challenges in bioavailability and delivery. Nonetheless, anti-TLR4 therapies present a promising alternative for future innovative treatments for both IBD and GIM. In the future, there is a need for tissue specific studies looking into these anti-TLR4 therapies in order to mimic the therapeutic setting of IBD and GIM.

Chapter 3: Structural insight and analysis of TLR4 interactions with IAXO-102, TAK-242 and SN-38: An *in silico* approach

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Statement of Authorship

Statement of Authorship

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Name of Principal Author (Candidate)	Shu Yie Janine Tam
Contribution to the Paper	Planned, researched and wrote the first draft of paper. Edited subsequent drafts.
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Jinxin Pei
Contribution to the Paper	Assisted with the *in silico* docking analysis
Signature	Date 12/04/2022

Name of Co-Author	Dr Janet Coller
Contribution to the Paper	Provided critical feedback of the manuscript for important intellectual content that shaped the research.
Signature	Date 12/4/2022

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Prof Clive Prestidge		
Contribution to the Paper	Provided critical feedback of the manuscript for important intellectual content that shaped the research.		
Signature		Date	

Name of Co-Author	Prof Joanne Bowen		
Contribution to the Paper	Provided feedback and critical revision of the manuscript for important intellectual content, final approval of the manuscript.		
Signature		Date	11/04/2022

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Abstract

Introduction: Toll-like receptor 4 (TLR4) has attracted interest due to its role in chemotherapy-induced gastrointestinal inflammation. This structural study aimed to provide in silico rational of the recognition and potential binding of TLR4 ligands IAXO-102, TAK-242, and SN-38 (the toxic metabolite of the chemotherapeutic irinotecan hydrochloride), which could contribute to rationale development of therapeutic anti-inflammation drugs targeting TLR4 in the gastrointestinal tract.

Methods: *In silico* docking was performed between the human TLR4/MD-2 complex and ligands (IAXO-102, TAK-242, SN-38) using Autodock Vina, setting the docking grids to cover either the upper or the lower bound of TLR4. The conformation having the lowest binding energy value (kcal/mol) was processed for post-hoc analysis of the best-fit model. Hydrogen bonding was calculated by using ChimeraX.

Results: Binding energies of IAXO-102, TAK-242 and SN-38 at the upper bound of TLR4/MD-2 ranged between −3.8 and −3.1, -6.9 and -6.3, and -9.0 and -7.0, respectively. Binding energies of IAXO-102, TAK-242 and SN-38 at the lower bound ranged between −3.9 and −3.5, -6.5 and -5.8, and -8.2 and -6.8, respectively. Hydrogen bonding at the upper bound of TLR4/MD-2 with IAXO-102, TAK-242 and SN-38 was to aspartic acid 70, cysteine 133 and serine 120, respectively. Hydrogen bonding at the lower bound of TLR4/MD-2 with IAXO-102, TAK-242 and SN-38 was to serine 528, glycine 480 and glutamine 510, respectively.

Conclusion: The in silico rational presented here supports further investigation of the binding activity of IAXO-102 and TAK-242 for their potential application in the prevention of gastrointestinal inflammation caused by SN-38.

Introduction

Gastrointestinal (GI) toll-like receptor 4 (TLR4) promotes mucosal integrity and microbial tolerance while able to rapidly induce an inflammatory response to provide protection from invading bacteria [45]. Lipopolysaccharides (LPS) and other microbial-associated molecular patterns (MAMPs) activate TLR4 expressed on intestinal epithelium as well as immune cells in the lamina propria [44]. TLR4 activation controls cellular responses through downstream signalling pathways including NF-κB and IRF3 [160]. While elimination of noxious stimuli and repair of damaged structures is the ultimate goal in response to TLR4 activation, excessive TLR4 or dysregulated TLR4 signalling is associated with many inflammatory conditions such as cancer treatment-related intestinal inflammation, referred to clinically as gastrointestinal mucositis (GIM) [42].

Chemotherapeutic agents, such as irinotecan hydrochloride (specifically its active component SN-38), cause direct injury to intestinal epithelial cells, allowing luminal antigens to enter the lamina propria [28]. The role of TLR4 in intestinal inflammatory conditions has been investigated thoroughly in genetic (knockout and over-expression) mouse models and patient-derived tissue [42, 161]. This previous work has shown strong associations with disease onset, damage severity and even cancer development [42, 161]. A limitation of knockout models is the reliance on TLR4 signalling to repair the colon following inflammatory insult [58]. A further barrier is an inability to evaluate the contribution of non-MAMP TLR4 agonists in the development of injury, especially during cancer treatment. Potential agonists include moieties of chemotherapy agents from the taxane and camptothecin classes [162]. As such, development of TLR4-targeted pharmacological interventions is required to overcome these limitations.

While targeting TLR4 is a biologically supported approach to gastrointestinal (GI) inflammatory conditions, there has been little progress in the field, which may be due to a lack of specific inhibitors that selectively target the TLR4 protein and/or its associated co-receptors, MD-2 and CD-14. The integration between biological systems and computational techniques provides the possibility to explore drug development opportunities in order to rapidly provide structural, chemical, and biological data to improve understanding of potential drug targets. For example, the interactive association between various methods, such as *in silico* molecular docking and protein binding studies has been employed by researchers for the development of pharmacologically active drugs [56].

IAXO-102 was recently developed and has been shown to have inhibitory binding properties against the TLR4/MD-2 complex at both CD-14 and MD-2 sites [54]. However, IAXO-102 has only been used in studies with a focus on aortic aneurysms [55]. TAK-242, a cyclohexene derivative, is a novel small-molecule compound with a proposed inhibitory action through cysteine 747 that selectively inhibits TLR4 signalling and has been studied in a variety of inflammatory models such as sepsis and neuroinflammation [57, 148]. However, these antagonists have not been extensively studied in an intestinal inflammation model [163]. Similarly, it is currently unclear if the active metabolite of irinotecan, SN-38, is able to selectively bind to TLR4 and its co-receptors and mimic the agonistic action of LPS [164]; or if it poses antagonistic properties at specific concentrations [165]. Therefore, determining if SN-38 could directly interact with TLR4 and co-receptors will help explain whether it has the capacity to modulate LPS-dependent inflammation.

In the current study, we investigated the interactions of the TLR4 ligands, IAXO-102 and TAK-242, and SN-38 with the TLR4-MD-2 complex by in silico analyses of molecular docking. The purpose of this structural study is to provide a better

understanding of the recognition and potential binding of these compounds which may contribute to rationale development of therapeutic anti-inflammation drugs targeting TLR4 in the gastrointestinal tract.

Materials and Methods

Protein/chemical structures and docking programs

In silico modelling was performed using similar methods reported previously [166]. The human TLR4/MD-2 protein crystal structure (PDB ID:3FXI) was obtained from the National Institutes of Health NCBI Structure database (https://www.ncbi.nlm.nih.gov/Structure/pdb/3FXI accessed in October 2021). The SMILES codes (Figure 3.1A) for the ligands were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov accessed in October 2021) and converted into software-compatible 3D structures in .pdb format using the online SMILES Translator and Structure File Generator (National Cancer Institute, U.S. Department Health and Human, Washington DC).

Docking Estimations

MGLtools was used to prepare both TLR4/MD2 and ligand docking coordinates. The docking was performed using Autodock Vina [167, 168], setting the docking grids to cover either the upper (blue) or the lower (red) bound of the TLR4/MD-2 complex (Figure 3.1B). Assessment of the top 9 conformations from upper and lower bounds were completed and the conformation having the lowest atomic energy value (kcal/mol) was processed for post-hoc analysis of the best-fit model.

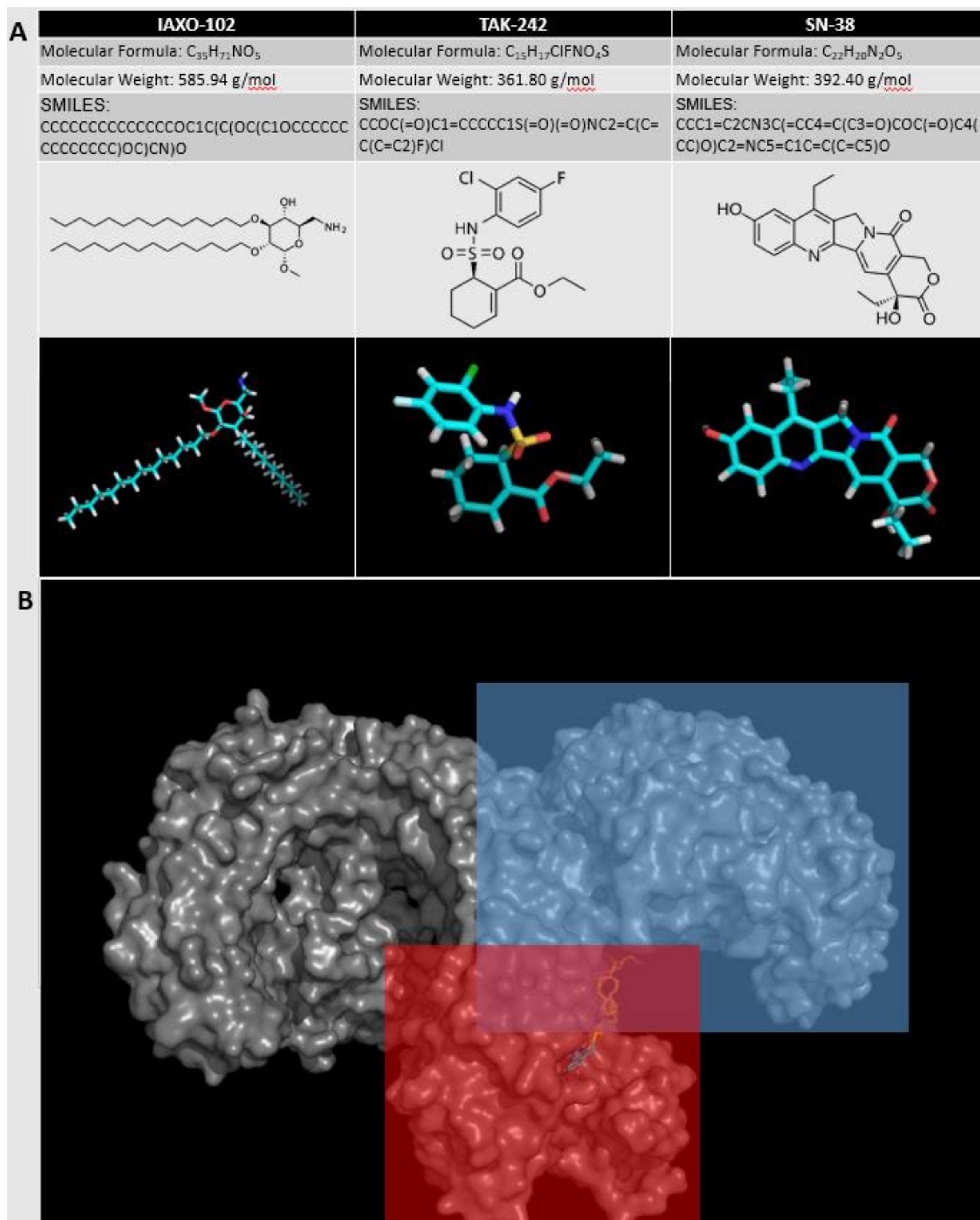


Figure 3.1: Details of TLR4 ligands used in the present study. **(A)** Basic chemical and 3D structures of the TLR4 ligands, IAXO-102, TAK-242, and SN-38. **(B)** Docking grid of the TLR4/MD-2 complex (grey), which was divided into 2 sections labelled upper bound (blue) and lower bound (red).

Root mean square deviation (RMSD) values measured the average distance between atoms of a position relative to the best fitting position and were calculated using only movable heavy atoms. RMSD upper bound (rmsd/ub) matches each atom in one conformation with itself in the other conformation, ignoring any symmetry. RMSD lower bound (rmsd/lb) matches each atom in one conformation with the closest atom of the same element type in the other conformation and is defined as follows: rmsd/lb (c_1, c_2) = max (rmsd' (c_1, c_2), rmsd' (c_2, c_1)), with c_n = conformation number. The first results of rmsd/lb is compared to itself, hence it will always be 0. Subsequent results are then compared to the first. Any small deviation indicates minor changes in the conformation of the ligand compared to the first result conformation.

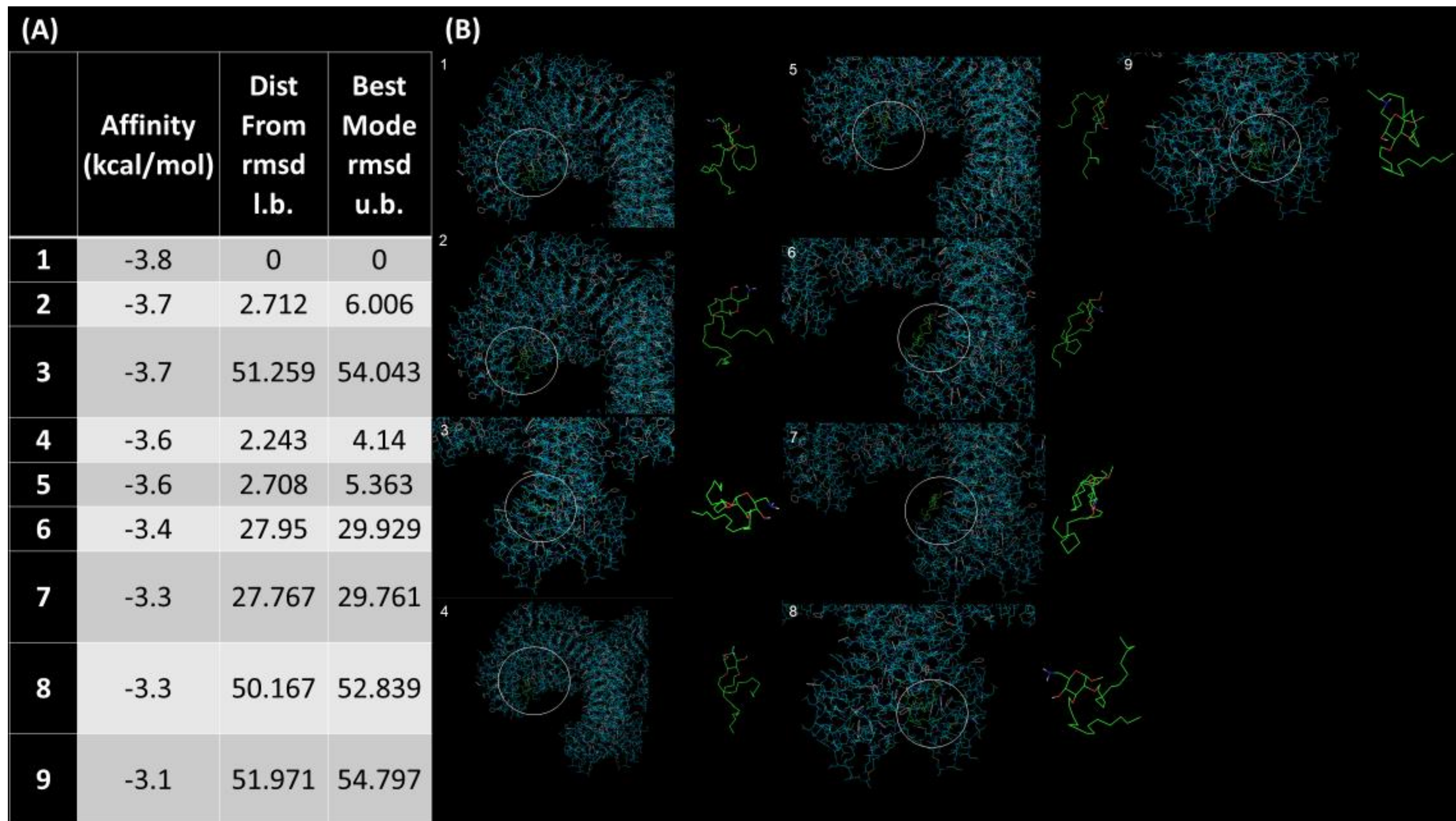
Hydrogen Bonding

Hydrogen bonding is critical for the determination of the binding affinity of a ligand (in a specific binding pose) within a specific target protein. Hence the presence of hydrogen bonds between the ligands IAXO-102, TAK-242 and SN-38 with TLR4/MD-2 was investigated using ChimeraX [169, 170].

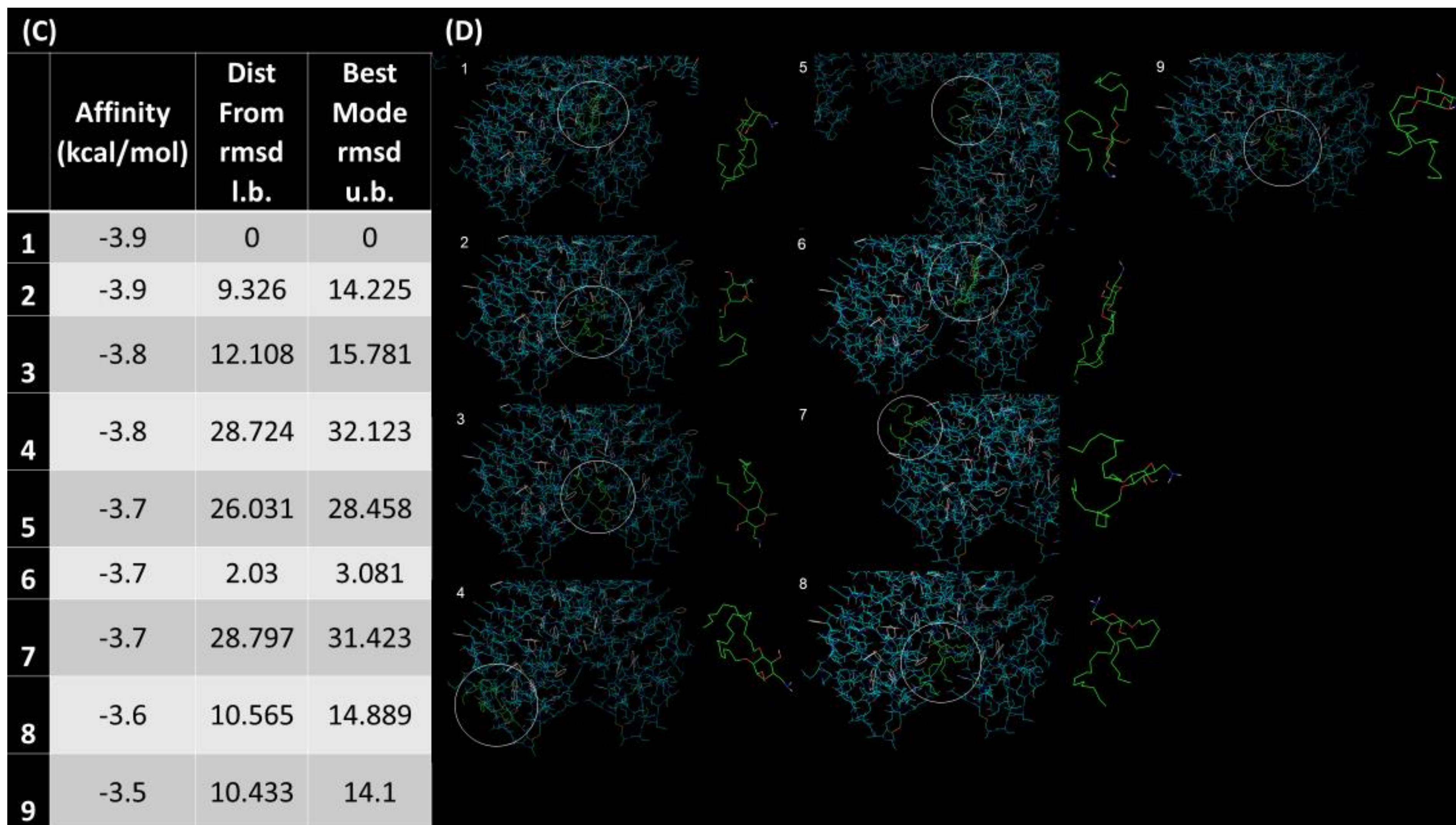
Results

In silico docking of IAXO-102, TAK-242, and SN-38 with the upper and lower bound of TLR4/MD-2 Complex

The docking poses were ranked according to their docking scores and both the ranked list of docked ligands and their corresponding binding poses. This ranking of the compounds was based on their binding energy with TLR4/MD-2. If the binding energy of the ligand was less, then the particular ligand is classified as being more active in nature and has a stronger binding affinity. The binding energies of IAXO-102, TAK-242 and SN-38 with upper bound TLR4/MD-2 ranged between −3.8 to −3.1 kcal/mol (Figure 3.2A), -6.9 to -6.3 kcal/mol (Figure 3.3A) and -9 to -7 kcal/mol (Figure 3.4A), respectively. Figures 3.2B, 3.3B and 3.4B show docked poses of upper bound TLR4/MD-2 with the ligands IAXO-102, TAK-242 and SN-38, respectively; with the binding positions of the ligands identified. The binding energies of IAXO-102, TAK-242 and SN-38 with lower bound TLR4/MD-2 ranged between −3.9 to −3.5 kcal/mol (Figure 3.2C), -6.5 to -5.8 kcal/mol (Figure 3.3C) and -8.2 to -6.8 kcal/mol (Figure 3.4C), respectively. Figures 3.2D, 3.3D and 3.4D show docked poses of lower bound TLR4/MD-2 with the ligands IAXO-102, TAK-242 and SN-38, respectively.

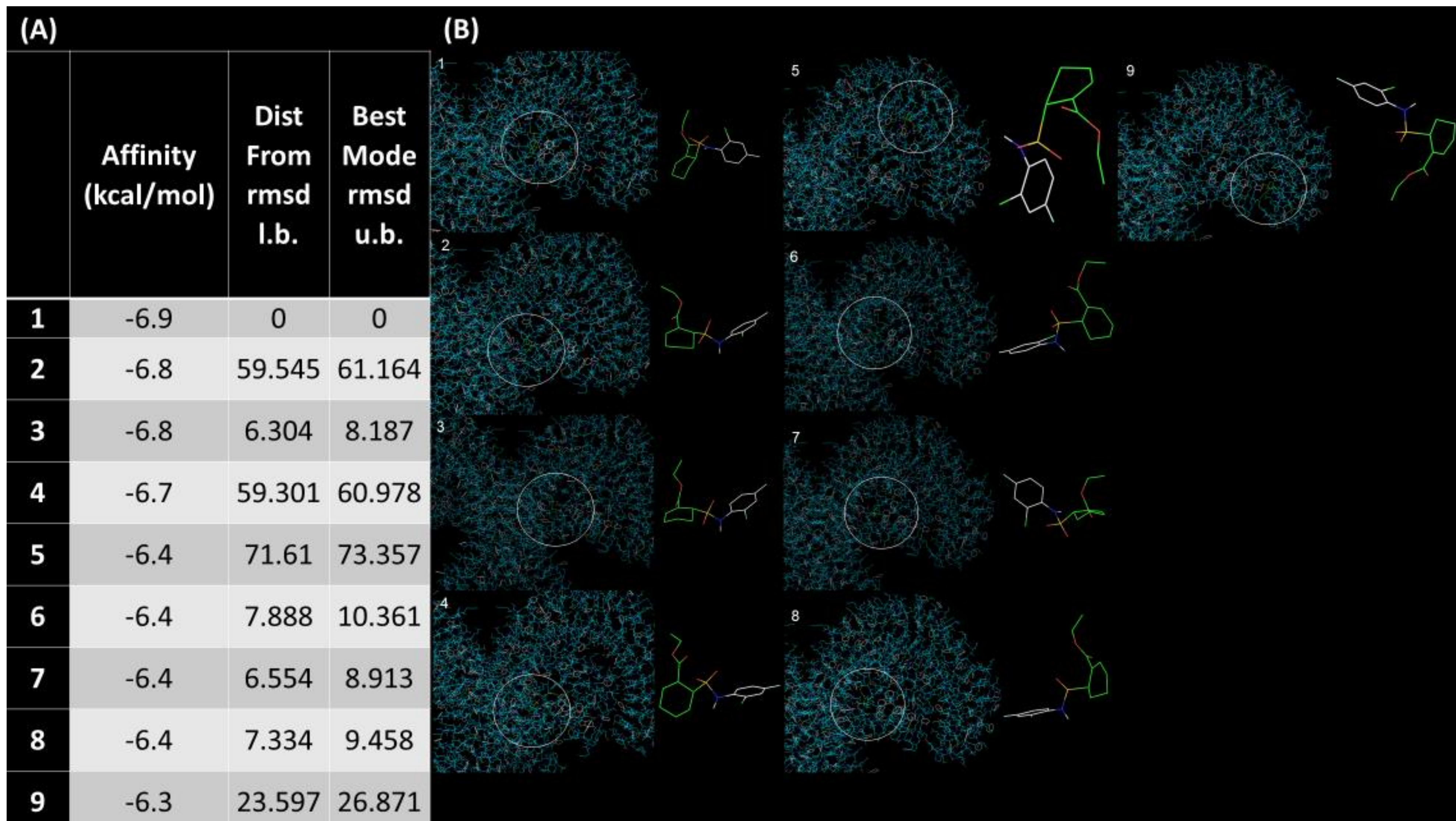


	Affinity (kcal/mol)	Dist From rmsd l.b.	Best Mode rmsd u.b.
1	-3.8	0	0
2	-3.7	2.712	6.006
3	-3.7	51.259	54.043
4	-3.6	2.243	4.14
5	-3.6	2.708	5.363
6	-3.4	27.95	29.929
7	-3.3	27.767	29.761
8	-3.3	50.167	52.839
9	-3.1	51.971	54.797


(C)

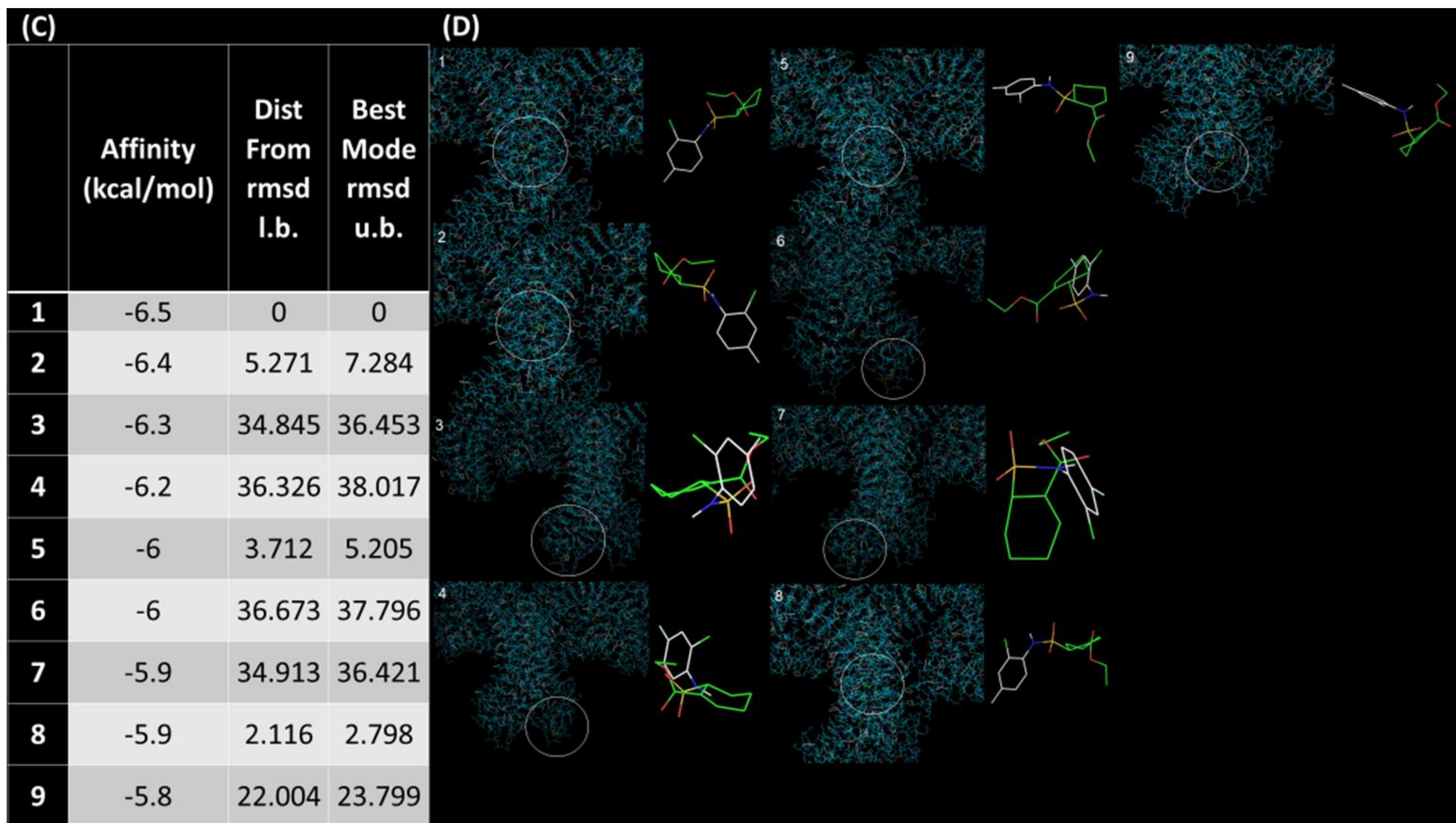
	Affinity (kcal/mol)	Dist From rmsd l.b.	Best Mode rmsd u.b.
1	-3.9	0	0
2	-3.9	9.326	14.225
3	-3.8	12.108	15.781
4	-3.8	28.724	32.123
5	-3.7	26.031	28.458
6	-3.7	2.03	3.081
7	-3.7	28.797	31.423
8	-3.6	10.565	14.889
9	-3.5	10.433	14.1

Figure 3.2: Docking results of IAXO-102 in upper and lower bound of TLR4/MD-2. Energy values describing the affinity of interaction between IAXO-102 and TLR4/MD-2 at (A) upper bound and (C) lower bound. Computer generated views of the predicted binding sites for IAXO-102 on TLR4/MD-2 at (B) upper bound and (D) lower bound. Left section in panel shows binding location and right section in panel shows conformational changes of IAXO-102 on TLR4/MD-2.



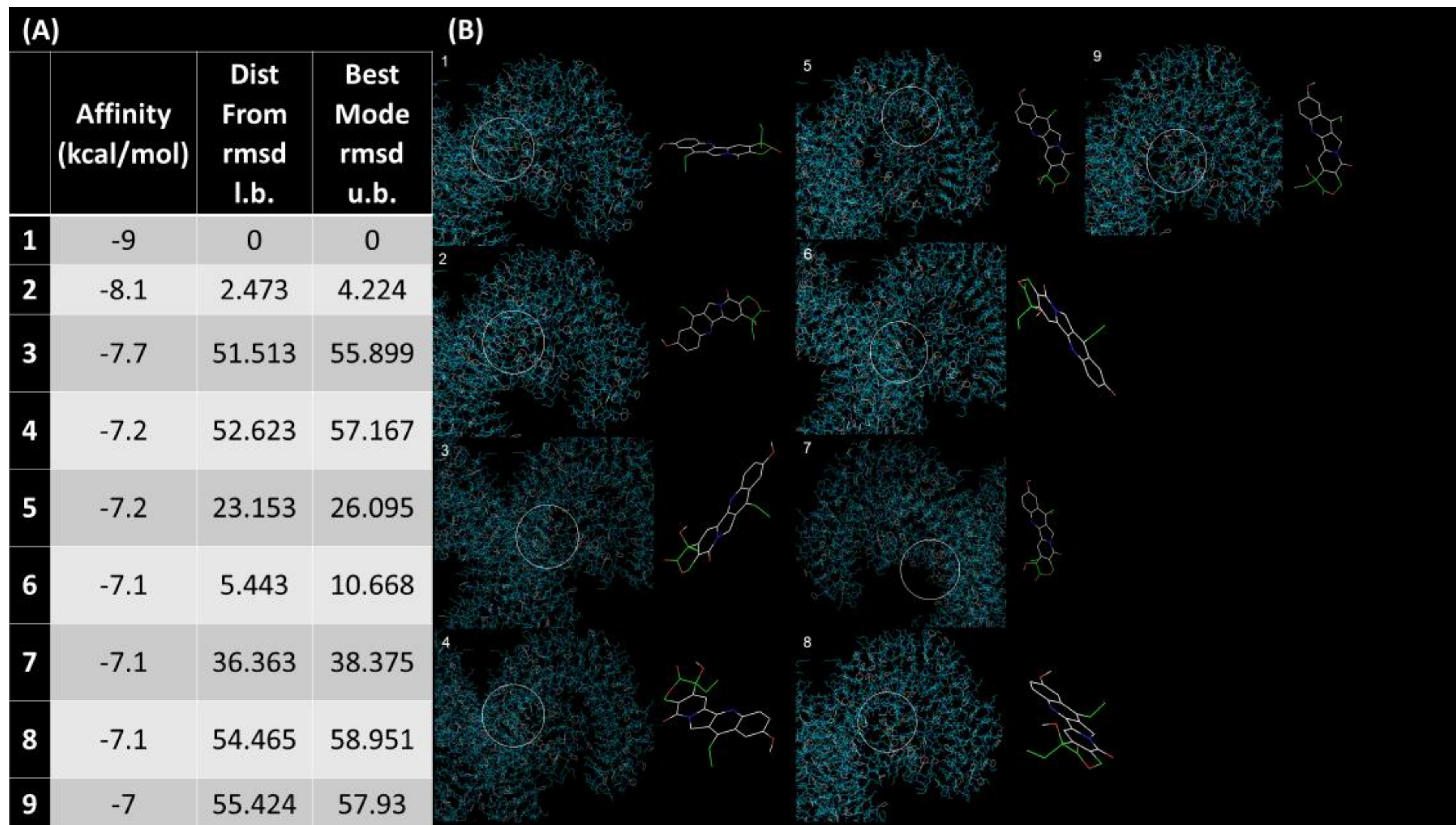
(A)

	Affinity (kcal/mol)	Dist From rmsd l.b.	Best Mode rmsd u.b.
1	-6.9	0	0
2	-6.8	59.545	61.164
3	-6.8	6.304	8.187
4	-6.7	59.301	60.978
5	-6.4	71.61	73.357
6	-6.4	7.888	10.361
7	-6.4	6.554	8.913
8	-6.4	7.334	9.458
9	-6.3	23.597	26.871



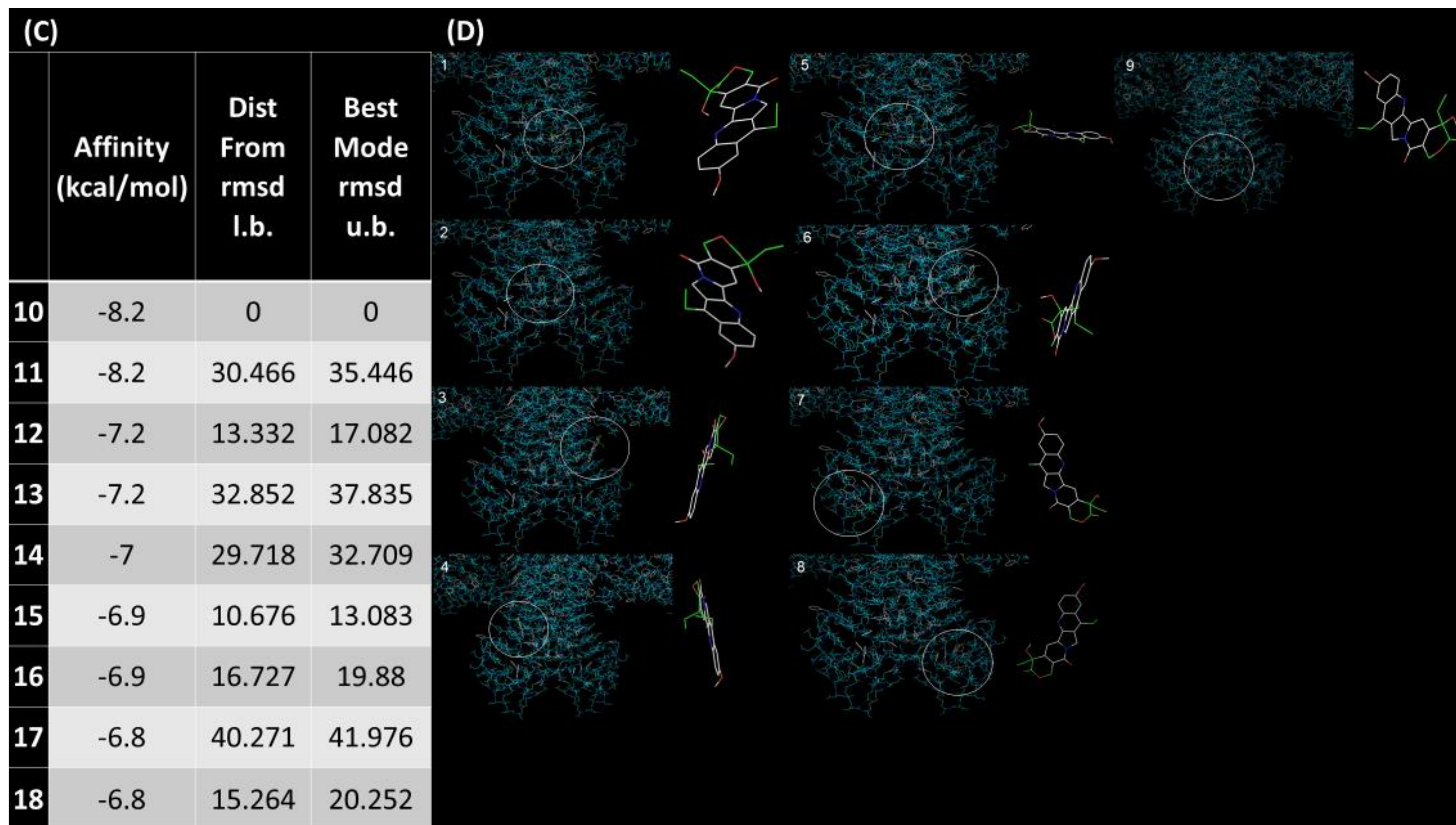
	Affinity (kcal/mol)	Dist From rmsd l.b.	Best Mode rmsd u.b.
1	-6.5	0	0
2	-6.4	5.271	7.284
3	-6.3	34.845	36.453
4	-6.2	36.326	38.017
5	-6	3.712	5.205
6	-6	36.673	37.796
7	-5.9	34.913	36.421
8	-5.9	2.116	2.798
9	-5.8	22.004	23.799

Figure 3.3: Docking results of TAK-242 between the upper and lower domain of TLR4/MD-2. Energy values describing the affinity of interaction between TAK-242 and TLR4/MD-2 at (A) upper bound and (C) lower bound. Computer generated views of the predicted binding sites for TAK-242 on TLR4/MD-2 at (B) upper bound and (D) lower bound. Left section in panel shows binding location and right section in panel shows conformational changes of TAK-242 on TLR4/MD-2.



(A)

	Affinity (kcal/mol)	Dist From rmsd l.b.	Best Mode rmsd u.b.
1	-9	0	0
2	-8.1	2.473	4.224
3	-7.7	51.513	55.899
4	-7.2	52.623	57.167
5	-7.2	23.153	26.095
6	-7.1	5.443	10.668
7	-7.1	36.363	38.375
8	-7.1	54.465	58.951
9	-7	55.424	57.93



(C)

	Affinity (kcal/mol)	Dist From rmsd l.b.	Best Mode rmsd u.b.
10	-8.2	0	0
11	-8.2	30.466	35.446
12	-7.2	13.332	17.082
13	-7.2	32.852	37.835
14	-7	29.718	32.709
15	-6.9	10.676	13.083
16	-6.9	16.727	19.88
17	-6.8	40.271	41.976
18	-6.8	15.264	20.252

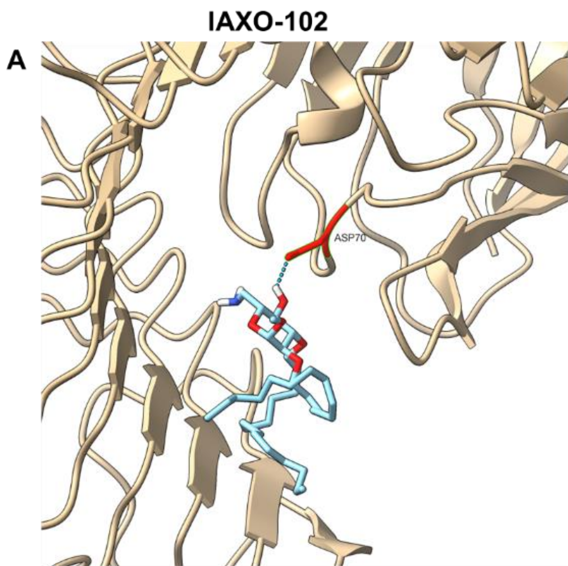
Figure 3.4: Docking results of SN-38 between the upper and lower domain of TLR4/MD-2. Energy values describing the affinity of interaction between SN-38 and TLR4/MD-2 at (A) upper bound and (C) lower bound. Computer generated views of the predicted binding sites for SN-38 on TLR4/MD-2 at (B) upper bound and (D) lower bound. Left section in panel shows binding location and right section in panel shows conformational changes of SN-38 on TLR4/MD-2.

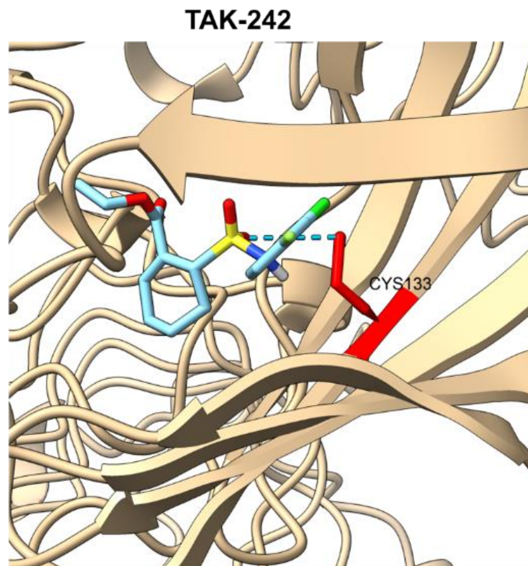
Hydrogen bonding of the ligands IAXO-102, TAK-242 and SN-38 with TLR4/MD-2

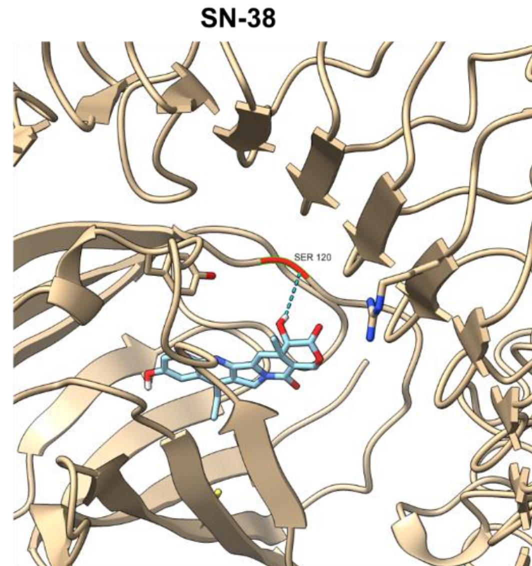
To model likely sites of drug interaction with the TLR4/MD-2 complex we next modelled hydrogen bonding between the ligands and TLR4/MD-2. Figure 3.5A shows the presence of hydrogen bonding between upper bound TLR4/MD-2 with IAXO-102, TAK-242 and SN-38 at the following amino acid residues: aspartic acid 70, cysteine 133 and serine 120, respectively. While Figure 3.5B shows the presence of hydrogen bonding between lower bound TLR4/MD-2 with IAXO-102, TAK-242 and SN-38 at the following amino acid residues: serine 528, glycine 480 and glutamine 510, respectively.

IAXO-102 **TAK-242** **SN-38**

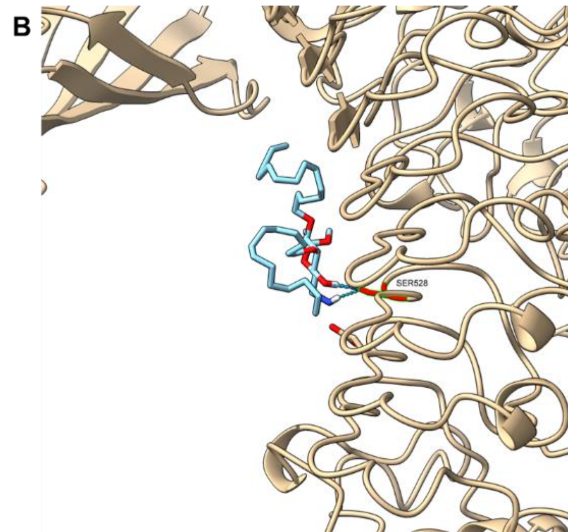
A

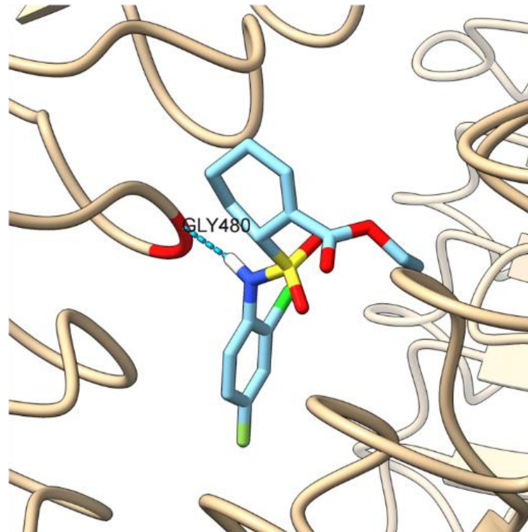






B





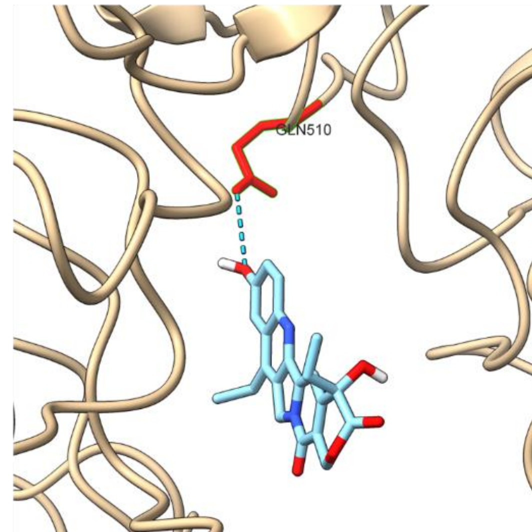


Figure 3.5: Magnified views of hydrogen bonding interactions of IAXO-102, TAK-242 and SN-38 with TLR4, calculated by using ChimeraX. (A) The hydrogen bonding (blue dotted line) between upper bound TLR4/MD-2 with IAXO-102, TAK-242 and SN-38 occurs at the following amino acid residues: aspartic acid 70, cysteine 133 and serine 120, respectively. (B) The hydrogen bonding (blue dotted line) between lower bound TLR4/MD-2 with IAXO-102, TAK-242 and SN-38 is with serine 528, glycine 480 and glutamine 510, respectively.

Discussion

While targeting TLR4 is a biologically supported approach to GI inflammatory conditions, there has been little progress in the field due to lack of specific inhibitors that selectively target the TLR4 protein and its associated co-receptor MD-2. The MD-2 protein is responsible for LPS binding in the TLR4/MD-2 complex [45]. It is characterised by a wide hydrophobic pocket that hosts the fatty acid chains from LPS [171]. The binding energy between the ligands TAK-242, IAXO-102 and SN-38 to TLR4/MD-2 obtained from analysis of molecular docking supports possible binding at TLR4/MD-2. From the tabulated binding affinities, SN-38 had the strongest binding affinity followed by TAK-242 and IAXO-102, in both upper and lower bound TLR4/MD-2.

Hydrogen bonding plays a crucial role in the determination of protein structure and is equally central in many aspects of biological function [52]. For this reason, a crucial objective in *in silico* docking in biological systems is an accurate description of hydrogen bonding. These results may provide further information on the strength and stability of a drug-receptor interaction. An enormous variety of hydrogen bonds, both between various side-chain functional groups and involving the backbone peptide group, are possible. In our study, we found at the upper bound TLR4/MD-2, IAXO-102, TAK-242 and SN-38 forms hydrogen bonds with the amino acids aspartic acid 70, cysteine 133 and serine 120, respectively. While at the lower bound TLR4/MD-2, IAXO-102, TAK-242 and SN-38 forms hydrogen bonds with different amino acids, serine 528, glycine 480 and glutamine 510, respectively.

The TLR4/MD-2 complex is found on the extracellular matrix of cells which means these ligands would not need to cross the cell membrane to become active. Compared to other inflammatory drugs such as adalimumab and infliximab that inhibit TNF-α

receptors (TNFR1 and TNFR2) located intracellularly [172]. This would be an advantage as there should be less anatomical barriers for drug transport which may lead to better pharmacokinetics. Additionally, the hydrogen bonding of all 3 ligands are located on different areas of the TLR4/MD-2 complex. We can therefore infer that all 3 ligands do not bind in the same positions and would not impede or compete for binding spaces.

These *in silico* docking studies provide evidence for the ability of the ligands investigated to bind to TLR4/MD-2 with binding energy values, docking scores, and protein-receptor interactions. Additionally, it supports the hypothesis that SN-38 has a strong binding affinity to TLR4 as previously reported by Wong *et al* [165]. However, a limitation of the approach is an inability to determine if this interaction is antagonistic or agonistic. Nonetheless, this strong affinity may explain additional off-target actions of SN-38 due to interaction with TLR4/MD-2 to modulate LPS-dependent inflammation. These findings also further expand the current knowledge concerning the pathogenesis of intestinal mucositis which was previously thought to occur only through direct DNA damage by SN-38 via it's topoisomerase I inhibitory activity [165]. Ultimately, this may support the hypothesis that TLR4 signalling pathways play key roles in the development of irinotecan-induced GI inflammation.

Carrying out *in vitro* functional protein binding tests would be the next logical step to further define the binding effects of IAXO-102 and TAK-242 compared to the potential agonist effects of SN-38 at TLR4/MD-2. These types of studies may also be able to determine the specificity and selectiveness of TAK-242 and IAXO-102 with TLR4/MD-2 which have not been elucidated using the *in silico* approach.

If proven to be specific and selective, these ligands may be able to overcome the limitations of other drugs such as naloxone and amitriptyline that have been used

previously to target TLR4 in models of mucositis but have failed due to multi-target receptor actions [173, 174]. In comparison, there is some evidence that TAK-242 has multi-target actions at the cytokine receptor common subunits beta and gamma that functions as a receptor for interleukin-3, interleukin-5 and granulocyte-macrophage colony-stimulating factors, respectively [175]. Despite this, TAK-242 has less off-target actions compared to both naloxone and amitriptyline [175]. Whilst IAXO-102 has only one reported off-target action at CD-14 [54]. CD-14 is another part of the LPS signalling complex with TLR4/MD-2 that is structurally characterised by a bent solenoid typical of leucine-rich repeat (LRR) proteins with a large hydrophobic pocket and is found on the surface of many TLR4 expressing cells. Both CD-14 and MD-2 pockets share a similar topology in terms of solvent accessible surface area and volume [176]. Since IAXO-102 was designed with a similar structure to LPS as seen by the long phospholipid chains, it is able to bind to the TLR4/MD-2 complex [176]. In addition, due to CD-14's similarity to MD-2, IAXO-102 is also able to bind via TLR4/CD-14 [54] that is also capable of recognising other microbial and cellular molecular determinants such as bacteria and glycans, in addition to LPS [54]. The major difference between MD-2 and CD-14 is the polarity of the rim which may allow MD-2 to be more selective than CD-14 in the recognition of LPS [177], suggesting that IAXO-102 is similarly more selectively recognised by TLR4/MD-2 compared to TLR4/CD-14. However, since both TLR4/MD-2 and TLR4/CD-14 utilise the same downstream signalling pathway, any off-target action with CD-14 may ultimately be beneficial.

A limitation of our study was that there were no available crystal structure poses and binding scores of the ligands IAXO-102, TAK-242 and SN-38. Therefore, comparisons between this study and others could not be made which makes validation of the docking protocol difficult. However, this study is beginning to address this knowledge gap and to allow others to continue with further research.

Conclusion

In summary, this study evaluated the potential binding sites and affinity of IAXO-102, TAK-242 and SN-38 to the human TLR4-MD-2 complex, identifying specific amino acid residues of interaction and 3D structural analysis. The evidence presented here supports further investigation of the binding activity of IAXO-102 and TAK-242 for their potential application in the prevention of GI toxicity caused by irinotecan and its toxic metabolite, SN-38.

Chapter 4: Investigation of TLR4 antagonists for prevention of intestinal inflammation

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Contribution to the Paper	Planned, researched and wrote the first draft of paper. Edited subsequent drafts.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/04/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Introduction: Activation of toll-like receptor 4 (TLR4) has been shown to be a major influence on the inflammatory signalling pathways in gastrointestinal mucositis (GIM), as demonstrated by TLR4 knock-out mice. Pharmacological TLR4 inhibition has thus been postulated as a potential new therapeutic approach for the treatment of GIM but specific TLR4 inhibitors have yet to be investigated. As such, we aimed to determine whether direct TLR4 antagonism prevents inflammation in pre-clinical experimental models of GIM.

Methods: The non-competitive and competitive TLR4 inhibitors, TAK-242 (10 μM) and IAXO-102 (10 μM), respectively, or vehicle were added to human T84, HT-29 and U937 cell lines and mouse colonic explants 1h before the addition of lipopolysaccharide (LPS) (*in vitro*: 100 μg/mL; *ex vivo*: 10 μg/mL), SN-38 (*in vitro*: 1 μM or 1 nM; *ex vivo*: 2 μM) and/or tumour necrosis factor-alpha (TNF-α) (5 μg/mL). Supernatant was collected for human IL-8 and mouse IL-6 enzyme-linked immunosorbent assays (ELISAs), as a measure of inflammatory signalling. Cell viability was measured using XTT assays. Explant tissue was used in histopathological and RT-PCR analysis for genes of interest; TLR4, MD2, CD14, MyD88, IL-6, IL-6R, CXCL2, CXCR1, CXCR2.

Results: SN-38 increased cytostasis compared to vehicle ($P < 0.0001$). However, this was not prevented by either antagonist ($P > 0.05$) in any of the 3 cell lines. Quantitative histological assessment scores showed no differences between vehicle and treatment groups ($P > 0.05$). There were no differences in *in vitro* IL-8 ($P > 0.05$, in all 3 cells lines) and *ex vivo* IL-6 ($P > 0.05$) concentrations between vehicle and treatment groups. Transcript expression of all genes was similar across vehicle and treatment groups ($P > 0.05$).

Conclusions: TLR4 antagonism using specific inhibitors TAK-242 and IAXO-102 was not effective at blocking GIM in these pre-clinical models of mucositis. This work indicates that specific epithelial inhibition of TLR4 with these compounds is insufficient to manage mucositis-related inflammation. Rather, TLR4 signalling through immune cells may be a more important target to prevent GIM.

Introduction

Toll-like receptors (TLRs) are an important class of pattern recognition receptors of the innate immune system and are expressed on a variety of immune cells (macrophages, dendritic cells) and non-immune cells (epithelial cells) in the intestine [178-181]. Each TLR family member contains a ligand-specific extracellular domain and conserved intracellular domain, which allows highly selective responses to intestinal environmental stimuli, including homeostatic, pathogenic, and damage-associated signals [182, 183]. However, TLRs can also amplify immune responses under stress conditions which leads to chronic inflammation [184, 185].

TLR4, the best studied TLR family member in the context of infection and inflammation, is primarily beneficial to the intestine as it induces an inflammatory response to provide protection from invading bacteria and promotes mucosal integrity [100]. However, TLR4 can also be overexpressed in chronic inflammatory conditions such as inflammatory bowel disease (IBD), whereby people with ulcerative colitis (UC) have a 2.3-fold increase ($P = 0.02$) and people with Crohn's disease (CD) have a 1.7-fold increase ($P = 0.04$), compared to people who have normal colonic mucosal tissue [94]. Signal transmission mediated by the upregulation of TLR4 promotes the sustained release of pro-inflammatory cytokines [186] (e.g. interleukin-1 beta (IL-1B), IL-6 and tumour necrosis factor-alpha (TNF-α)). This in turn, develops and persists as intestinal inflammation, and has also been associated with risk of inflammation-associated colon cancer [187, 188].

The pathobiology of acute intestinal inflammation as seen in gastrointestinal mucositis (GIM) in people with cancer following chemotherapy with irinotecan has also been linked to the activation of TLR4. In GIM, TLR4 activation upregulates the pro-inflammatory cytokines, TNF-α and IL-6 [189]. This occurs via a downstream signalling

pathway whereby chemotherapeutic agents cause direct injury to the intestinal epithelial cells, allowing the luminal antigens to enter the lamina propria. Lipopolysaccharides (LPS), or endotoxins, are a product of luminal antigens, which activate TLR4 expressed on the intestinal epithelial layer and mucosa-associated immune cells when the luminal antigens cross over the damaged epithelial layer [28]. Subsequently, causing inflammation and ulceration. Ulceration then leads to enhanced translocation of luminal contents and increases the risk of bacteraemia in immunocompromised patients [28]. A previous study has shown that the genetic deletion of TLR4 renders mice resistant to chemotherapy-induced mucositis [42]. However, due to limitations of genetically modified animals in research translation, research efforts are now targeted at tailoring methods of inhibiting TLR4 pharmacologically.

Currently, TLR4 antagonists are being investigated for their potential in treating inflammatory-based diseases such as sepsis and arthritis [57, 190]. TAK-242 is a small-molecule TLR4 inhibitor that interferes with the down-stream signalling mediated by the CD-14/TLR4 complex without directly inhibiting the binding of LPS to TLR4 [191]. It had previously undergone clinical trials as a treatment for severe sepsis [192]. While IAXO-102 is a synthetic glycolipid that modulates TLR4 activation and signalling by interfering selectively with the TLR4 co-receptors CD-14 and MD-2 [193]. IAXO-102 has only been used in experimental studies in abdominal aortic aneurysms [55]. There is a significant lack in studies using these antagonists (such as TAK-242 and IAXO-102) in IBDs such as GIM. This study therefore aimed to investigate the potential of the TLR4 antagonists, TAK-242 and IAXO-102, to attenuate intestinal inflammation using *in vitro* and *ex vivo* models.

Materials and Methods

Chemicals

TLR4 antagonists TAK-242 (Sapphire Bioscience, Australia) and IAXO-102 (Innaxon, UK) and TLR4 agonists and inflammatory mediators, LPS O55:B5 (Sigma-Aldrich, USA), SN-38 (Tocris Bioscience, United Kingdom) and TNF-α (Research and Diagnostic Systems, United States), were reconstituted according to manufacturer's instructions for *in vitro* and *ex vivo* experiments: TAK-242: DMSO; IAXO-102: DMSO and ethanol; LPS: sterile MilliQ water; SN-38: DMSO; TNF-α: sterile PBS and 0.1% bovine serum albumin.

In vitro human cell culture

Human colorectal adenocarcinoma cell lines, T84 and HT-29, were grown in DMEM (Thermo Fisher Scientific, United States), supplemented with 10% FBS (Scientifix Pty Ltd, Australia) and 1% penicillin-streptomycin (Sigma-Aldrich) to simulate intestinal colonocytes. In contrast, the pro-monocytic, human myeloid leukaemia cell line U937 which are innate monocyte-like immune cells, were grown in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% FBS (Scientifix Pty Ltd) and 1% penicillin-streptomycin (Sigma-Aldrich). All cells were grown in 75 cm^2 flasks in a 37 °C incubator with 5% CO_2. For all experiments, cells were used between passages 3 and 20.

T84, HT-29 and U937 cell viability were evaluated using a Cell Proliferation Kit II (XTT) (Merck & Co., United States). 100 µL of suspension containing T84: 5×10^4; HT-29: 1×10^4; U937: 3×10^4 cells were seeded in 96-well plates (Corning, USA). The plates were then incubated for 24 h at 37 °C in 5% CO_2. After 24 h incubation, the medium was replaced, and the cells were treated with TAK-242 (10 µM) and IAXO-102 (10 µM) and incubated at 37 °C with CO_2 for 1 h. Cells were then treated with LPS (100 ng/mL), active metabolite of irinotecan SN-38 (T84 and HT-29: 1 µM; U937: 1 nM) and TNF-α

(5 µg/mL) and incubated at 37 °C with CO_2 for 36 h. Concentrations of all treatments used as well as incubations times were from previous [55, 61] and extensive dose finding studies (supplementary data). DMSO (0.1%) was used as the vehicle treatment. After the 36 h incubation period, the media was replaced with 100 µL media and 50 µL of XTT solution (composed of 5 mL XTT labelling reagent and 100 µL of electron coupling reagent). The plate was then incubated for 4 h at 37 °C with 5% CO_2. Then, the cell viability was measured using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek, Vermont, United States) at 490 nm. Percentage (%) of cell cytostasis was calculated using the following equation:

$$\text{Cytostasis (\%)} = \left[\frac{\text{A490 Vehicle} - \text{A490 Treated}}{\text{A490 Vehicle}}\right] \text{X } 100$$

Ex vivo culture of mouse colonic explants

The study was approved by the University of Adelaide Animal Ethics Committee and complied with the National Health and Research Council Australia Code of Practice for Animal Care in Research and Training (2013). 12 wild-type C57Bl6 mice (Animal Resource Centre, Australia) were culled via CO_2 inhalation and cervical dislocation. The entire gastrointestinal tract was removed, and the colons were flushed with chilled 1 × phosphate buffered saline (PBS) (Thermo Fisher Scientific) to remove contents. The colon was then divided into 9 equal pieces and stored in chilled 1 x PBS. Each piece was cut longitudinally along the mesentery line, flattened onto a piece of manila paper and placed lumen side down in a 24-well plate (Corning) containing RPMI (400 µL) media and stored in an incubator at 37 °C at 5% CO_2 for 10 m to equilibrate. Tissue was pre-treated with TAK-242 (10 µM) and IAXO-102 (10 µM) for 1 h before the administration of LPS (100 ng/ml) and SN-38 (2 µM) for 3 h at 37 °C at 5% CO_2. Concentrations of all treatments used as well as incubation times were taken from the *in vitro* study. DMSO was used as the vehicle treatment. Following treatment, all

explant supernatant was collected and stored at -20 °C for enzyme-linked immunosorbent assays (ELISAs). In addition, the explant tissue was either fixed in 10% neutral buffered formalin (ChemSupply Australia Pty Ltd, Australia) for 24 h, transferred to 70% ethanol (ChemSupply Australia Pty Ltd) and embedded in paraffin wax (ChemSupply Australia Pty Ltd) for histopathological analysis, or immediately snap-frozen in liquid nitrogen and stored at -80 °C for real-time polymerase chain reaction (RT-PCR) analysis.

Histopathological analysis of distal colonic explant tissue

Haematoxylin and eosin (H&E) staining was performed using 5 μm sections of the embedded explant tissue, cut on a rotary microtome and mounted onto glass microscope slides (Thermo Fisher Scientific). Slides were scanned and assessed (100 × magnification) using a NanoZoomer 2.0-HT slide scanner (Hamamatsu Photonics, Shizuoka Pref., Japan). All slides underwent quantitative histopathological assessment to generate an injury score. The histological criteria used in the assessment were as follows: epithelia disruption; crypt loss; crypt abscesses; goblet cell loss; oedema; submucosal thickening; muscularis externa thickening; and polynuclear cell infiltration [194]. Each parameter was scored as: 0 = absent; 1 = mild; 2 = moderate; or 3 = severe, with a possible maximum score of 24.

Immunohistochemistry assessment of cellular markers of apoptosis and proliferation.

Immunohistochemistry (IHC) was carried out on 5 μm sections of explant tissue, cut on a rotary microtome and mounted onto FLEX IHC microscope slides (Flex Plus Detection System, Dako; #K8020). IHC analysis was performed for caspase-3 (Abcam; #ab4051), a marker of apoptosis, and Ki67 (Abcam; #ab16667), a marker of proliferation. Changes in both parameters are validated markers for altered tissue kinetics and an excellent way to assess the subclinical severity of toxicity [195]. IHC

analysis was performed using Dako reagents on an automated machine (AutostainerPlus, Dako; #AS480) following standard protocols supplied by the manufacturer. Briefly, sections were deparrafinised in xylene and rehydrated through graded ethanols before undergoing heat-mediated antigen retrieval using an EDTA/Tris buffer (0.37 g/L EDTA, 1.21 g/L Tris; pH 9.0). Retrieval buffer was preheated to 65°C using the Dako PT LINK (pretreatment module; Dako; #PT101). Slides were immersed in the buffer, and the temperature was raised to 97°C for 20 minutes. After returning to 65°C, slides were removed and placed in the Dako AutostainerPlus (Dako; #AS480) and stained following manufacturer's guidelines. Negative controls had the primary antibody omitted. Slides were scanned using the NanoZoomer (Hamamatsu Photonics) and assessed with NanoZoomer Digital Pathology software view.2 (Histalim). The criteria used in the assessment were as follows according to percentage (%) of area positively stained for either Ki67 or Caspase 3: 0 - 25% = 0; 26% - 50% = 1; 51% - 75% = 2; 76% - 100% = 3.

RT-PCR of human cell culture and colonic explant tissue

RNA was isolated from T84, HT-29, U937 and snap frozen colonic intestinal explant tissue using the NucleoSpin RNA Plus kit (Scientifix Pty Ltd, Victoria, Australia) following the manufacturer′s protocol. RNA was quantified using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek, Vermont, United States) and reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, California, United States) according to the manufacturer′s protocol. cDNA was quantified using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek, Vermont, United States) and diluted to a working concentration of 100 ng/µL. Primers for genes of interest were designed using web-based primer design program, PRIMER 3 (v. 0.4.0) [196, 197] and manufactured by Sigma-Aldrich (Missouri, United states). A list of all the primers used is shown in Table

4.1. Amplified transcripts were detected by SYBR Green (Quantitect, Qiagen Pty Ltd., Victoria, Australia) in a Rotor-Gene Q Series Rotary Cycler (Qiagen Pty Ltd., Victoria, Australia). All reactions were completed in triplicate. Fold change in mRNA expression was calculated using the $2^{(\text{delta CT})}$ ($2^{\Delta Ct}$) method using GAPDH as the housekeeper gene [198].

Table 4.1: RT-PCR primer sequences designed by PRIMER 3, version 0.4.0.

Mouse TLR4	Forward: 5‘-CTC TGC CTT CAC TAC AGA GAC-3’ Reverse: 5’-TGG ATG ATG TTG GCA GCA ATG-3’
Mouse MD2	Forward: 5‘-GTC CGA TGG TCT TCC TGG CGA GT-3’ Reverse: 5’-GCT TCT CAG ATT CAG TCA ATA TGG G-3’
Mouse CD14	Forward: 5‘-GTC AGG AAC TCT GGC TTT GC-3’ Reverse: 5’-GGC TTT TAC CCA CTG AAC CA-3’
Mouse MyD88	Forward: 5‘-GGA GCC AGA TTC TCT GAT GC-3’ Reverse: 5’-TGT CCC AAA GGA AAC ACA CA-3’
Mouse IL-6	Forward: 5‘-AGT TGC CTT CTT GGG ACT GA-3’ Reverse: 5’-TCC ACG ATT TCC CAG AGA AC-3’
Mouse IL-6 Receptor	Forward: 5‘-TGA ATG ATG ACC CCA GGC AC-3’ Reverse: 5’-ACA CCC ATC CGC TCT CTA CT-3’
Mouse CXCL2	Forward: 5‘-AAG TTT GCC TTG ACC CTG AA-3’ Reverse: 5’-AGG CAC ATC AGG TAC GAT CC-3’
Mouse CXCR1	Forward: 5‘-GGG TGA AGC CAC AAC AGA TT-3’ Reverse: 5’-GCA GAC CAG CAT AGT GAG CA-3’
Mouse CXCR2	Forward: 5‘-GCA GAG GAT GGC CTA GTC AG-3’ Reverse: 5’-TCC ACC TAC TCC CAT TCC TG-3’
Mouse GAPDH (housekeeper)	Forward: 5‘-CCT CGT CCC GTA GAC AAA ATG-3’ Reverse: 5’-TCT CCA CTT TGC CAC TGC AA-3’

ELISAs of human cell culture and mouse colonic explant supernatants

Human IL-8 production was measured in cell culture supernatant using an ELISA kit (Abcam, Cambridge, United Kingdom) following the manufacturer's instructions. Mouse IL-6 production was measured in intestinal explant culture supernatant using an ELISA kit (Invitrogen, Massachusetts, United States) following the manufacturer's instructions. Absorbance was measured at 450 nm using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek, USA). Absorbance output was calculated and converted into protein concentration using a standard curve from the ELISA kit (IL-8: 1000 – 31.25 pg/mL; IL-6: 500 – 4 pg/mL) and GraphPad Prism Software version 9.0 (GraphPad® Software, USA).

Statistical Analysis

Data was graphed and analysed using GrahPad Prism Software 9.0 (GraphPad® Software, San Diego, USA). A Kruskal-Wallis test with Dunn’s multiple comparisons test was performed on non-parametric data to compare between the treatment groups. An ordinary one-way ANOVA with Tukey's multiple comparisons test was performed on parametric data to compare between the treatment groups. Any data point that had a higher value than 3 times the standard deviation from the mean was excluded. P-values of < 0.05 were considered statistically significant.

Results

Effect of TAK-242 and IAXO-102 treatment on cell viability (cytostasis)

Since a hallmark feature of GIM leading to inflammation is cell loss, we measured cytostasis in three different cell lines: T84, HT-29 and U937. The TLR4 antagonists, TAK-242 (10 μM) and IAXO-102 (10 μM), alone did not cause cytostasis ($P > 0.05$, Figure 4.1). However, they also did not provide protection against cytostasis following treatments with LPS (100 μg/mL), TNF-α (5 μg/mL) and SN-38 treatment (T84 and HT-29: 1 μM; U937: 1 nM) in any of the cell lines ($P > 0.05$, Figure 4.1).

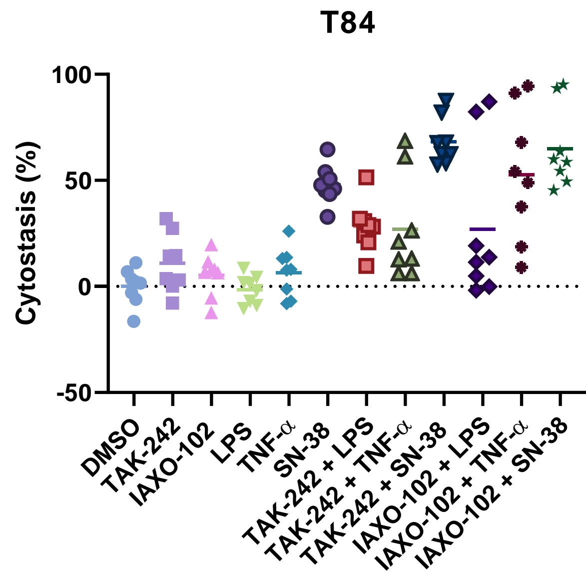
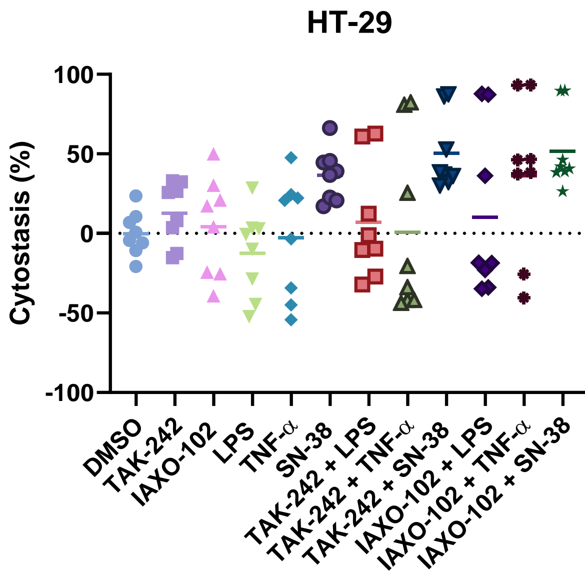
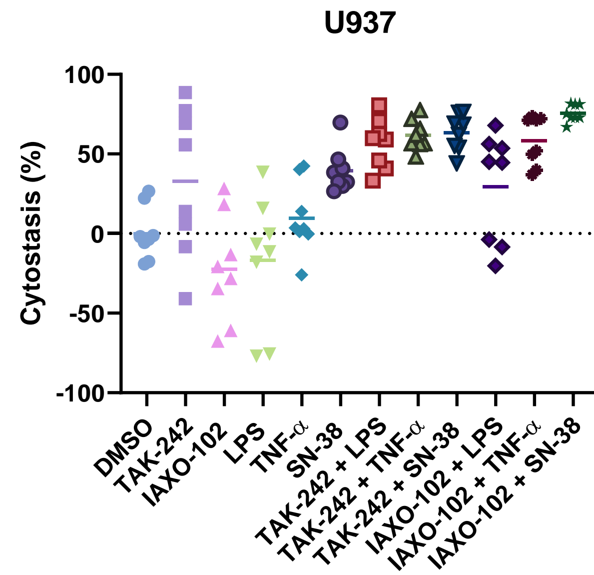

T84
Cytostasis (%)
100
50
0
-50
DMSO
TAK-242
IAXO-102
LPS
TNF-α
SN-38
TAK-242 + LPS
TAK-242 + TNF-α
TAK-242 + SN-38
IAXO-102 + LPS
IAXO-102 + TNF-α
IAXO-102 + SN-38

HT-29
Cytostasis (%)
100
50
0
-50
-100
DMSO
TAK-242
IAXO-102
LPS
TNF-α
SN-38
TAK-242 + LPS
TAK-242 + TNF-α
TAK-242 + SN-38
IAXO-102 + LPS
IAXO-102 + TNF-α
IAXO-102 + SN-38

U937
Cytostasis (%)
100
50
0
-50
-100
DMSO
TAK-242
IAXO-102
LPS
TNF-α
SN-38
TAK-242 + LPS
TAK-242 + TNF-α
TAK-242 + SN-38
IAXO-102 + LPS
IAXO-102 + TNF-α
IAXO-102 + SN-38

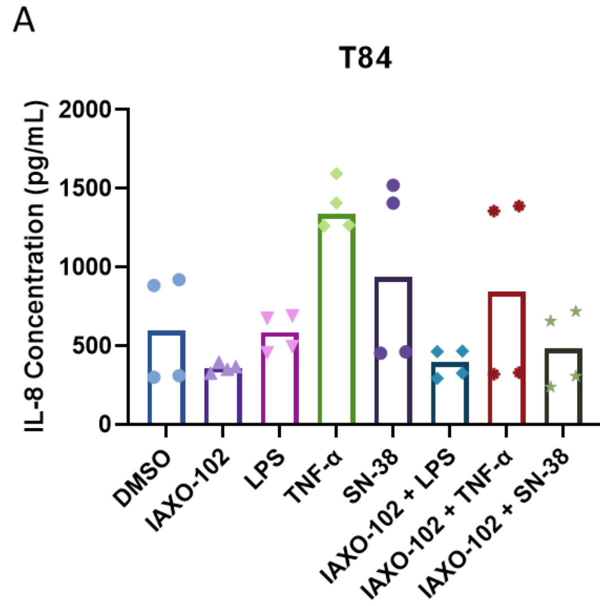
Figure 4.1: Percentage cytostasis of cells following treatment. Effect of TLR4 antagonism on cell cytostasis following LPS (100 µg/mL), TNF-α (5 µg/mL) and SN-38 treatment (T84 and HT-29: 1 µM; U937: 1 nM) in (A) T84, (B) HT-29 and (C) U937 cell lines. 0.1 % DMSO was used as the vehicle. **(A) T84**: DMSO vs SN-38 (P<0.0001), DMSO vs. TAK-242 + SN-38 (P<0.0001), DMSO vs. IAXO-102 + TNF-α (P<0.0001), DMSO vs. IAXO-102 + SN-38 (P<0.0001), TAK-242 vs. SN-38 (P<0.01), TAK-242 vs. TAK-242 + SN-38 (P<0.0001), TAK-242 vs. IAXO-102 + TNF-α (P<0.01), TAK-242 vs. IAXO-102 + SN-38 (P<0.0001), IAXO-102 vs. SN-38 (P<0.001), IAXO-102 vs. TAK-242 + SN-38 (P<0.0001), IAXO-102 vs. IAXO-102 + TNF-α (P<0.001), IAXO-102 vs. IAXO-102 + SN-38 (P<0.0001), LPS vs. SN-38 (P<0.0001), LPS vs. TAK-242 + SN-38 (P<0.0001), LPS vs. IAXO-102 + TNF-α (P<0.0001), LPS vs. IAXO-102 + SN-38 (P<0.0001), TNF-α vs. SN-38 (P<0.01), TNF-α vs. TAK-242 + SN-38 (P<0.0001), TNF-α vs. IAXO-102 + TNF-α (P<0.001), TNF-α vs. IAXO-102 + SN-38 (P<0.0001), TAK-242 + LPS vs. TAK-242 + SN-38 (P<0.01), TAK-242 + LPS vs. IAXO-102 + SN-38 (P<0.0001), TAK-242 + TNF-α vs. TAK-242 + SN-38 (P<0.01), TAK-242 + TNF-α vs. IAXO-102 + SN-38 (P<0.0001), TAK-242 + SN-38 vs. IAXO-102 + LPS (P<0.01), IAXO-102 + LPS vs. IAXO-102 + SN-38 (P<0.0001). **(B) HT-29**: LPS vs. TAK-242 + SN-38 (P<0.05), LPS vs. IAXO-102 + SN-38 (P<0.05). **(C) U937**: DMSO vs. TAK-242 + LPS (P<0.01), DMSO vs. TAK-242 + TNF-α (P<0.001), DMSO vs. TAK-242 + SN-38 (P<0.001), DMSO vs. IAXO-102 + TNF-α (P<0.001), DMSO vs. IAXO-102 + SN-38 (P<0.0001), TAK-242 vs. IAXO-102 (P<0.01), TAK-242 vs. LPS (P<0.05), IAXO-102 vs. SN-38 (P<0.001), IAXO-102 vs. TAK-242 + LPS (P<0.0001), IAXO-102 vs. TAK-242 + TNF-α (P<0.0001), IAXO-102 vs. TAK-242 + SN-38 (P<0.0001), IAXO-102 vs. IAXO-102 + LPS (P<0.01), IAXO-102 vs. IAXO-102 + TNF-α (P<0.0001), IAXO-102 vs. IAXO-102 + SN-38 (P<0.0001), LPS vs. SN-38 (P<0.01), LPS vs. TAK-242 + LPS (P<0.0001), LPS vs. TAK-242 + TNF-α (P<0.0001), LPS vs. TAK-242 + SN-38 (P<0.0001), LPS vs. IAXO-102 + LPS (P<0.05), LPS vs. IAXO-102 + TNF-α

($P<0.0001$), LPS vs. IAXO-102 + SN-38 ($P<0.0001$), TNF-α vs. TAK-242 + LPS ($P<0.05$), TNF-α vs. TAK-242 + TNF-α ($P<0.01$), TNF-α vs. TAK-242 + SN-38 ($P<0.01$), TNF-α vs. IAXO-102 + TNF-α ($P<0.05$), TNF-α vs. IAXO-102 + SN-38 ($P<0.001$), IAXO-102 + LPS vs. IAXO-102 + SN-38 ($P<0.05$). Data are presented as mean (n = 8 per group).

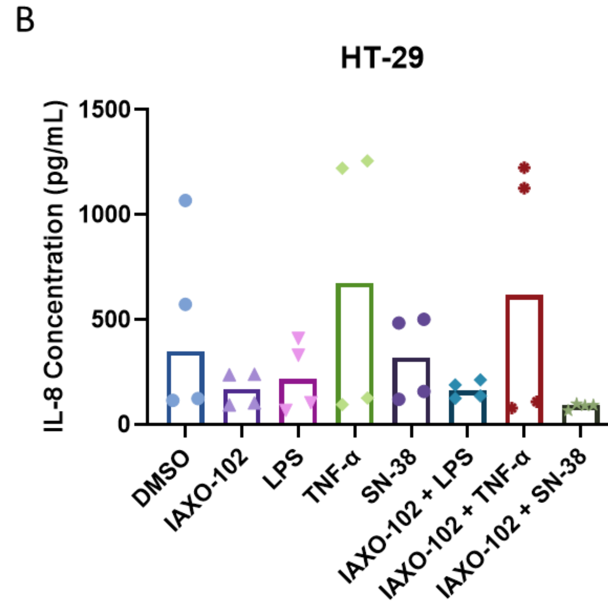
Effect of IAXO-102 treatment on IL-8 production

In human models of inflammation, IL-8 is a key downstream cytokine released following TLR4 activation. As such, IL-8 secretion was tested in intestinal and immune cell lines. All 3 cell lines produced an IL-8 secretory response following treatment (Figure 4.2). Due to the similarity observed in the cytostasis results of all 3 cell lines, we proceeded to focus only on IAXO-102 due to its novel aspect compared to TAK-242 which is already a popular research compound. We found the TLR4 antagonist IAXO-102 (10 μM), alone did not cause an IL-8 secretory response ($P > 0.05$, Figure 4.2). However, they also did not suppress any IL-8 secretory responses following treatments with LPS (100 μg/mL), TNF-α (5 μg/mL) and SN-38 treatment (T84 and HT-29: 1 μM; U937: 1 nM) in any of the cell lines ($P > 0.05$, Figure 4.2).

A



B



C

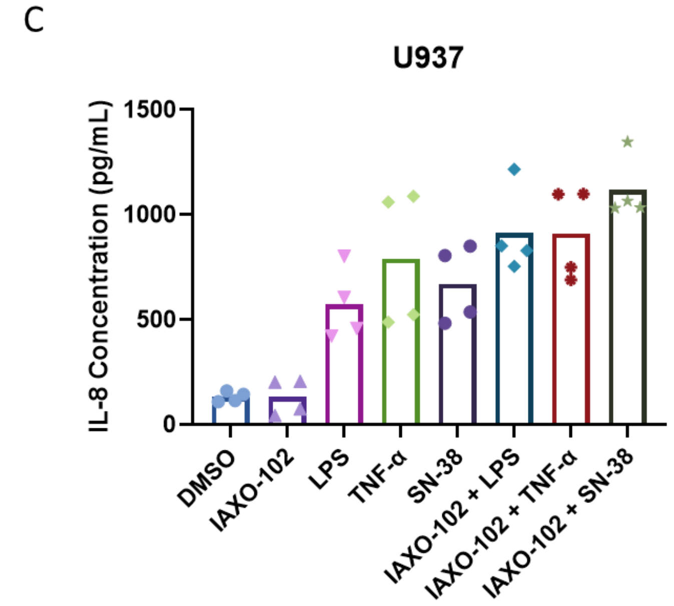
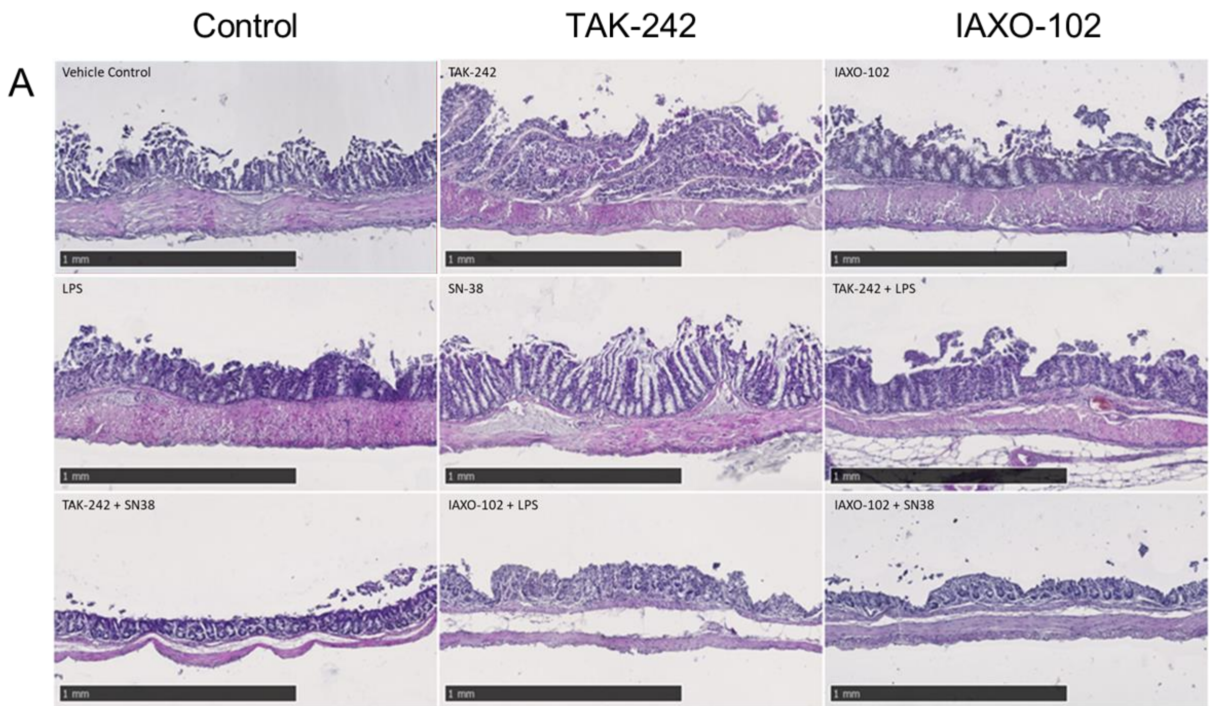


Figure 4.2: IL-8 concentration in cell supernatant following treatment. Effect of the TLR4 antagonist IAXO-102 (10 μM) on suppression of IL-8 secretion following LPS (100 μg/mL), TNF-α (5 μg/mL) and SN-38 treatment (T84 and HT-29: 1 μM; U937: 1 nM) in (A) T84, (B) HT-29 and (C) U937 cell lines. 0.1 % DMSO was used as the vehicle. There was no significant difference in IL-8 secretion between the treated and vehicle groups in the T84 and HT-29 cell lines ($P>0.05$). (C) U937: DMSO vs. TNF-α ($P<0.01$), DMSO vs. SN-38 ($P<0.05$), DMSO vs. IAXO-102 + LPS ($P<0.001$), DMSO vs. IAXO-102 + TNF-α ($P<0.001$), DMSO vs. IAXO-102 + SN-38 ($P<0.0001$), IAXO-102 vs. TNF-α ($P<0.01$), IAXO-102 vs. SN-38 ($P<0.05$), IAXO-102 vs. IAXO-102 + LPS ($P<0.001$), IAXO-102 vs. IAXO-102 + TNF-α ($P<0.001$), IAXO-102 vs. IAXO-102 + SN-38 ($P<0.0001$), LPS vs. IAXO-102 + SN-38 ($P<0.01$), SN-38 vs. IAXO-102 + SN-38 ($P<0.05$). Data are presented as median (T84 and HT-29) and mean (U937) ($n = 4$ per group).

Histopathological analysis of mouse colonic explants

The ability to model inflammation in single cell lines is limited, thus we adapted a colonic explant model to further examine TLR4 signalling in mucositis development. No histological changes were observed in the mouse colon explants after treatment with DMSO (0.2%), TAK-242 (10 μM), IAXO-102 (10 μM), LPS (100 μg/mL) and SN-38 (2 μM) (Figure 4.3A). All sections showed infiltration of neutrophils and disruption of the epithelial layer with no distinguishable differences observed between colon tissue treated with the TLR4 antagonists, TAK-242 and IAXO-102, or pro-inflammatory mediators (Figure 4.3A). This is supported by no differences in the quantitative histopathological scores in the colonic explants following any treatments ($P > 0.05$, Figure 4.3B).



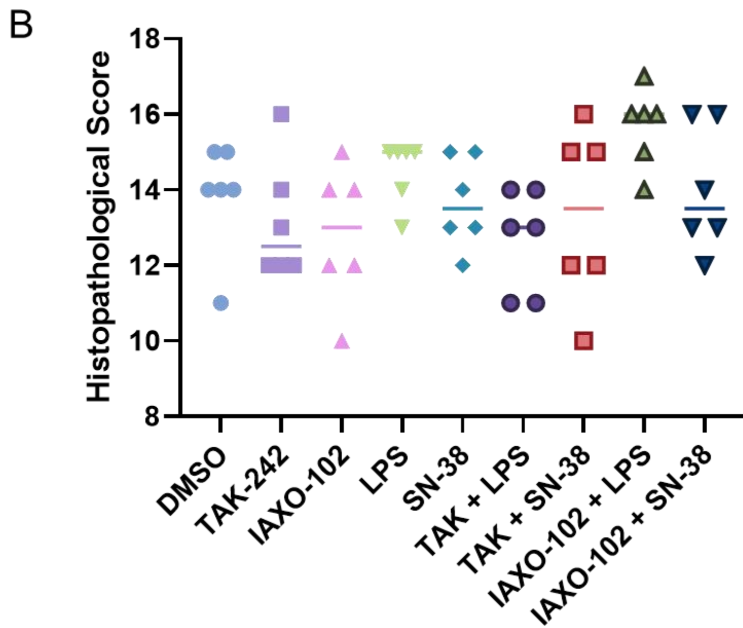
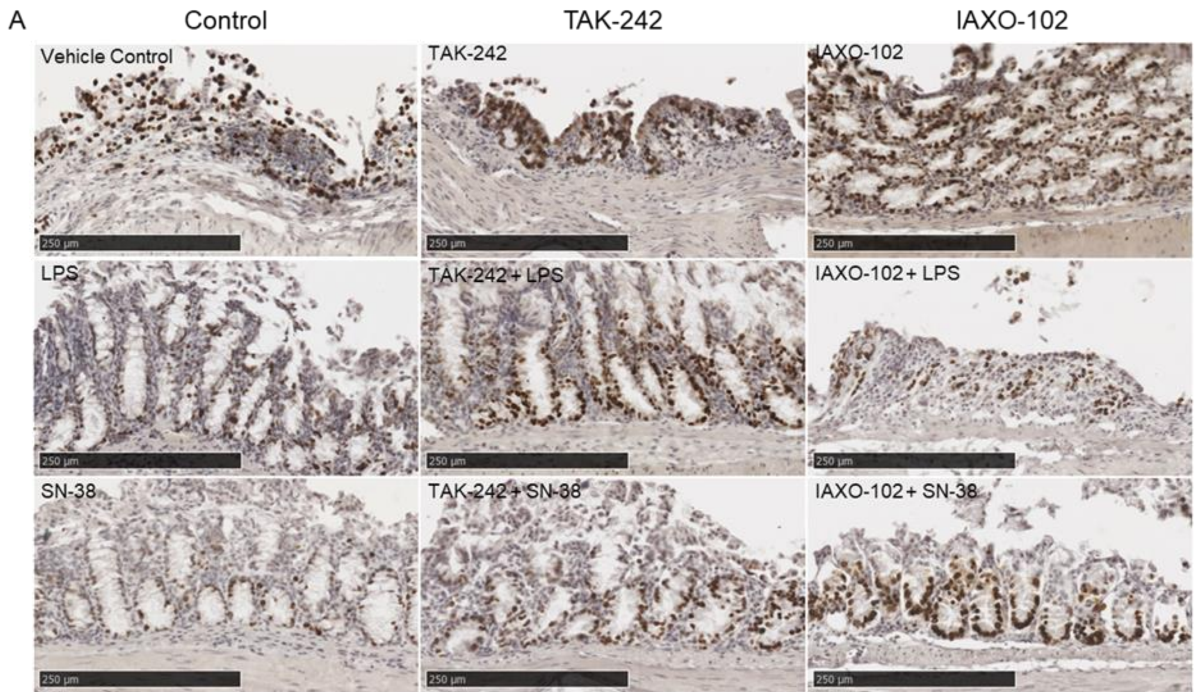


Figure 4.3: H&E staining results. (A) Representative H&E stained colonic explants following treatments. 0.2% DMSO was used as the vehicle. (B) Histopathological analysis of the H&E images from multiple mice. 0.2% DMSO was used as the vehicle. No significant differences in histopathological scores in the colonic explants between the treated and vehicle tissue was observed ($P > 0.05$). Data are presented as median ($n = 6$ per group).

Immunohistochemistry (IHC) assessment of cellular markers of apoptosis and proliferation

To follow up on the cell viability assay, Ki67 IHC staining was used to determine cell proliferation while caspase-3 IHC staining was used to determine presence of apoptotic cells in the explants treated with DMSO (0.2%), TAK-242 (10 μM), IAXO-102 (10 μM), LPS (100 μg/mL) and SN-38 (2 μM) (Figure 4.4A and 4.5A, respectively). All sections displayed widely distributed staining of Ki67 with no distinguishable differences observed between colon tissue treated with the TLR4 antagonists, TAK-242 and IAXO-102, or pro-inflammatory mediators (Figure 4.4A). This is supported by no differences in the quantitative scores in the colonic explants following any treatments ($P > 0.05$, Figure 4.4B). As for caspase-3, apoptosis was observed to be decreased in the explant tissues treated with both the antagonist and inflammatory mediator compared to explants tissues treated with either DMSO only, antagonist only or inflammatory mediator only (Figure 4.5B).



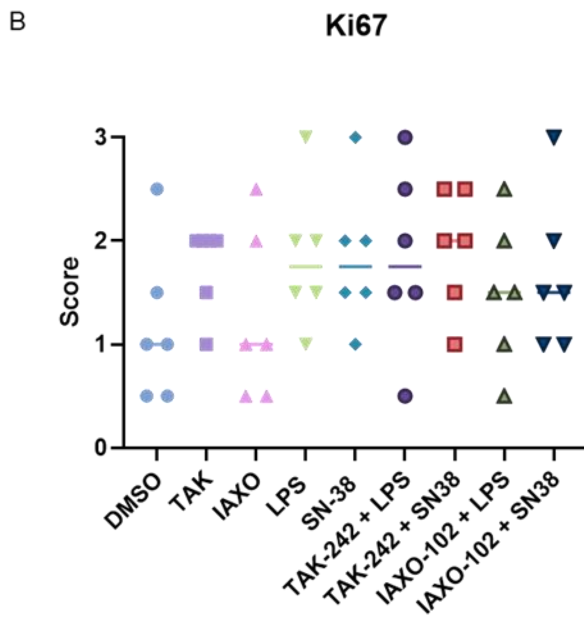
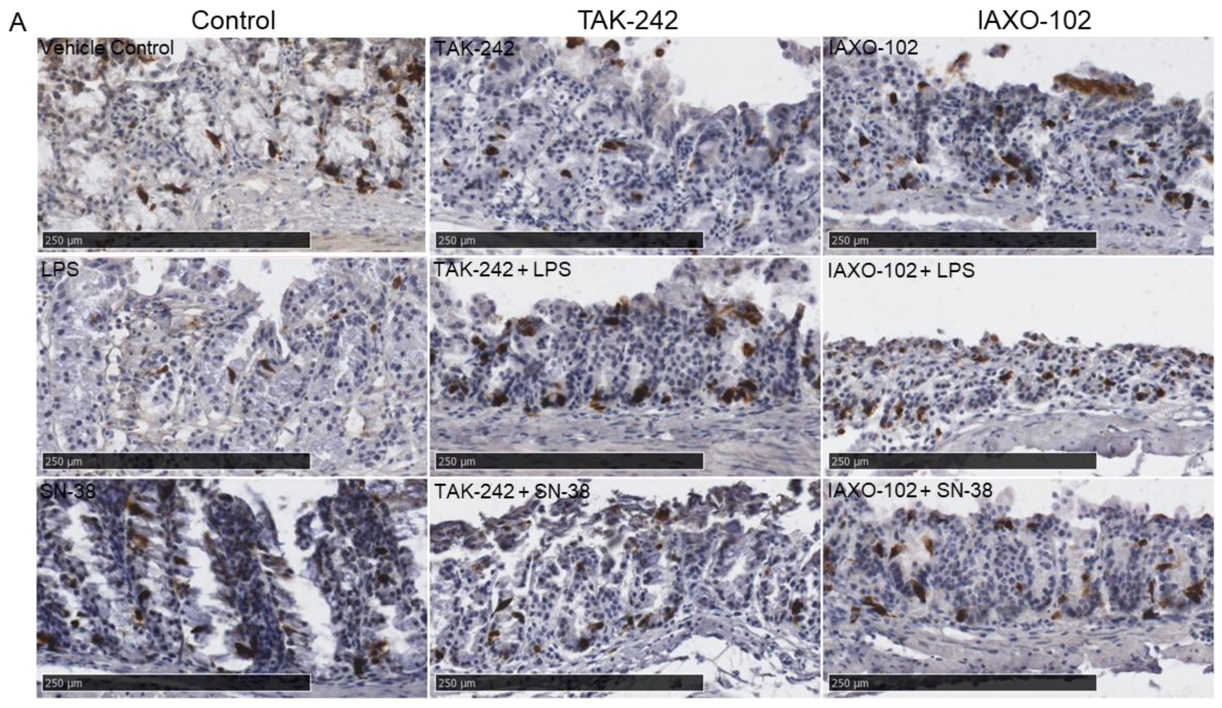


Figure 4.4: Ki67 staining results. (A) Representative images of colonic explants following treatments stained with Ki67 (brown staining). (B) Analysis and scoring of the colonic explants stained with Ki67. No significant differences in scores in the colonic explants between the treated and vehicle tissue was observed ($P > 0.05$). Data presented as median, n = 6 per group.


A
Control
TAK-242
IAXO-102
Vehicle Control
TAK-242
IAXO-102
LPS
TAK-242 + LPS
IAXO-102 + LPS
SN-38
TAK-242 + SN-38
IAXO-102 + SN-38
250 µm

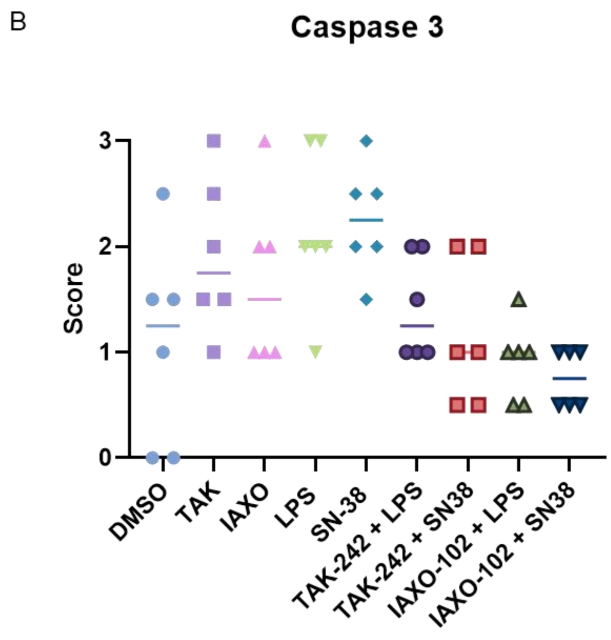

B
Caspase 3
Score
0
1
2
3
DMSO
TAK
IAXO
LPS
SN-38
TAK-242 + LPS
TAK-242 + SN38
IAXO-102 + LPS
IAXO-102 + SN38

Figure 4.5: Caspase 3 staining results. (A) Representative images of colonic explants following treatments stained with Caspase 3 (brown staining). (B) Analysis and scoring of the colonic explants stained with Caspase 3. DMSO vs TAK-242 ($P < 0.01$), DMSO vs LPS ($P < 0.0001$), DMSO vs SN-38 ($P < 0.0001$), TAK-242 vs TAK-242 + SN-38 ($P < 0.01$), TAK-242 vs IAXO-102 + LPS ($P < 0.0001$), TAK-242 vs IAXO-102 + SN-38 ($P < 0.0001$), IAXO-102 vs IAXO-102 vs LPS ($P < 0.01$), IAXO-102 vs IAXO-102 vs SN-38 ($P < 0.001$), LPS vs TAK-242 + LPS ($P < 0.01$), LPS vs TAK-242 + SN38 ($P < 0.0001$), LPS vs IAXO-102 + LPS ($P < 0.0001$), LPS vs IAXO-102 + SN38 ($P < 0.0001$), SN-38 vs TAK-242 + LPS ($P < 0.01$), SN-38 vs TAK-242 + SN38 ($P < 0.0001$), SN-38 vs IAXO-102 + LPS ($P < 0.0001$), SN-38 vs IAXO-102 + SN38 ($P < 0.0001$). 0.2% DMSO was used as the vehicle. Data presented as median, n = 6 per group. Data presented as median, n = 6 per group.

Secretion of pro-inflammatory cytokine IL-6 from mouse colonic explants

Histological visualisation is not sufficient to evaluate release of pro-inflammatory signals that may contribute to mucositis development. As such, we measured secretion of the key inflammatory cytokine linked to intestinal tissue inflammation in mucositis, IL-6. Inflammatory mediators and TLR4 agonists, LPS (100 μg/mL) and SN-38 (2 μM), did not significantly increase the IL-6 secretion in the explant media ($P > 0.05$, Figure 4.6). In addition, the TLR4 antagonists TAK-242 (10 μM) and IAXO-102 (10μM) did not cause IL-6 secretion alone or alter the secretion of IL-6 following treatments ($P > 0.05$, Figure 4.6).

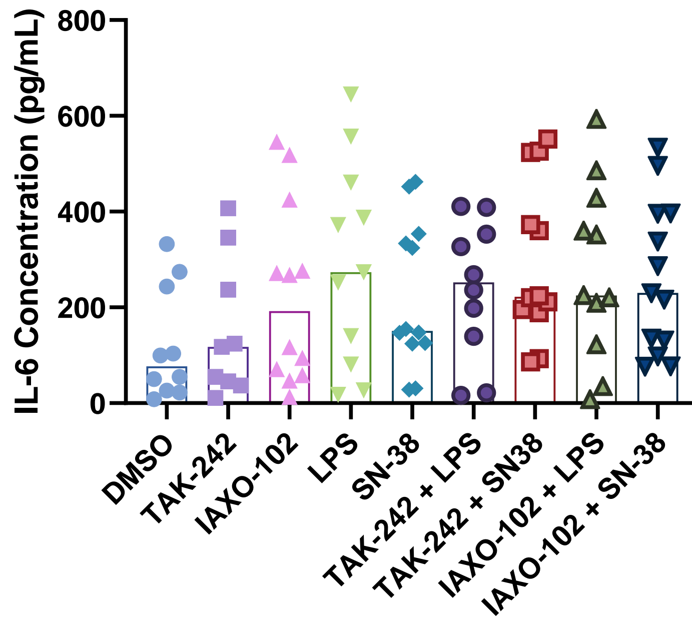


Figure 4.6: IL-6 secretion from mouse colonic explant supernatant following various treatments: DMSO (0.2%), TAK-242 (10 µM), IAXO-102 (10 µM), LPS (100 µg/mL) and SN-38 (2 µM). TAK-242 and IAXO-102 did not significantly inhibit IL-6 concentration after treatment with inflammatory mediators and TLR4 agonists LPS and SN-38 ($P > 0.05$). Data are presented as median (n = 13 per group).

Effect of TAK-242 and IAXO-102 treatment on gene expression in colonic mouse explants

We decided to look at the levels of expression of genes associated with the TLR4/MD-2 downstream signalling pathway. LPS (100 µg/mL) and SN-38 (2 µM) stimulation did not result in higher transcription levels of the associated genes TLR4, MD-2, MyD88, CD-14, IL-6, IL-6R, CXCL2, CXCR1 and CXCR2 ($P > 0.05$, Figure 4.7). In addition, there was no change observed in gene expression when the explants had been pre-treated with the TLR4 antagonists TAK-242 (10 µM) and IAXO-102 (10 µM) ($P > 0.05$, Figure 4.7).

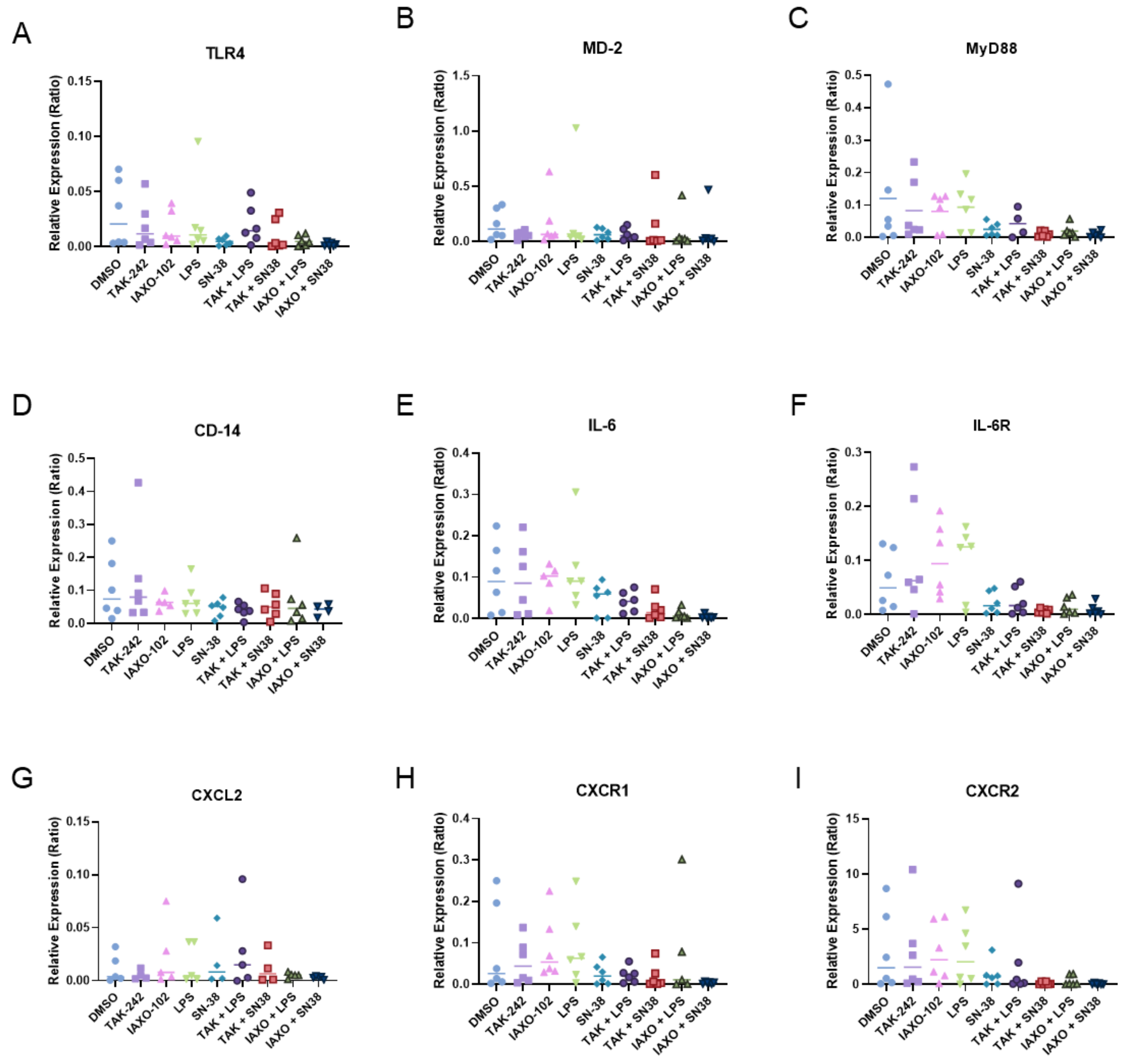

A
TLR4
Relative Expression (Ratio)
0.15
0.10
0.05
0.00
DMSO
TAK-242
IAXO-102
LPS
SN-38
TAK + LPS
TAK + SN38
IAXO + LPS
IAXO + SN38
B
MD-2
1.5
1.0
0.5
0.0
C
MyD88
0.5
0.4
0.3
0.2
0.1
0.0
D
CD-14
E
IL-6
0.4
F
IL-6R
G
CXCL2
H
CXCR1
I
CXCR2
15
10
5
0

Figure 4.7: Relative gene expression in explant tissue: (A) TLR4, (B) MD-2, (C) MyD88, (D) CD-14, (E) IL-6, (F) IL-6R, (G) CXCL2, (H) CXCR1 and (I) CXCR2 from mouse colonic explants following various treatments: TAK-242 (10 μM), IAXO-102 (10 μM), LPS (100 μg/mL) and SN-38 (2 μM). 0.2% DMSO was used as the vehicle. No significant upregulation of the genes was observed in the tissue treated with LPS and SN-38 ($P > 0.05$). No significant downregulation of the genes was observed in tissue pre-treated with TAK-242 and IAXO-102 ($P > 0.05$). Data are presented as median ($n = 6$ per group).

Discussion

GIM is defined as inflammation of the mucosa of the intestinal tract and is a side-effect of high dose chemotherapy. Patients who develop GIM will often suffer from pain, nausea and diarrhoea. These symptoms can be so debilitating that potentially life-saving treatments need to be stopped, which considerably affects their overall survival. TLR4 signalling has been strongly implicated in the development of intestinal mucositis. Evidence from *in vitro* studies using human cells and *in vivo* studies using animal models supports the hypothesis that the activation of TLR4 is related to the pathogenesis of intestinal inflammation [42, 58]. Studies have also shown evidence that when TLR4 is inhibited, there is a decrease in inflammatory infiltrate and protection against damage. For example, a study by Ungaro *et al* showed that mice with intestinal inflammation that were pre-treated with TAK-242 had a decreased disease activity index (DAI) score and IL-6 secretion ($P < 0.05$ vs disease only) [100]. A study by Fort *et al* (2005) found that the DAI and histological score was significantly decreased ($P < 0.05$ vs vehicle) in mice pre-treated with a synthetic TLR4 antagonist CRX-526 (50 µg) [47].

Therefore, TLR4 activation and signalling provides a strong target for pharmacological intervention. This study therefore aimed to determine how TLR4 inhibition protects against intestinal mucositis using preclinical models of inflammation. It was the first time the specific TLR4 antagonists, TAK-242 and IAXO-102, have been investigated in such models. We observed that these TLR4 antagonists did not display any toxic side effects in the preclinical models of inflammation.

In our *in vitro model*, the cell lines T84, HT-29 and U937 had different relative expression levels of TLR4, but this did not impact their response to TLR4 antagonists. The concentration of TLR4 antagonists used in this study was determined by previous

studies using the same compounds [55, 199]. All three cell lines displayed a relatively similar reaction as seen in our results of cell cytostasis and secretion of inflammatory mediator IL-8. However, the TLR4 antagonists were not able to prevent any inflammation or cell death induced by LPS, TNF-α and SN-38. Although it was observed that the TLR4 antagonists did not protect against SN-38 in our cell cytostasis results, this is consistent with studies showing that TLR4 is required for healing in colitis [100].

When we observed the results from the human IL-8 ELISA, it was observed that all 3 cell lines secreted the inflammatory cytokine after being treated with LPS, TNF-α and SN-38. However, when the cell lines with LPS, TNF-α and SN-38 were with pre-treated with IAXO-102, there was no decrease in IL-8 observed. Compared to a study by a study by Huggins *et al* (2015) stimulated human umbilical cord vein endothelial cells (HUVEC) with LPS (100 ng/mL) with and without pre-treatment of IAXO-102 (1 and 10 μM), with IAXO-102 (10 μM) inhibiting the secretion of IL-8 ($P < 0.01$ against LPS only) [55].

One possible reason for the contradictory *in vitro* results is that the intestinal epithelium, i.e. the T84 and HT-29 cells, must remain mute to the presence of commensal flora and bacterial pathogens to avoid a constant need to defend the intestinal environment against invading pathogens. The colonic epithelial cell types would also be exposed heavily to LPS as they are the main protective barrier between the lumen and the lamina propria. Due to the constant exposure to LPS, these cell types may limit their response to LPS and bacterial pathogens by down-regulating the TLR4/MD2 expression [58]. Another limitation is that immortalised cells do not mimic a human intestinal tract as they are derived from human tumour cells. As such, TLR4 signalling may not reflect the healthy intestine and primary cell lines may need to be considered for future work.

This limitation was why an explant model was used in the present study, to better mimic a healthy intestinal tract system. However, similar results were observed in the explant model of intestinal inflammation as were observed in the *in vitro* cell lines; no significant protection against inflammation was observed in colonic explants treated with TLR4 antagonists and the inflammatory mediators or TLR4 agonists. This indicates that inhibition of TLR4 was not able to suppress the inflammatory response.

Possible explanations for these results may be that it was too late to inhibit TLR4 to see a significant reaction. Due to the nature of explants, inflammatory signals would have been released and cells would undergo apoptosis when the intestine was removed from the mouse. When the tissue was divided into the wells, the media would have been saturated with pro-inflammatory mediators as seen in the increase in IL-6 secretion in our colonic explant model. When the TLR4 antagonist was finally added to the tissue, a difference in cytokine secretions would be unidentifiable. Which may be why TLR4 knockout models of mice are so effective at preventing mucositis. A similar reason can be used to explain the variability seen in the gene expression of the explant tissue. Whereby due to the degradation of the colonic explants, the DNA extracted from the tissue may have been compromised.

However, previous studies using intestinal explants have been quite successful. For example, a study by Guabiraba *et al* (2014) reported no increase in the pro-inflammatory cytokine IL-33 in their colonic explants, but when SN-38 was added, there was a significant increase in the IL-33 levels ($P < 0.001$ vs vehicle) [61]. Mouse intestinal explants are a popular model to use in a variety of studies [200, 201]. However, none of these studies have provided any histology on the tissue. As such, our study extends knowledge in the field and limitation of explant tissue models. As such, whole animal studies are preferable.

Conclusion

In conclusion, the results demonstrated no protective capabilities of TLR4 antagonism against the *in vitro* and *ex vivo* intestinal inflammation models. Although T84 and HT-29 cells mimic the colonic epithelial cell phenotype in many regards, these cell lines cannot replicate all stages of colonic epithelial cell differentiation and lack key microbiomes that may be important for TLR4 signalling [202]. The explant models would also need to be refined to prevent the tissue from degrading rapidly in order to provide more consistent results or more viable, long-term models such as organoids, or other co-culture systems considered. Further investigation on the specific binding sites of these TLR4 antagonists should also be considered. Therefore, our data must be interpreted with the considerations of the inherent limitations of these systems. However, we were able to fill a knowledge gap in the explant model with our histology findings.

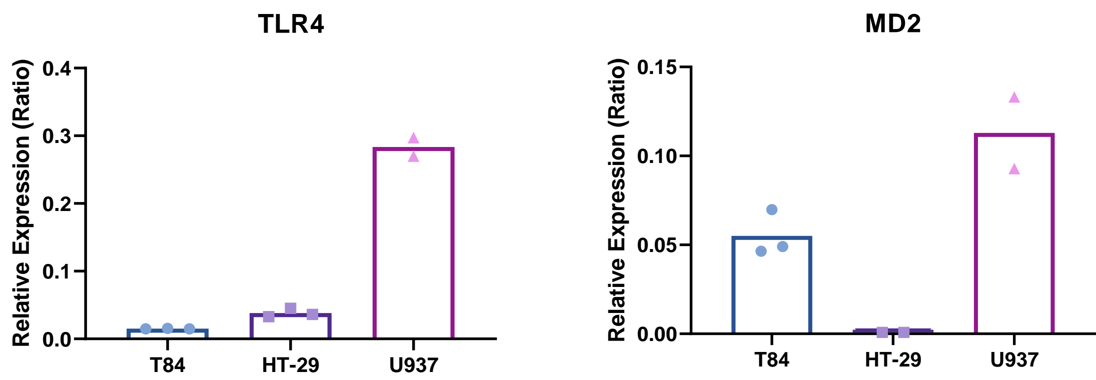
Supplementary Data

Expression of TLR4 and MD-2 genes in T84, HT-29 and U937 cell lines.

Relative expression of the genes TLR4 and MD-2 was confirmed through RT-PCR in the cell lines, T84, HT-29 and U937 (Supplementary Figure 4.1). All reactions were completed in triplicate. Fold change in mRNA expression was calculated using the $2^{(\text{delta CT})}$ ($2^{\Delta Ct}$) method using GAPDH as the housekeeper gene [198]. A list of all the primers used is shown in Supplementary Table 4.1.

Supplementary Table 4.1: RT-PCR primer sequences designed by PRIMER 3, version 0.4.0.

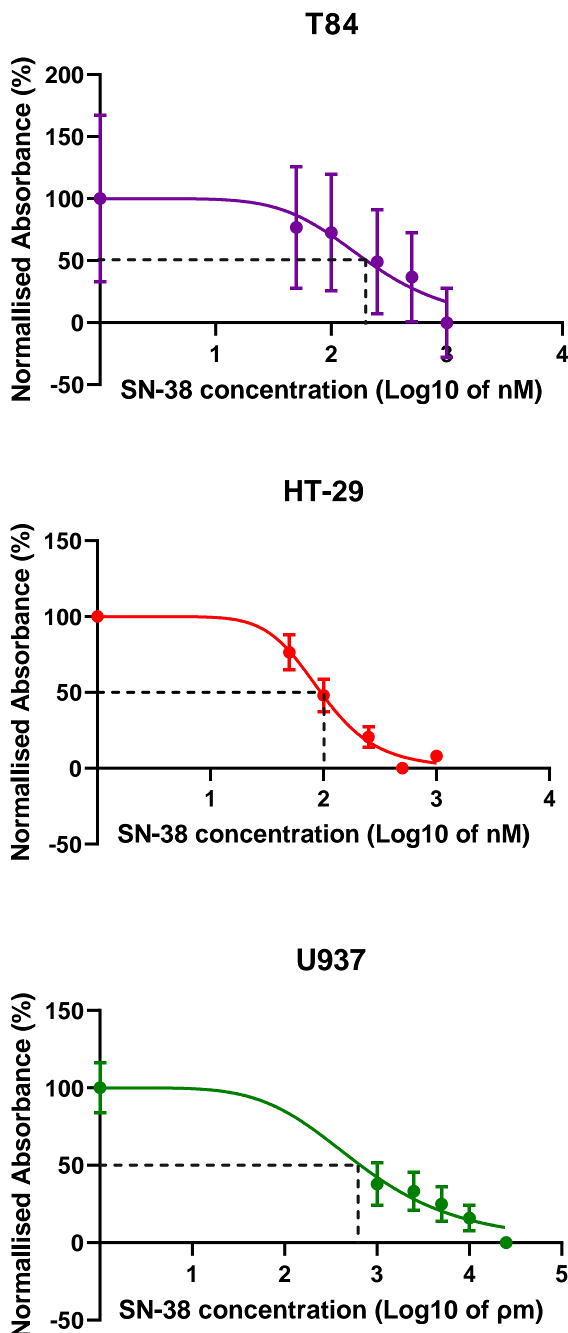
Human TLR4	Forward: 5‘-TGA GCA GTC GTG CTG GTA TC-3’ Reverse: 5’-CAG GGC TTT TCT GAG TCG TC-3’
Human MD-2	Forward: 5‘-ATT GGG TCT GCA ACT CAT CC-3’ Reverse: 5’-CGC TTT GGA AGA TTC ATG GT-3’
Human GAPDH (housekeeper)	Forward: 5‘-CTC TCT GCT CCT CCT GTT CGA C-3’ Reverse: 5’-TGA GCG ATC TGG CTC GGC T-3’



Supplementary Figure 4.1: Relative gene expression in cell lines: T84, HT-29 and U937 cell lines. Data presented as mean (n = 3).

IC_{50} of SN-38 for the cell lines T84, HT-29 and U937

The IC_{50} of SN-38 for T84 and HT-29 was 1 µM, and for U937 was 1 nM as seen in Supplementary Figure 4.2.



Supplementary Figure 4.2: IC_{50} graphs of SN-38 for cell lines: T84, HT-29 and U937. Data presented as mean ± SEM. IC_{50} for T84 and HT-29 was 1 µM. IC_{50} for U937 was 1 nM.

Chapter 5: Effects of a novel Toll-like receptor 4 antagonist IAXO-102 in a murine model of chemotherapy-induced gastrointestinal toxicity.

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Statement of Authorship

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Publication Details	Tam JSY, Crame EE, Elz AS, Coller JK, Wignall A, Prestidge CA, and Bowen JM (2022) Effects of a novel toll-like receptor 4 antagonist IAXO-102 in a murine model of chemotherapy-induced gastrointestinal toxicity. Cancer Chemotherapy and Pharmacology 10.1007/s00280-022-04463-x

Principal Author

Name of Principal Author (Candidate)	Shu Yie Janine Tam		
Contribution to the Paper	Conceived and designed the analysis; Conducted the animal study; Collected data; performed analysis; wrote the paper.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	10/04/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Helped conduct the animal study		
Signature		Date	11/04/2022

Name of Co-Author	Aurelia Elz		
Contribution to the Paper	Helped conduct the animal study		
Signature		Date	11/4/2022

Name of Co-Author	Janet Coller		
Contribution to the Paper	Aided in interpreting the results; Provided critical feedback and helped shape the research.		
Signature		Date	12/4/2022

Name of Co-Author	Anthony Wignall		
Contribution to the Paper	Performed analysis.		
Signature		Date	11/4/2022

Name of Co-Author	Prof Clive Prestidge		
Contribution to the Paper	Provided critical feedback and helped shape the research.		
Signature		Date	

Name of Co-Author	Prof Joanne Bowen		
Contribution to the Paper	Conceived the original idea; Aided in interpreting the results; Provided critical feedback and helped shape the research		
Signature		Date	11/04/2022

Please cut and paste additional co-author panels here as required.

Abstract

Introduction: Gastrointestinal mucositis (GIM) is a side effect of high-dose irinotecan (CPT-11), causing debilitating symptoms that are often poorly managed. The role of TLR4 in the development of GIM has been clearly demonstrated. We therefore aimed to investigate the potential of the TLR4 antagonist, IAXO-102, to attenuate gastrointestinal inflammation as well as supress tumour activity in a colorectal-tumour bearing mouse model of GIM induced by CPT-11.

Methods: 24 C57BL/6 mice received a vehicle, daily i.p. IAXO-102 (3 mg/kg), i.p. CPT-11 (270 mg/kg) or a combination of CPT-11 and IAXO-102. GIM was assessed using validated clinical markers. At 72 h, colon and tumour tissue were collected and examined for histopathological changes and RT-PCR for genes of interest; TLR4, MD-2, CD-14, MyD88, IL-6, IL-6R, CXCL2, CXCR1, and CXCR2.

Results: IAXO-102 prevented diarrhoea in mice treated with CPT-11. Tumour volume in IAXO-102 treated mice was lower compared to vehicle at 48 h ($P < 0.05$). There were no differences observed in colon and tumour weights between the treatment groups. CPT-11 treated mice had a lower spleen weight compared to IAXO-102 mice ($P < 0.01$). Mice who received the combination treatment had improved tissue injury score ($P < 0.05$) in the colon but did not show any improvements in cell proliferation or apoptotic rate. Expression of all genes was similar across all treatment groups in the tumour ($P > 0.05$). In the colon, there was a difference in transcript expression in vehicle vs. IAXO-102 ($P < 0.05$) and CPT-11 vs. combination ($P < 0.01$) in MD-2 and IL-6R, respectively.

Conclusion: IAXO-102 was able to attenuate symptomatic parameters of GIM induced by CPT-11 as well as reduce tissue injury in the colon. However, there was no effect on cell proliferation and apoptosis. As such, TLR4 activation plays a partial

role in GIM development but further research is required to understand the specific inflammatory signals underpinning tissue-level changes.

Introduction

Gastrointestinal mucositis (GIM) is a difficult to manage complication of cancer treatment characterised by inflammation of the mucosa of the intestinal tract that leads to immunological, functional, and structural changes [113]. It is a common side effect of high-dose irinotecan (CPT-11) and remains one of the most debilitating side effects of cancer treatment despite decades of research. GIM has also been known to cause other symptoms such as pain, nausea, vomiting and diarrhoea [203]. These symptoms significantly reduce patient quality of life, as well as survival, as GIM can negatively impact tolerance of chemotherapy which leads to discontinuation or de-escalation of treatment.

The pathobiology of acute intestinal inflammation as seen in GIM following CPT-11 has been linked to the activation of innate immune receptor, TLR4. In GIM, TLR4 activation upregulates pro-inflammatory cytokines TNF-α and IL-6 [113]. This occurs via a downstream signalling pathway whereby CPT-11 causes direct injury to the intestinal epithelial cells, allowing the luminal antigens to enter the lamina propria. Antigen-derived lipopolysaccharides (LPS), or endotoxins, then activate TLR4 expressed on the basal membrane of epithelial cells and mucosa-associated immune cells [28]. Subsequently, these interactions lead to inflammation and eventual ulceration. Ulceration then leads to enhanced translocation of luminal contents and increases the risk of bacteraemia in immunocompromised patients [28]. Previous studies have shown that the genetic deletion of TLR4 had contradicting results in different strains. It either renders mice resistant or enhances CPT-11-induced GIM in BALB/c vs C57Bl/6, respectively [42, 204]. A study by Boeing *et al* reported that the colon of wild-type mice treated with CPT-11 displayed an increase in histoarchitecture loss, inflammatory infiltrate and the presence of cryptitis compared to the colon of vehicle treated mice [205]. Mice that are germ-free, thus lacking LPS signals are also

protected from CPT-11 GI injury [206]. However, due to limitations of genetically modified animals in research translation, research efforts are now targeted at tailoring methods of inhibiting TLR4 pharmacologically to confirm its role in GIM.

Previous experiments have also shown that TLR4 expression by tumour cells can be a contributing factor that promotes tumour cell proliferation, survival, migration, and metastasis [207]. Research has shown that tumours activated the suppression of T-cell and natural killer cell activity, but when TLR4 was inhibited, this tumour-mediated suppression of T-cell and natural killer cells was prevented, which delayed tumour growth and increased survival of the tumour bearing mice [208]. Another study showed LPS stimulation of the TLR4/MD-2 complex can activate downstream signalling pathways that promotes the adhesiveness and metastatic capacity of colorectal cancer (CRC) cells [51]. These findings have shown the impact TLR4 has in CRC progression. While TLR4 activation can increase tumour growth and immunosuppression, it can also promote anti-tumour activity. For example, a study has shown that TLR4 expressed on dendritic cells plays an important role in promoting anti-tumour immune responses following chemotherapy [41].

Any treatment that modifies TLR4 signalling may have protective effects for the intestine while also increasing anti-tumour activity during chemotherapy. However, there has yet to be a specific TLR4 antagonist used in a tumour bearing preclinical model to investigate the impact on GIM and tumour growth, simultaneously. IAXO-102 is a highly specific ligand that interferes selectively with TLR4 and its co-receptors, MD-2 and CD-14. IAXO-102 has been investigated in experimental studies of abdominal aortic aneurysms to date displaying its ability to inhibit TLR4 and subsequent downstream effects in an inflammatory disease [55]. This study therefore aimed to investigate the potential of IAXO-102 to attenuate gastrointestinal

inflammation as well as suppress tumour activity in a colorectal tumour-bearing mouse model of GIM induced by CPT-11.

Methods

Animal Model and Ethics

The study was approved by the University of Adelaide Animal Ethics Committee (M-2021-033) and complied with the National Health and Research Council Australia (Australia) Code of Practice for Animal Care in Research and Training (2013) [209]. Mice were group housed in ventilated cages (n = 3-6 mice/cage) with a 12 h light/dark cycle, while food and water were provided *ad libitum*.

Experimental Design

All mice were on a C57BL/6 background. Female and male mice (n_{total} = 24) weighing between 15 and 25 g (6-13 weeks of age) were bred in the University of Adelaide Laboratory Animal Service (SA, Australia). Mice were subcutaneously transplanted in the right flank with MC-38 cells, a murine colon adenocarcinoma cell line derived from C57BL/6 mice. MC-38 cells were kindly provided by A/Professor Michele Teng of the Cancer Immunoregulation and Immunotherapy Laboratory, QIMR Berghofer Medical Research Institute, Australia. When tumour growth reached approximately 0.2 cm^3, the mice were treated with either of the following: 3 days of daily 3 mg/kg intraperitoneal (i.p.) dose of the TLR4 antagonist IAXO-102 (MedChemExpress, USA) in a diluent of 10% EtOH, 40% PEG400, 5% Tween-80 and 45% saline; a single 270 mg/kg i.p. dose of CPT-11 (kindly provided by Pharmacia/Pfizer, USA) prepared in a sortibol/lactic acid buffer (45 mg/mL sorbitol/0.9 mg/mL lactic acid; pH 3.4; Sigma-Aldrich, USA); the combination of CPT-11 and IAXO-102; or the sorbitol/lactic acid buffer only (vehicle mice). Mice were randomly assigned to treatment groups and culled by cervical

dislocation at 72 h after being anaesthetised by isoflurane inhalation (1 L/min O_2 with 4% isofluorane). The study timeline is shown in Figure 5.1A.

Clinical assessment of intestinal toxicity

Mice were weighed daily to track weight loss/gain. All mice were monitored twice daily for the presence of diarrhoea (scored as present or absent) and other clinical parameters: ruffled coat, dehydration, hunched posture, rectal bleeding, and reluctance to move. Mice were killed if they displayed ≥ 15% weight loss or significant distress and clinical deterioration, in compliance with animal study ethical requirements.

Tissue preparation

Gastrointestinal tract: The entire gastrointestinal tract from pyloric sphincter to rectum was dissected and flushed with chilled 1 × phosphate buffered saline (PBS, pH 7.4, Thermo Fisher Scientific, USA) to remove contents. The large intestine was weighed immediately after resection. Samples (1 cm in length) of colon were collected and (i) drop-fixed using 10% neutral buffered saline for processing and embedding into paraffin wax, or (ii) snap frozen in liquid nitrogen and stored at −80°C for molecular analyses.

Histopathologic analysis

Haematoxylin and eosin (H&E) staining was performed on 5 μm sections of colon cut on a rotary microtome and mounted onto glass Menzel-Gläser Superfrost microscope slides (Thermo Fisher Scientific). Slides were scanned using the NanoZoomer (Hamamatsu Photonics, Japan) and assessed with NanoZoomer Digital Pathology software.view2 (NDP.view2, Version 2.7.39) (Hamamatsu Photonics). The occurrence of eight histological criteria in the colon was examined to generate a total tissue injury score [210]. These criteria were disruption of brush border, architectural disruption,

disruption of crypt cells, and infiltration of polymorphonuclear leukocytes cells, dilation of lymphatics and capillaries, oedema, reduction in goblet cell number and thickening of muscularis externa. Each parameter was scored as present = 1 or absent = 0 in a blinded fashion by two independent assessors (J.S.Y. Tam/A. Wignall). Concordance on all scores was confirmed between assessors.

Immunohistochemistry assessment of cellular markers of apoptosis and proliferation

Immunohistochemistry (IHC) was carried out on 5 µm sections of colon and tumour, cut on a rotary microtome and mounted onto FLEX IHC microscope slides (Agilent, USA). IHC analysis was performed for Ki67 (Abcam; #ab16667), a marker of proliferation and caspase-3 (Abcam; #ab4051), a marker of apoptosis. Changes in both parameters are validated markers for altered tissue kinetics and an excellent way to assess the subclinical severity of toxicity [195]. IHC analysis was performed using Agilent reagents on an automated machine (AutostainerPlus, Agilent) following standard protocols supplied by the manufacturer. Briefly, sections were deparaffinised in xylene and rehydrated through graded ethanols before undergoing heat-mediated antigen retrieval using an EDTA/Tris buffer (0.37 g/L EDTA, 1.21 g/L Tris; pH 9.0). Retrieval buffer was preheated to 65°C using the Dako PT LINK (pretreatment module; Agilent; #PT101). Slides were immersed in the buffer, and the temperature was raised to 97°C for 20 min. After returning to 65°C, slides were removed and placed in the Agilent AutostainerPlus (Agilent; #AS480) and stained following manufacturer's guidelines. Negative controls had the primary antibodies omitted. Slides were scanned using the NanoZoomer (Hamamatsu Photonics) and assessed with NDP.view2 software (Hamamatsu Photonics). Cell proliferation data were represented as the percentage of positively stained cells relative to total cells in the intestinal crypts. Apoptosis was quantified by counting the number of positively stained cells for 15

crypts and the data were presented as average positively stained cells per crypt. Only well-oriented, non-oblique crypts were included for analysis. A scoring system of percentage area of cells stained brown in the tissue: : 0 - 25% = 0; 26% - 50% = 1; 51% - 75% = 2; 76% - 100% = 3 was used to analyse the tumour tissue stained with Ki67 and caspase-3. Two blinded investigators (J.S.Y Tam/A. Wignall) independently scored each stained section and mean score from both investigators were calculated. Concordance was confirmed between investigators on all scoring results.

RT-PCR for markers of TLR4 signalling

RNA was isolated from snap frozen tumour and colonic tissue using the NucleoSpin® RNA Plus kit (Macherey-Nagel, Germany) following the manufacturer′s protocol. RNA was quantified using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek, USA) and reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA) according to the manufacturer′s protocol. cDNA was quantified using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek) and diluted to a working concentration of 100 ng/μL in nuclease-free water. Expression of key markers of TLR4/MD-2 downstream signalling pathway were investigated. Primers for genes of interest were designed using web-based primer design program, PRIMER 3 (v. 0.4.0) and manufactured by Sigma-Aldrich (Table 5.1). Amplified transcripts were detected by SYBR Green (Qiagen Pty Ltd., Australia) in a Rotor-Gene Q Series Rotary Cycler (Qiagen Pty Ltd.). All reactions were completed in triplicate including a non-template control to determine presence of contamination. The relative ratio of mRNA expression was calculated using $2^{\Delta Ct}$ method using β-actin as the normalising housekeeper gene [198]. β-actin has been shown to have stable expression levels across cell types and treatments [211].

Statistical Analysis

Data was graphed and analysed using GrahPad Prism Software 9.0 (GraphPad® Software, San Diego, USA). A D'Agostino and Pearson normality tests were conducted to determine if data was parametric or non-parametric. A Kruskal-Wallis test with Dunn's multiple comparisons test was performed on non-parametric data to compare between the treatment groups. A two-way ANOVA with Tukey's multiple comparisons test was performed on parametric data to compare between the treatment groups. Any data point that had a value more than 3 times the standard deviation from the mean was excluded as an outlier. P-values of < 0.05 were considered statistically significant.

Table 5.1: Mouse RT-PCR primer sequences designed by PRIMER 3 (v. 0.4.0).

TLR4	Forward: 5‘-CTC TGC CTT CAC TAC AGA GAC-3’ Reverse: 5’-TGG ATG ATG TTG GCA GCA ATG-3’
MD-2	Forward: 5‘-GTC CGA TGG TCT TCC TGG CGA GT-3’ Reverse: 5’-GCT TCT CAG ATT CAG TCA ATA TGG G-3’
CD-14	Forward: 5‘-GTC AGG AAC TCT GGC TTT GC-3’ Reverse: 5’-GGC TTT TAC CCA CTG AAC CA-3’
IL-6	Forward: 5‘-AGT TGC CTT CTT GGG ACT GA-3’ Reverse: 5’-TCC ACG ATT TCC CAG AGA AC-3’
IL-6R	Forward: 5‘-TGA ATG ATG ACC CCA GGC AC-3’ Reverse: 5’-ACA CCC ATC CGC TCT CTA CT-3’
CXCR2	Forward: 5‘-GCA GAG GAT GGC CTA GTC AG-3’ Reverse: 5’-TCC ACC TAC TCC CAT TCC TG-3’
CXCL1	Forward: 5‘-GGG TGA AGC CAC AAC AGA TT-3’ Reverse: 5’-GCA GAC CAG CAT AGT GAG CA-3’
CXCL2	Forward: 5‘-GCA GAG GAT GGC CTA GTC AG-3’ Reverse: 5’-TCC ACC TAC TCC CAT TCC TG-3’
β-actin	Forward: 5‘-CTC TTC CAG CCT TCC TTC CT-3’ Reverse: 5’-AGC ACT GTG TTG GCG TAC AG-3’

Results

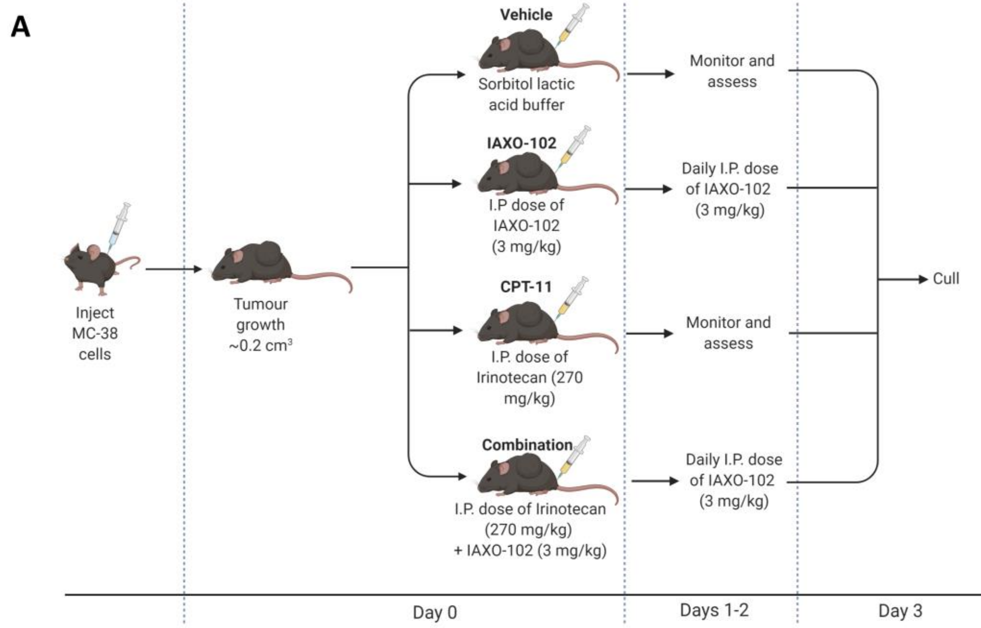
Mice treated with IAXO-102 were protected from CPT-11-induced GIM symptom of diarrhoea

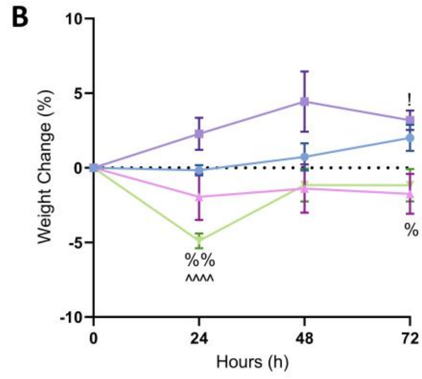
Weight loss following CPT-11 treatment was most severe at 24 h in the combination group (-4.90% ± 1.22% vs baseline) (Figure. 5.1B). The IAXO-102 group gained the most weight at 24 h (2.28% ± 2.65% vs baseline) and 48 h (4.43% ± 4.95 vs baseline). The weight loss in the combination group was different compared to the vehicle ($P < 0.0001$) at 24 h, and the IAXO-102 group at 24 h ($P <0.01$) and 72 h ($P < 0.05$). While the CPT-11 group had a difference in weight compared to the IAXO-102 group at 72 h ($P < 0.05$).

CPT-11 caused diarrhoea in 50% of mice within 6 h of administration and 100% at 24 h (Table 5.2). However, IAXO-102 treatment attenuated diarrhoea related to CPT-11 induced GIM in the combination group (Table 5.2). No diarrhoea was seen in any vehicle or IAXO-102 treated mice (data not presented).

IAXO-102 slowed colorectal tumour growth

Tumours were measured daily and expressed as a change in volume from the day of CPT-11 injection. From 24 h to 72 h, tumour volume of the vehicle group was higher compared to the CPT-11 group (24 h: $P < 0.05$; 48 and 72 h: $P < 0.0001$) and combination group (24 h: $P < 0.01$; 48 and 72 h: $P < 0.0001$) (Figure 5.1C). Tumour volume of the IAXO-102 group was different at 48 h and 72 h compared to the vehicle group (48 h and 72 h: $P < 0.05$), the CPT-11 group (48 h: $P < 0.05$; 72 h: $P < 0.05$) and the combination group (48 h: $P < 0.001$; 72 h: $P < 0.0001$) (Figure 5.1C). There were no differences in tumour volume of the CPT-11 and combination group from 24 h to 72 h (24 h and 48 h: $P = 0.99$; 72 h: $P = 0.69$) (Figure 5.1C).


A
Vehicle
Sorbitol lactic acid buffer
Monitor and assess
IAXO-102
I.P dose of IAXO-102 (3 mg/kg)
Daily I.P. dose of IAXO-102 (3 mg/kg)
Inject MC-38 cells
Tumour growth ~0.2 cm³
Cull
CPT-11
I.P. dose of Irinotecan (270 mg/kg)
Monitor and assess
Combination
I.P. dose of Irinotecan (270 mg/kg) + IAXO-102 (3 mg/kg)
Daily I.P. dose of IAXO-102 (3 mg/kg)
Day 0
Days 1-2
Day 3


B
Weight Change (%)
Hours (h)
%%
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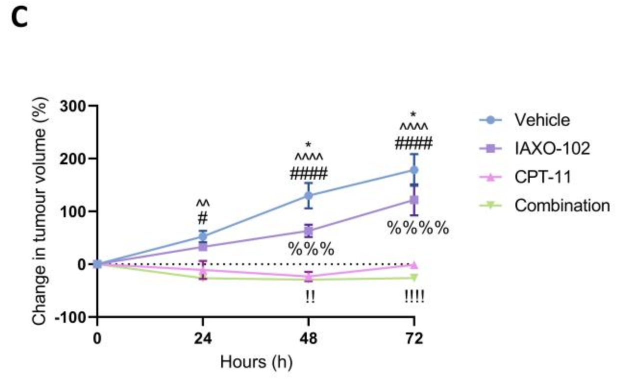
C
Change in tumour volume (%)
Hours (h)
Vehicle
IAXO-102
CPT-11
Combination
^^
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Figure 5.1: Experimental timeline and clinical assessment. (A) Experimental timeline showing the sequence of events and treatment timepoints. (B) Percentage change in weight over 72 h. Data displayed as a mean ± standard error of the mean (SEM) percentage weight change from baseline (0 h), n = 6 per group. (C) Tumour volume over 72 h. Data displayed as a mean ± SEM percentage change in tumour volume from baseline (0 h), n = 6 per group. Symbols indicate statistical significance: vehicle group vs. IAXO-102 group: * P < 0.05; vehicle group vs. CPT-11 group: # P < 0.05, #### P < 0.0001; vehicle group vs. combination group: ^^ P < 0.01, ^^^^ P < 0.0001; IAXO-102 group vs. CPT-11 group: ! P < 0.05, !! P < 0.01, !!!! P < 0.0001; IAXO-102 group vs. combination group: % P < 0.05, %% P < 0.01, %%% P < 0.001, %%%% P < 0.0001.

Table 5.2: Toxicity symptoms over 72 h. Data presented as total number of animals (per time point). Clinical parameters includes: ruffled coat, dehydration, hunched posture, rectal bleeding and reluctance to move.

Clinical Parameter	**CPT-11 (number of animals)**				**Combination (number of animals)**			
	6 h	24 h	48 h	72 h	6 h	24 h	48 h	72 h
Diarrhoea	5/6	6/6	0/6	0/6	0/6	0/6	0/6	0/6
Clinical Parameters	3/6	3/6	1/6	0/6	5/6	2/6	1/6	0/6

Mice treated with CPT-11 had a reduction in spleen weight compared to mice treated with IAXO-102

There was no difference in colon wet weights between treatment groups: vehicle vs. IAXO-102: P = 0.81; vehicle vs. CPT-11: P = 0.96; vehicle vs. combination: P=0.99; IAXO-102 vs. CPT-11: P=0.53, IAXO-102 vs. combination: P = 0.79; CPT-11 vs. combination: P = 0.99 (Figure 5.2A).

The CPT-11 group had lower spleen weights compared to the IAXO-102 group (P < 0.01) (Figure 5.2B). There were no differences in spleen weights between the other treatment groups (vehicle vs. IAXO-102, vehicle vs. combination and CPT-11 vs. combination: P > 0.99; vehicle vs. CPT-11: P = 0.15; IAXO-102 vs. combination: P = 0.25).

There were also no differences observed in tumour weights between the treatment groups (vehicle vs. IAXO-102: P = 0.91; vehicle vs. CPT-11: P = 0.55; vehicle vs. combination: P = 0.84; IAXO-102 vs. CPT-11: P = 0.22; IAXO-102 vs. combination: P = 0.47; CPT-11 vs. combination: P = 0.95) (Figure 5.2C).

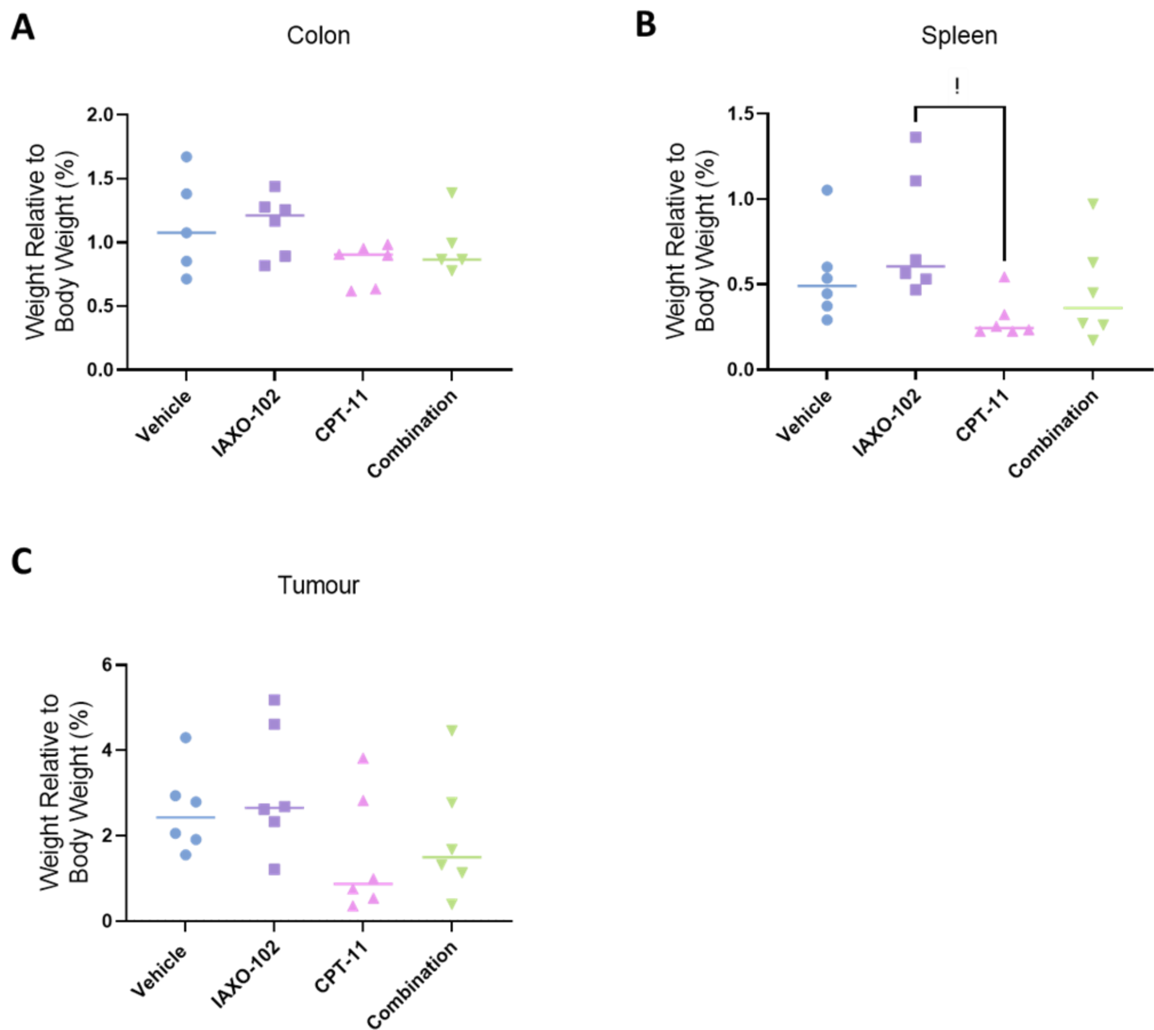


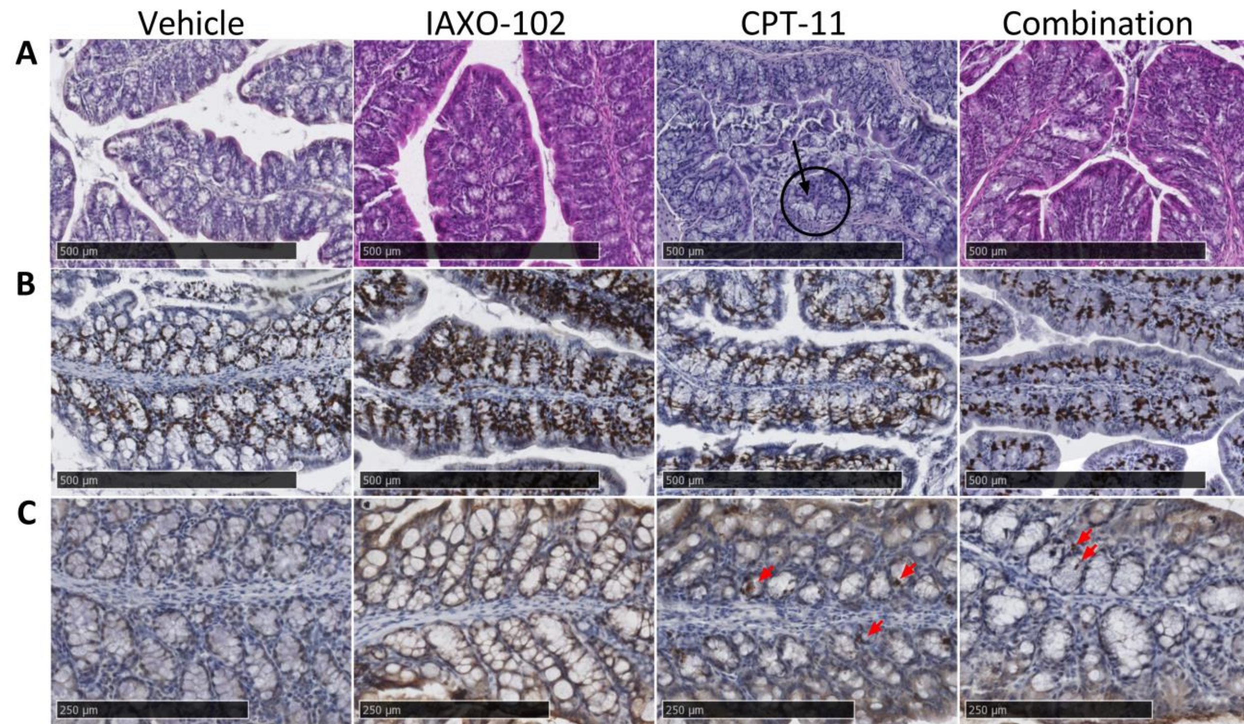
Figure 5.2: Organ wet weight of all treatment groups. (A) Colon wet weights. (B) Spleen wet weights. (C) Tumour wet weights. All data displayed as a percentage of weight relative to body weight and lines represent group median, n = 5 - 6 per group. Symbols indicate statistical significance: IAXO-102 vs. CPT-11: ! $P < 0.05$.

IAXO-102 protects against CPT-11-induced colonic histopathology independent of cell death and turnover

Representative H&E images (Figure 5.3A) show minimal damage in vehicle, IAXO-102 and combination groups. CPT-11 treatment caused epithelial disruption (black arrow) and inflammatory infiltrate (black circle). Histopathological analysis (Figure 5.3D) showed that combination treated mice were protected against CPT-11-induced mucosal tissue injury in the colon, with a lower histopathological score compared to the CPT-11 group ($P < 0.05$). The IAXO-102 group also had a difference in tissue injury score compared to the CPT-11 group ($P < 0.01$). There were no other differences observed between the groups (vehicle vs. IAXO-102 and IAXO-102 vs. combination: $P = 0.54$; vehicle vs. combination: $P > 0.99$).

Representative images show Ki67 positive cells (stained brown, Figure 5.3B). Analysis of the Ki67 images (Figure 5.3E) showed that CPT-11 group had a decrease in proliferating cells compared to vehicle and IAXO-102 groups ($P < 0.01$). Combination group also had a lower number of proliferating cells compared to vehicle and IAXO-102 groups ($P < 0.05$). There were no differences observed between the other groups (vehicle group vs. IAXO-102 group and CPT-11 group vs. combination group: $P > 0.99$).

Representative images of caspase-3 positive cells in the colonic crypts are shown (red arrow, Figure 5.3C). Analysis of the caspase-3 images (Figure 5.3F) showed that CPT-11 ($P < 0.05$) and combination ($P < 0.01$) groups had a higher apoptotic rate compared to the vehicle group. There were no other differences observed between the groups (vehicle vs. IAXO-102: $P = 0.52$; IAXO-102 vs. CPT-11: $P = 0.19$; CPT-11 vs. combination: $P = 0.83$).



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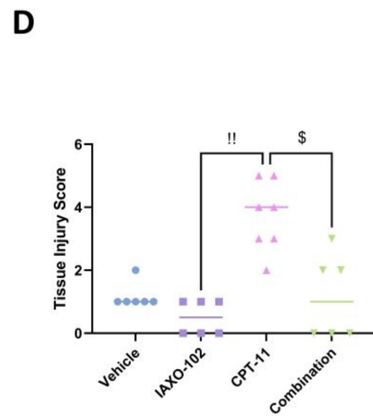
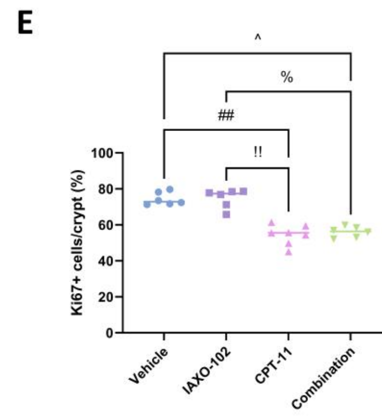


E



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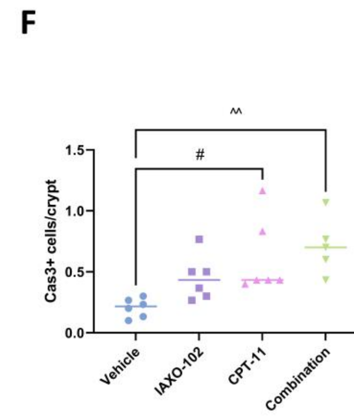

Figure 5.3: H&E and IHC staining results in the colon. (A) Representative H&E images showing epithelial disruption (black arrow) and inflammatory infiltrate (black circle). Scale bars, 1 mm. 40 x original magnification. (B) Representative immunostaining of Ki67 cells in colonic crypts. Proliferating cells are stained brown. Scale bars, 500 µm. 40 x original magnification. (C) Representative immunostaining of caspase-3 cells in colonic crypts. Apoptotic cells are stained brown (red arrow). Scale bars, 250 µm.40 x original magnification. (D) Histopathological tissue injury scores in the colon of mice. Data presented as median, n = 6 per group. (E) Percentage of Ki67 positively stained cells in the colonic crypts. Data presented as median, n = 6 per group. (F) Number of caspase-3 positively stained cells in the colonic crypts. Data presented as median, n = 5 - 6 per group. Symbols indicate statistical significance: vehicle vs. CPT-11: # P < 0.05, ## P < 0.01; vehicle vs. combination: ^ P < 0.05, ^^ P < 0.01; IAXO-102 vs. CPT-11: !! P < 0.01; IAXO-102 vs. combination: % P < 0.05; CPT-11 vs. combination: $ P < 0.05.

Tumours in mice treated with CPT-11 had a higher apoptotic score compared to mice treated with vehicle

A scoring system of percentage area of cells stained brown in the tissue: 0 = < 25%; 1 = 25%; 2 = 50%; 3 = >75% was used to analyse the tumour tissue stained with Ki67 and caspase-3.

Representative images of proliferating cells (Ki67 positive cells stained brown) in tumour tissue (Figure 5.4A) revealed no differences in scores for positively stained proliferating cells between the groups (all $P = 0.99$) (Figure 5.4C).

Representative images of apoptotic cells (caspase-3 positive cells stained brown) in tumour tissue (Figure 5.4B) revealed that the CPT-11 group had a higher score for positively stained apoptotic cells compared to the vehicle group ($P < 0.01$) (Figure 5.4D). There were no other differences observed between the other groups (vehicle vs. IAXO-102 and IAXO-102 vs. combination: $P > 0.99$; vehicle vs. combination: $P = 0.42$; IAXO-102 vs. CPT-11: $P=0.15$; CPT-11 vs. combination: $P=0.69$) (Figure 5.4D).

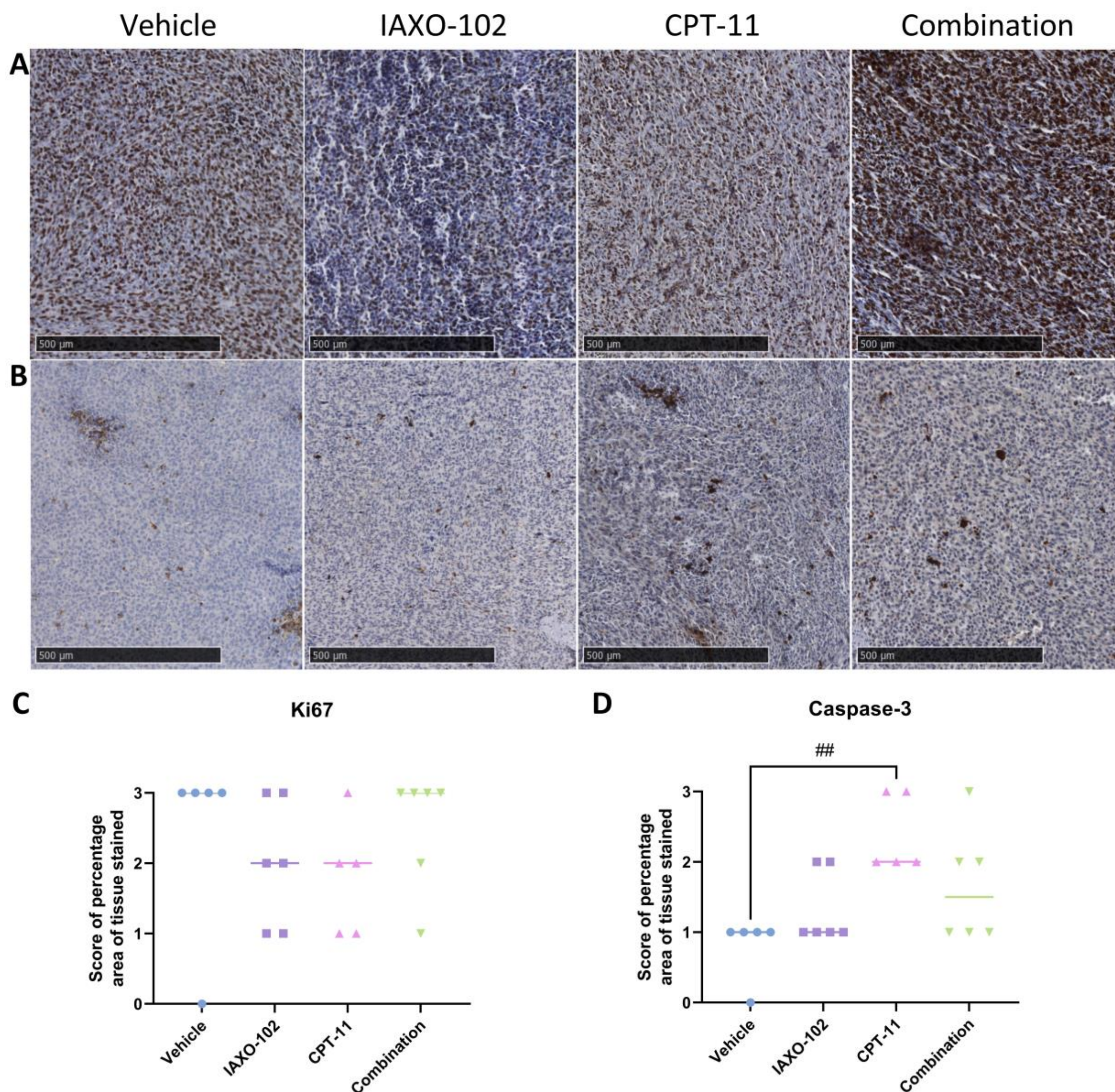

Figure 5.4: IHC staining results in the tumour. (A) Representative immunostaining of Ki67 cells in tumour tissue. Proliferating cells are stained brown. Scale bars, 500 µm. 40 x original magnification. (B) Representative immunostaining of caspase-3 cells in tumour tissue. Apoptotic cells are stained brown (red arrow). Scale bars, 500 µm. Original magnification, 40 x. (C) Analysis and scoring of tumour tissue stained with Ki67. Data presented as median, n = 5 - 6 per group. (D) Analysis and scoring of tumour tissue stained with Caspase-3. Data presented as median, n = 5 - 6 per group. Symbols indicate statistical significance: vehicle vs. CPT-11: ## $P < 0.01$.

Effect of IAXO-102 treatment on gene expression in mouse colonic tissue

There was no change in transcript levels between treatment groups for **TLR4** (Figure 5.5A, vehicle vs. IAXO-102 and vehicle vs. combination: P = 0.39; vehicle vs. CPT-1 and IAXO-102 vs. combination: P > 0.99; IAXO-102 vs. CPT-11 and CPT-11 vs. combination: P = 0.29), **CD-14** (Figure 5.5C, vehicle vs. IAXO-102, vehicle vs. CPT-11 and IAXO-102 vs. CPT-11: P>0.99; vehicle vs. combination: P=0.0639; IAXO-102 vs. combination: P = 0.07; CPT-11 vs. combination: P = 0.13) and **CXCR2** (Figure 5.5E, all groups: P > 0.99). A difference was observed in **MD-2** transcript levels between vehicle and IAXO-102 groups (P < 0.05); no other differences were observed between the groups (vehicle vs. CPT-11, vehicle vs. combination and CPT-1 vs. combination: P > 0.99; IAXO-102 vs. CPT-11: P = 0.56; IAXO-102 vs. combination: P = 0.21) (Figure 5.5B). A difference was also observed in **IL-6R** transcript levels between CPT-11 and combination groups (P < 0.01); no other differences were observed between the groups (vehicle vs. IAXO-102, vs. CPT-11, vehicle vs. CPT-11 and IAXO-102 vs. CPT-11: P > 0.99; vehicle vs. combination: P = 0.09; IAXO-102 vs. combination: P = 0.16) (Figure 5.5D). The transcript expression of IL-6, CXCL1 and CXCR1 was investigated in the colon but there was no expression in any of the treatment groups.

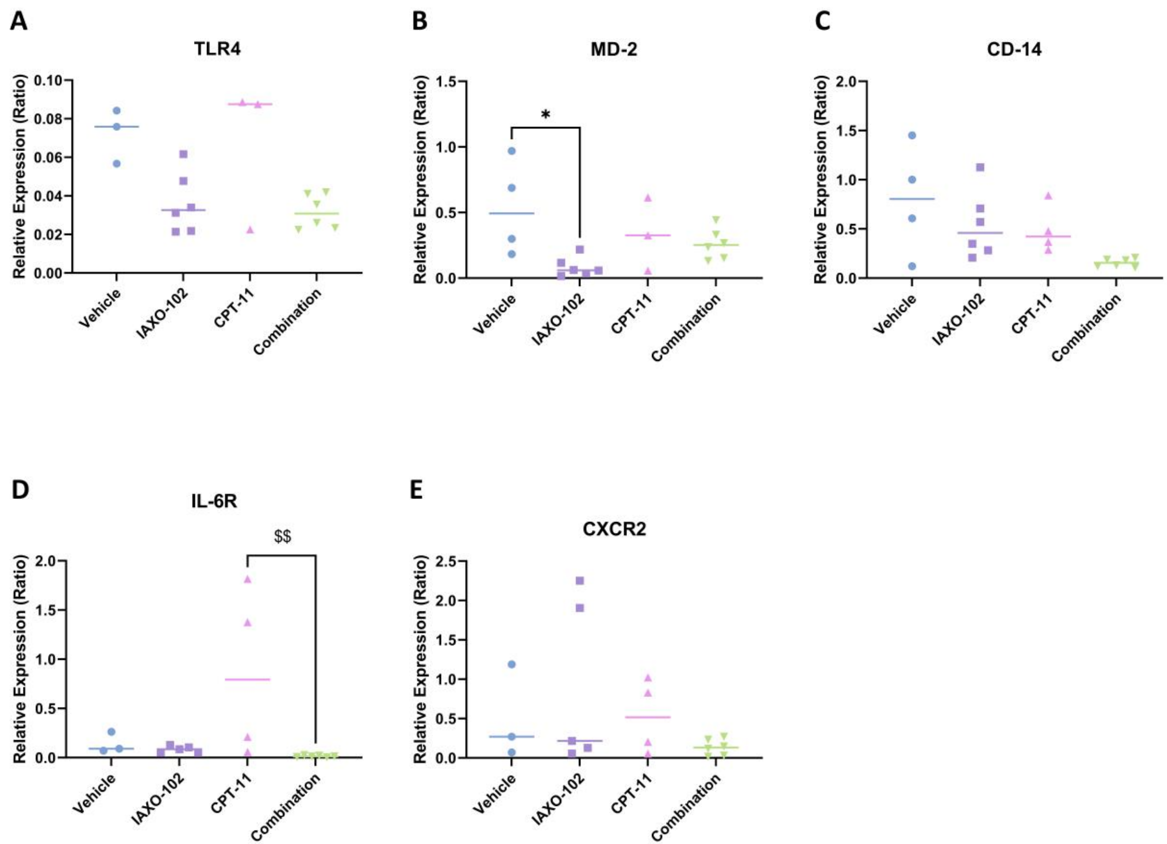

Figure 5.5: Transcript expression in the colon. (A) TLR4, (B) MD-2, (C) CD-14, (D) IL-6R, and (E) CXCR2 from colonic tissue relative to the housekeeper β-actin. Data are presented as median, n = 3 - 6 per group. Symbols indicate statistical significance: vehicle vs. IAXO-102: * P < 0.05; CPT-11 vs. combination: $$ P < 0.01.

Effect of IAXO-102 treatment on gene expression in mouse tumour tissue

Levels of transcript expression in the tumour tissue of all the groups were also analysed. There was no change in transcript levels across any groups in any of the genes of interest; **TLR4** (vehicle vs. IAXO-102: P = 0.32, vehicle vs. CPT-11: P = 0.96, vehicle vs. combination: P = 0.99, IAXO-102 vs. CPT-11: P = 0.63, IAXO-102 vs. combination: P = 0.43, CPT-11 vs. combination: P = 0.99) (Figure 5.6A); **MD-2** (vehicle vs. CPT-11, vehicle vs. combination, IAXO-102 vs. combination, CPT-11 vs. combination: P > 0.99, vehicle vs. IAXO-102: P = 0.75, IAXO-102 vs. CPT-11: P = 0.38) (Figure 5.6B); **CD-14** (Vehicle vs. CPT-11, IAXO-102 vs. combination, CPT-11 vs. combination: P > 0.99, vehicle vs. IAXO-102: P = 0.14, vehicle vs. combination: P = 0.96, IAXO-102 vs. CPT-11: P = 0.24) (Figure 5.6C); **IL-6** (vehicle vs. CPT-11, vehicle vs. combination, CPT-11 vs. combination: P > 0.99, vehicle vs. IAXO-102: P = 0.42, IAXO-102 vs. CPT-11: P = 0.22, IAXO-102 vs. combination: P = 0.63) (Figure 5.6D); **IL-6R** (vehicle vs. IAXO-102: P = 0.96; vehicle vs. CPT-11, vehicle vs. combination, IAXO-102 vs. CPT-11, IAXO-102 vs. combination, CPT-11 vs. combination: P > 0.99) (Figure 5.6E); **CXCL2** (all groups P > 0.99) (Figure 5.6F); **CXCR1** (vehicle vs. IAXO-102, vehicle vs. CPT-11, vehicle vs. combination, IAXO-102 vs. combination, CPT-11 vs. combination: P > 0.99; IAXO-102 vs. CPT-11: P= 0.81) (Figure 5.6G); **CXCR2** (vehicle vs. IAXO-102, vehicle vs. CPT-11, vehicle vs. combination, IAXO-102 vs. combination, CPT-11 vs. combination: P > 0.99; IAXO-102 vs. CPT-11: 0.47) (Figure 5.6H).

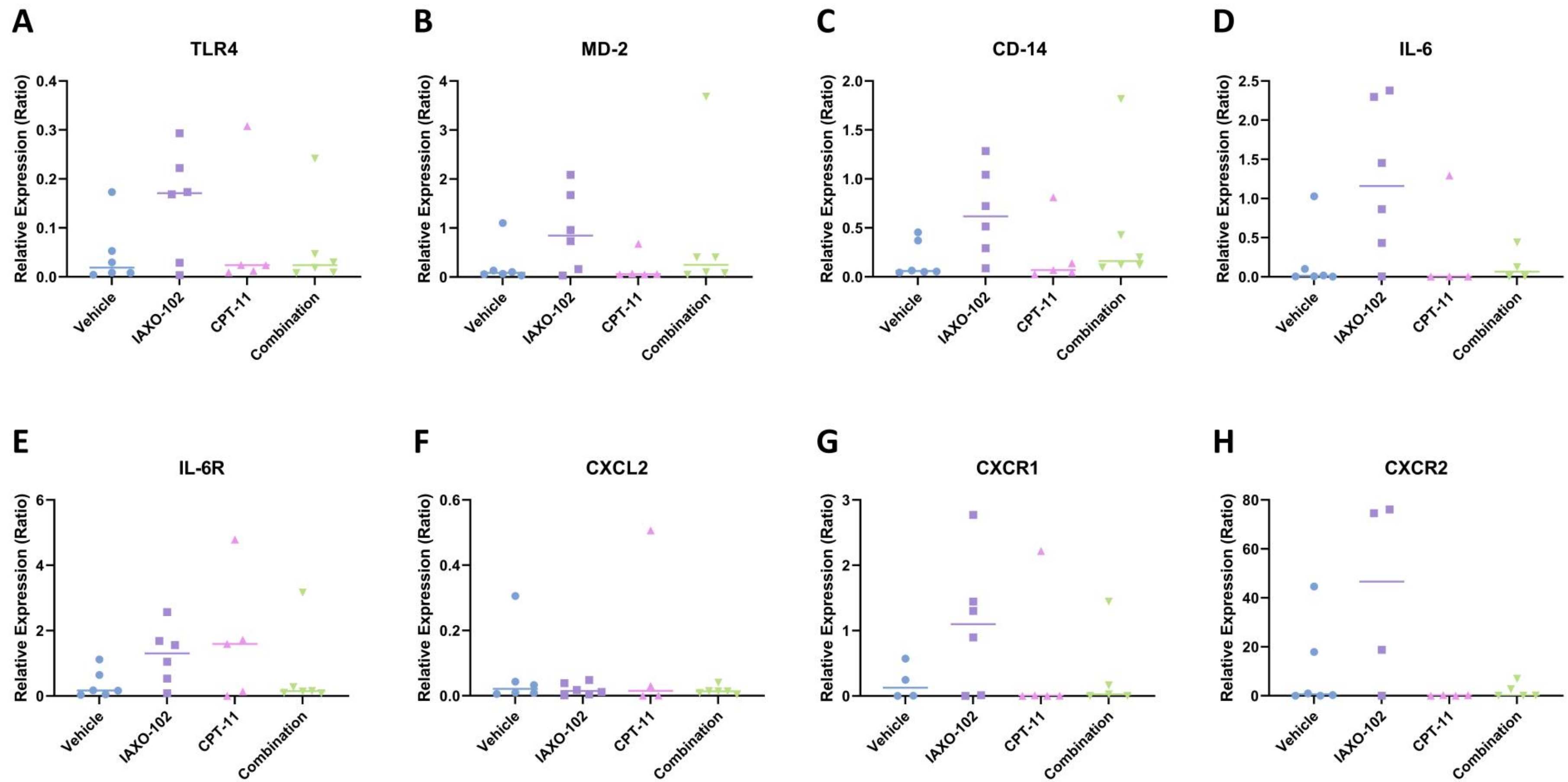

Figure 5.6: Transcript expression in the tumour. (A) TLR4, (B) MD-2, (C) CD-14, (D) IL-6, (E) IL-6R, (F) CXCL2, (G) CXCR1 and (H) CXCR2 from tumour tissue relative to the housekeeper β-actin. Data are presented as median, n = 4 - 6 per group.

Discussion

Inflammation of the mucosa of the intestinal tract during cancer treatment is known as GIM and is most severe during high-dose chemotherapy. TLR4 signalling has been strongly implicated in the development and treatment of CRC and GIM through its regulation of inflammation. This study explored how interruption of TLR4 signalling using a pharmacological intervention modulates these outcomes.

It was found that the TLR4 antagonist IAXO-102 was able to prevent diarrhoea in mice treated with CPT-11. This was associated with improved histopathological scores, indicating retention of colonic morphology and subsequent function. Work by others have shown similar protective effects using non-specific TLR4 antagonists. For example a study by Fakiha *et al* reported that amitriptyline was able to prevent CPT-11-induced diarrhoea and colonic apoptosis in rats but did not see any protective effects in histological architecture in the intestinal tract [174]. Although not using a TLR4 antagonist, a study by Wardill *et al* found that TLR4 knock-out mice were protected against CPT-11-induced mucosal tissue injury in the small intestine and also displayed a reduction in CPT-11-induced diarrhoea [42]. Another study has also shown that pharmacological inhibition of TLR4 was able to reduce disease activity and prevent morphological damage in an inflamed colon [47]. In contrast, a study using tumour bearing rats reported that naloxone did not improve GIM following CPT-11 treatment. Naloxone treatment also did not reduce any weight loss and even increased tumour growth in the rats [173]. Collectively this provides evidence that targeting TLR4 signalling interferes with development of GIM and warrants further investigation.

The mechanisms by which TLR4 inhibition protects colonic tissue and prevents diarrhoea was then further investigated using well-established tissue markers. The typical markers of CPT-11-induced injury, apoptosis and reduced proliferation of crypt epithelial cells [195], were not significantly affected by IAXO-102. The lack of

measurable change may be due to the kinetics of cell death and halting of the cell cycle following chemotherapy exposure. Previous studies have shown that apoptosis may be an early indicator of intestinal damage with rates peaking at 6 h after administration of CPT-11 [42, 174]. Although slightly slower than apoptosis, halting of the cell cycle and reduced proliferation is known to peak between 24 and 48 h after exposure to chemotherapy [174, 212, 213]. Collectively, this may account for the lack of difference between the CPT-11 and combination groups where tissue was collected at 72 h. Conversely, this lack in difference may also suggest that TLR4 downstream signalling may not play a major role in apoptosis seen in GIM, however early time points coinciding with maximal protection from diarrhoea such as 24 h would need to be investigated to confirm both possibilities. As it was observed in previous study that apoptosis was decreased after 6 h in the colon [42, 174].

TLR4 signalling in the colon has been long associated with inflammatory conditions. As such, we next investigated TLR4-realted transcripts known to play key roles in inflammatory responses. There were no differences in transcript expression of TLR4 and CD-14 in the colon between the groups. However, there was a decrease in expression of the co-receptor MD-2 in the colon of the IAXO-102 group compared to the vehicle group. These results are unexpected as previous studies have found increased TLR4 expression in the colon following chemotherapy [214, 215]. IL-6R and CXCR2 are both receptors associated with pro-inflammatory cytokines which are upregulated during inflammation [216, 217]. There was no difference between the groups in levels of CXCR2 expression, but a more interesting observation was the effect of the combination treatment on the levels of IL-6R transcript expression in the colon. A decrease in IL-6R levels was observed in the colon of the combination group compared to the CPT-11 group, which may indicate a mechanism by which IAXO-102 is protective. Il-6 has been extensively studied in chemotherapy-induced GIM [217,

218] and mice that are TLR4 knock-out lack an IL-6 response [42, 219]. Collectively this alludes to IAXO-102 protecting against GIM through TLR4-depedent IL-6 regulation.

We also wanted to test whether TLR4 antagonism modulated CRC tumour growth and response to CPT-11 in our model. In the current study, IAXO-102 treatment alone led to a lower tumour volume compared to the vehicle group. A study by Pastille *et al* has reported similar findings. They observed that by inhibiting TLR4 with an antagonist during intestinal inflammation, the development and progression of colonic tumours was significantly reduced compared to control mice [220]. They also observed a decrease in infiltration of pro-inflammatory cells and cytokines compared to control mice [220]. CPT-11 prevented tumour growth equally well in both groups. Based on the findings in the IAXO-102-alone group, it was expected that the combination group would have a significant reduction in tumours compared to CPT-11 treatment alone, but this was not observed. As such, there are clearly different roles for TLR4 during development of tumours, compared to response to chemotherapy in our model. This is supported by other work showing conflicting roles of TLR4 in clinical response to cancer treatment [173, 174].

To explore the effect of TLR4 antagonsim on CRC tumours further, markers of cell proliferation and cell death were examined in all tumours at 72 h. Regarding levels of proliferation in the tumour, there were no differences between any of the groups, as such, the ability of IAXO-102 to decrease tumour growth is not attributable to increased cell turnover. As for levels of apoptosis, only the CPT-11 group had an increase in apoptosis levels compared to vehicle group. However, the results observed in both the proliferation and apoptosis scores were quite variable, which may be due to the heterogeneity of the tumour itself [221]. Consistent with the lack of significant effect of TLR4 antagonism on cell turnover, we were also unable to confirm any changes in

inflammatory targets between the groups. Depending on where the tumour was examined, there may be differences in cellular morphology, gene expression, metabolism, and proliferation. This may be what caused the variability observed in the results and may have also affected the targeted treatments on these tumours.

While this is the first study to explore the specific TLR4 antagonist, IAXO-102, for its ability to protect against GIM in a CRC mouse model, there were limitations to the final interpretation of our findings. Statistical significance was difficult to establish in the RT-PCR analysis due to issues with the quality of cDNA which did not amplify the target genes as well as the housekeeper. Therefore, these results and numbers were not included in the analysis causing a decrease in sample size which led to difficulty in determining significance in the results. Future work to confirm these findings will also need to include additional time points of tissue collection to look for changes coinciding with peak diarrhoea and weight change, as well as allowing longer growth trajectory of the tumours. Another limitation that needs to be noted in this study is that the diluent for IAXO-102 was not used as a vehicle. This was due to the limited time and number of animals. The diluent for CPT-11 was prioritised as it was determined to be the more toxic diluent compared to the IAXO-102 diluent. Although the diluents used to reconstitute IAXO-102 can cause toxicity, it is only at high concentrations for extended periods of time [222-224]. These diluents have also been diluted with 45% saline solution which would decrease the concentration and therefore toxicity.

Conclusion

In conclusion, the results demonstrated that IAXO-102 was able to attenuate CPT-11-induced diarrhoea as well as reduce tissue injury in the colon without impacting tumour response. However, given that there was no measurable impact on apoptosis or proliferation in either the colon or tumour, alternative mechanisms must account for these observations. Our work points to a downstream role for IL-6 in mediating the

protective effects of IAXO-102, whereas other inflammatory markers were not significantly altered. Research efforts can therefore be shifted towards targeting IL-6R in order to understand its relationship with inflammation and apoptosis within the GIT.

Chapter 6: General Discussion

Summary

Gastrointestinal mucositis (GIM) is an enduring problem that plagues chemotherapy treatment. The work described in this thesis was a detailed approach to investigating a novel intervention strategy. This thesis firstly investigated the interactions of the TLR4 antagonists, IAXO-102 and TAK-242, and the active metabolite of irinotecan, SN-38 with the TLR4/MD-2 complex by *in silico* analyses of molecular docking **(Chapter 3)**. The aim of this structural study was to provide a better understanding of the recognition and potential binding of these compounds which could contribute to the downstream analysis and development of therapeutic anti-GIM drugs that target TLR4 in the GI tract. The next step after these findings was to test both TLR4 antagonists' ability to inhibit the TLR4/MD-2 complex in an *in vitro* and *ex vivo* model of intestinal inflammation **(Chapter 4)**. The findings from this study determined which antagonist was used in the *in vivo* study. There was a significant lack in studies using these antagonists, TAK-242 and IAXO-102 in clinically relevant GIM models. The next study therefore aimed to investigate the potential of the TLR4 antagonist, IAXO-102, to attenuate intestinal inflammation as well as suppress tumour activity in a colorectal tumour-bearing mouse model with CPT-11-induced GIM **(Chapter 5)**.

Significance of findings

In silico docking is an important tool for the analysis of large databases of chemical compounds with which to identify possible drug candidates [168]. Information on the interactions responsible for binding can be extracted from the solutions generated by the docking programs and used to design even more successful compounds [168]. However, docking simulations can generate an avalanche of data that must be

carefully analysed and evaluated. The accuracy of the docking simulations is also highly dependent on the applied docking and scoring algorithms [167].

It was found that TLR4 antagonists TAK-242 and IAXO-102 are capable of binding with TLR4/MD-2, whereby the more negative the binding affinity value, the more favourable the binding of the ligand to the protein will be [167]. IAXO-102 was able to bind with TLR4/MD-2, however, it is also capable of binding with TLR4/CD-14 [54]. The present investigation also sheds a light on the potential interactions between TLR4 antagonists and TLR4/MD-2 using binding affinity and hydrogen bonds. Whereby, binding affinity is the strength of the binding interaction between a biomolecule to its ligand/binding partner [52]. All ligands showed good binding affinity to TLR4/MD-2 and thus hold a promising potential to be explored for their anti-TLR4 activity. Additionally, this study was able to provide information on the binding of SN-38 to TLR4/MD-2. However, the effect of this interaction (agonist or antagonist) is still unknown. A previous study has shown that SN-38 may have an antagonistic effect [165] however there may also be a possibility of an agonist effect. As other chemotherapeutics such as paclitaxel have shown agonist activity on TLR4/MD-2 [225]. Since SN-38 is able to bind with the TLR4/MD-2 complex, there is a likelihood that SN-38 may have an “off-target” effect and supports the hypothesis that TLR4 activation via SN-38 binding may be important in CPT-11-induced GIM.

Nonetheless, considering the data presented, the antagonist IAXO-102 was chosen as it was considered more novel compared to TAK-242 which has been used extensively in a variety of studies [149, 163, 190, 192, 226]. IAXO-102 is a synthetic glycolipid compound, similar to other cationic amphiphiles (such as: antidepressants and antibiotics) developed by Professor Peri and his research team at the University of Milano Bicocca, Italy [54]. The amphiphilic character of IAXO-102 contributes to its low water solubility which affects handling *in vitro* and *in vivo* as it is associated with poor

bioavailability (pharmacokinetic properties) [54, 227]. A previous pharmacokinetic study on IAXO-102 demonstrated that a dose of 3 mg/kg daily was sufficient to provide distribution to several organs [55]. A major limitation regarding the use of IAXO-102 was its poor water solubility, bioavailability and route of administration in **Chapter 5**. A suppository (oral/rectal) would have been ideal as it represents a direct administration of the TLR4 antagonist to the site of GIM. Unfortunately, poor absorption in the GI tract is caused by poor water solubility, leading to poor bioavailability [227], and as previously mentioned IAXO-102 has poor water solubility. Therefore, an oral suppository was ruled out and during the time of method development, a rectal suppository was not feasible due to concerns for animal welfare. An i.p. administration was then chosen due to its distribution through the blood stream and rapid absorption compared to a subcutaneous injection. Measurement of tissue distribution of IAXO-102 would support our assumptions regarding the preferred delivery route but was not conducted in the present study.

Additionally, an initial aim in the study was to determine the bioavailability of IAXO-102 in the serum of mice using high performance liquid chromatography mass spectrometry (HPLC/MS). A literature search was conducted to determine if there was a HPLC/MS methodology available, but none was available, and IAXO-102 suppliers were unable to share their HPLC/MS methodology due to intellectual property restrictions. A HPLC/MS methodology therefore had to be created and optimised to determine IAXO-102 concentration in the mouse serum. Unfortunately, this part of the study had many setbacks/hurdles with the methodology development. Firstly, IAXO-102 had very low limit of detection and a non-active IAXO-102 compound was required to act as a calibration internal standard and control, which also had a very low limit of detection. This in turn made creating and optimising the methodology very difficult with poor detection sensitivity. As part of the General Accreditation Guidance — Validation

and verification of quantitative and qualitative test methods written by the National Association of Testing Authorities (NATA), a non-standard and in-house-developed method requires method validation (https://nata.com.au/files/2021/05/Validation-and-Verification-of-Quantitative-and-Qualitative-Test-Methods.pdf) [228]. The method needs to be sensitive, selective and precise; able to assess and quantify the least amount of product that is detectable in the sample at an acceptable co-efficient of variation, differentiate the product of interest from endogenous and exogenous components within the sample and produce the same measurement each time, respectively [228]. Unfortunately, the HPLC/MS method developed to detect IAXO-102 did not meet any of these validation criteria and therefore had to be discontinued.

In the *in vitro* / *ex vivo* studies **(Chapter 4)**, IAXO-102 was not able to prevent cytostasis in IECs, or prevent structural damage or colonic apoptosis in the explant model. Although TLR4 is expressed on IECs, it is mainly expressed on immune cells during the initiation of inflammation and damage. This may underly the lack in reaction seen in the results of both the *in vitro* and *ex vivo* studies. Whole body circulation of inflammatory cells and mediators may be required for TLR4 antagonism to have an effect. Alternatively, an epithelial-immune co-culture model may be used. These models are derived from IEC's and macrophages to mimic either a healthy GIT or during inflammation [229]. A study by Li *et al* used IECs co-cultured with immune and glial cells to mimic the inflammatory conditions of the colon in UC. When they treated this co-culture with berberine, an anti-inflammatory drug, they found that berberine was able to modulate the co-culture interactions and thereby protecting the cells from inflammation [230]. A study by Spalinger *et al* also used a co-culture system to determine the relationship between IEC's and macrophages [231]. They reported that the gene known as protein tyrosine phosphatase non-receptor type 2 (PTPN2) regulates the interactions between IECs and macrophages to control the intestinal

barrier [231]. They also reported that people will have an increased risk in developing IBD if there is a loss of function in PTPN2 [231]. The use of co-culture systems may be the next steps in establishing the relationship between TLR4 antagonism and immune cells within a GIM setting.

Intestinal explants are used as a midway research method between *in vivo* and clinical trials. They also have the advantage of better mimicking the GIT compared to 2D cell cultures, as intestinal explants contain all the cell types present in the GIT including the immune cells. One major limitation of the explant model itself is the rapid degradation of the tissue after collection [232]. However, there have been improvements in explant cultures that minimise the rapid degradation of the tissue [232, 233]. Despite this, no study has ever conducted histology or IHC staining on the explant tissue [61]. In the study **(Chapter 4)**, histopathological analysis was conducted on explant tissue. Before this analysis, the explant model used was firstly optimised using various incubation time points as well as different culture approaches. The explants were incubated at 4, 6, 8, 16 and 24 h in either media or ringer's solution. Two different culture approaches were also used to determine which had the best culture conditions; 1) cultured in wells or 2) mounting in an Ussing chamber. There were no differences in explant tissue health between the 2 culture conditions and solutions. However, the later incubation time points resulted in major tissue destruction. Therefore, it was decided that the colon explants will be cultured in well plates filled with media for 4 h. Media was chosen for consistency with the *in vitro* studies and well plates were chosen as the Ussing chamber was a high-use apparatus and required time to set-up. Despite the optimisation of the explant model in **Chapter 4,** the results did not prove significant. However, this study fulfilled a knowledge gap in the field allowing others to pursue either alternative *ex vivo* models or to try different methods of *ex vivo* optimisation such as the addition of prostaglandin-inhibitors and antibiotics.

Besides the histological architecture of the explant tissue, the supernatant was also used to determine concentration of IL-6 secretion **(Chapter 4)**. A study by Phuong *et al* incubated colonic explants of DSS treated and untreated mice for 6 h [234]. They reported that the supernatant of colonic explants from DSS treated mice had a significantly higher concentration of IL-6 compared to the supernatant from colonic explants of untreated mice [234]. This may be why no differences were observed in IL-6 secretion in the colonic explant supernatant as well as IL-6 mRNA expression **(Chapter 4)**, IL-6 may only be produced/secreted at 6 h time points or later. As such, IL-6 secretion may not have been detected as the explants **(Chapter 4)** were only incubated for 4 h before tissue and supernatant were collected.

It may also explain why there were no differences in mRNA expression in the colon of mice treated with IAXO-102 and SN-38/CPT-11 in the *in vivo* study **(Chapter 5)**. The tissue collected from the mice was at 72 h after a single i.p. dose of CPT-11, which may have started the normal healing phase of GIM (Phase 5). A study by Wardill *et al* has shown that histopathological damage and apoptosis rates start to decrease 24 h after CPT-11 treatment in mice [42]. Therefore the acute inflammatory phase (phase 2), where there is an influx in secretion of pro-inflammatory cytokines may have been missed.

However, there was an increase in IL-6R mRNA expression in the colon of the CPT-11 group compared to the combination group (Chapter 5). These results mimic those seen in a study by Hosokawa *et al* where by IL-6R levels were highly expressed compared to IL-6 expression which was more muted [235]. However, these results are from the tissue of other colonic inflammatory conditions such pseudomembranous, ischaemic and antibiotic-associated haemorrhagic colitis [235]. In contrast, expression levels of IL-6 and IL-6R were both significantly increased in IBD tissue [235]. These

findings suggest that IL-6 and IL-6R expression on the GIT mucosa may be dependent on the type of colonic inflammation (auto-immune or infectious).

Previous literature has also shown that the protective effect of TLR4 is limited to the acute inflammatory phase [100, 136, 165]. A study using DSS to induce GI inflammation found that treatment with paeniflorin to inhibit TLR4 was able to decrease the secretion of TNF-α and IL-6 in the GI mucosa of mice [98]. Additionally, Ungaro *et al* reported that mice with DSS-induced GI inflammation and were treated early with a TLR4 antagonist had a delayed development in GI inflammation with significantly lower DAI scores [100]. However, the TLR4 antagonist had no effect in a more chronic setting of GI inflammation and prevented the GI mucosa from healing [100].

Another reason no differences were observed in mRNA expression of both the explant and *in vivo* study may be that the CPT-11/SN-38 is an inhibitor of TLR4. A study observed that SN-38 was able to compete with LPS for the same binding site in TLR4 to become an antagonist in the TLR4-mediated pro-inflammatory downstream signalling pathway as a decrease in pro-inflammatory cytokine secretion was observed [165]. This effect was also reversible when LPS concentration was increased, further indicating the antagonistic effect of SN-38 [165]. However, the study did not conduct any analysis on mRNA expression of TLR4 in SN-38-induced GIM. The further administration of a TLR4 antagonist in this environment would therefore not show any significant effect. However, another study has shown that when TLR4 is downregulated, TLR9 will be upregulated which enhances the damage caused by CPT-11-induced GIM [204]. This reaction was not observed in the *in vivo* study (**Chapter 5**) as the combination group did not show any severe GIM. Since IAXO-102 was able to prevent CPT-11-induced diarrhoea and colonic epithelial damage but did not show an effect in colonic proliferation or apoptosis **(Chapter 5),** it may be intervening via alternative mechanisms of action.

Previous studies [100, 136, 165] have clearly highlighted the involvement of TLR4 in the development of CPT-11-induced GIM and provides an opportunity to simultaneously treat these toxicities in an acute setting. In regards to anti-TLR4 therapies, its effect on the role of TLR4 and anti-tumour effect is important. As previous literature has suggested that inhibiting TLR4 may affect tumour growth. For example, a study using TLR4 KO mice had increased tumour growth in response to doxorubicin and under normal conditions [236]. In contrast, TLR4 inhibition has also been implicated in tumour regression. Studies have shown that cancer cell lines such as SW260 (colon) overexpress TLR4 and when these cell lines are stimulated with LPS, their growth rate increases [237]. It can therefore be theorised that when TLR4 is inhibited in cancer cells, their growth rate can be impeded. A study by Huang *et al* has shown that by inhibiting TLR4, LPS-stimulated production of IL-6 and IL-8 was down-regulated and tumour sizes were significantly smaller in tumour-bearing nude mice [238].

The pros and cons of TLR4 signalling and inhibition in GIM (Table 6.1) and tumours (Table 6.2) has been consolidated into 2 separate tables. Although IAXO-102 was able to reduce tumour volume, it did not affect tumour proliferation or apoptosis rates. Collectively, the work in this thesis showed that TLR4 inhibition was not able to prevent GI inflammation or ameliorate the structural damage.

Table 6.1: Pros and cons of colon TLR4 signalling and inhibition in GIM.

TLR4 (Colon)	Pro	Con
Signalling	• Regulation of IEC proliferation. • Important in colonic mucosal healing and recovery. • Important for recruiting inflammatory cells and production of inflammatory cytokines to prevent infection.	• Contributes to uncontrolled inflammation. • Plays a key role in the pathogenesis of inflammatory diseases.
Inhibition	• Prevents uncontrolled inflammation. • Reduces antigen-presenting cell infiltrate and pro-inflammatory chemokine and cytokine production.	• Impairs mucosal healing and repair. • Decreases host immunity.

Table 6.2: Pros and cons of TLR4 signalling and inhibition in tumours.

TLR4 (Tumour)	Pro	Con
Signalling	• Enhances host anti-cancer immunity.	• Promotes tumour cell proliferation, survival, migration, and metastasis.
Inhibition	• Inhibits tumour growth, adhesion and dysplasia by activating T-cell and natural killer cell activity.	• Prevents dendritic cells from promoting an anti-tumour immune response.

Future Directions

Although TLR4 plays a key physiological role in host response to bacterial infection, a prolonged TLR4 response can promote life-threatening pathology, such as septic shock. TLR4 activation has also been associated with certain autoimmune diseases, non-infectious inflammatory disorders, and neuropathic pain, suggesting a wide range of possible clinical settings for application of TLR4 antagonists and has proven to serve as inspiration for rational design of new TLR4 antagonists. Although these TLR4 antagonists were designed to have specific/selective interaction, it still cannot be completely excluded from other non-specific/off-target interactions.

Alternate binding sites or other possible non-specific interactions of these TLR4 antagonists are another area of importance that needs to be researched. As many of the drugs developed to treat GIM may have alternate binding sites. For example amitriptyline, as non-specific TLR4 antagonist, with activity at the u-opioid receptor has been investigated for management of cancer-associated pain [239]. It is also used in the treatment of other GI disorders such as IBD [240, 241]. The mechanism of action of amitriptyline's anti-inflammatory effects includes the inhibition of TLR4 and its downstream signalling activity [242]. However, amitriptyline is also an anti-depressant and has other off-target effects such as inhibiting ion channels, hERG channels and many others [243]. This severely limited the success of amitriptyline in the treatment of GIM [174, 244] as these off-target binding sites often cause negative side effects such as nausea, dry mouth and drowsiness [244].

When testing the antagonist IAXO-102 in an *in vitro* system, the cell lines T84 and HT-29 were used. These are human IEC lines derived from an adenocarcinoma; however, both cell lines were found to be broadly unresponsive or resistant to LPS stimulation [58] as well as SN-38 **(Chapter 4)**. In contrast, other studies have found T84, HT-29

and Caco-2 (another adenocarcinoma-derived cell line extensively studied in intestinal assays) to be responsive to LPS with an upregulation of TLR4 expression and increased secretion of IL-8 [245-247]. The difference in findings between **Chapter 4** and these other studies may be due to differences in LPS strain and concentrations used and incubation times. For example, in **Chapter 4,** the LPS strain: O55:B5, concentration: 100 ng/mL, incubation time: 36 h. compared to previous studies [245-247], the LPS strain: B8:0127 and O26:B6, concentration: 0.001 – 10 μg/mL, incubation time: 0 – 24 h. Although IECs are still relevant in the context of the *in vitro* study **(Chapter 4)**, perhaps using IECs derived from normal colon epithelial tissue cells (such as NCM460, and NCOL-1) may have been a better choice. However, the IECs derived from normal colon epithelial tissue were found to express low levels of TLR4/MD-2 [58]. It would be beneficial to address this gap in the literature to compare TLR4/MD-2/CD-14 levels of IECs derived from normal and adenocarcinoma tissue.

Another alternative of the explant model is the culture of intestinal organoids. Organoids are a 3-dimensional *in vitro* model that are able to mimic the human GI tract [248]. This model may be more relevant to identify the potential activity of IAXO-102 in a more complete system. As many other factors that may affect TLR4 antagonist drug activity such as the presence of dendritic cells and Peyer's patches not found in IEC cultures. The addition of a complete and functional immune system would prove critical in testing TLR4 antagonists due to the nature of GIM being studied as well as the TLR4 protein whose downstream activation has a direct impact on inflammation.

Organoids may also be used in place of the explant model **(Chapter 4)** which was mainly used to visualise any damage caused by an influx of LPS or the presence of SN-38. A study by Sprangers *et al* found that organoids are capable of modelling intestinal development, regeneration, and repair [249]. This may prove to be a better visualisation of GI damage than the explant model as the difference between damage

from the treatments vs degradation of the tissue proved difficult as seen in the histopathology and immunohistochemistry images in **Chapter 4**.

The innate immune system is strongly influenced by the microbial environment [250]. The gut microbiome is defined as the collection of microbes including bacteria, fungi and viruses that live in the GI tract. The GI microbiome is known to be inherently linked to the innate immune system [251]. However, there is growing evidence that the reverse is also true, and that the microbial environment is similarly influenced by the innate immune system which is regulated by TLR4 [252, 253]. A study has reported that germ-free mice had a lower incidence of diarrhoea as well as reduced GIM after being treated with CPT-11 [25]. Another study using doxorubicin to induce GIM reported a decrease in cell proliferation and crypt depth in the GI tract of WT mice, which was prevented in germ-free mice [254]. This was also reflected in another study that treated WT mice and germ-free mice with the same dose of CPT-11 [206]. Investigators observed that the WT mice displayed more lesions within the jejunal intestinal epithelium and higher GI permeability compared to the germ-free mice despite having the same dose of CPT-11 [206]. Additionally, clinical trials have also investigated the impact of removing the GI microbiome using antibiotics such as neomycin in combination with CPT-11, with results displaying a decrease in diarrhoea incidence/grade [255, 256]. Given the importance of the microflora in GI inflammation, and the fact that TLR4 plays a major role in the recognition of bacteria and inflammation, another factor that needs to be accounted for in *in vivo* studies **(Chapter 5)** is the presence of the microbiome. It is essential to determine if the effects of the IAXO-102 are from the IAXO-102 itself or through the innate immune system and the microbiome working in tandem.

Several studies that have reported that removal of the GI microbiome may be more harmful than it is beneficial, and that the restoration and maintenance of microbiome

diversity may be more important to maintain GI tract health and homeostasis. For example, a clinical study on patients being treated with CPT-11 who were also given the antibiotic neomycin to eliminate their gut microbiome, showed no differences in diarrhoea severity between patients that received neomycin and those that did not [257]. These contradicting results may be due to differences in the population of microorganisms in the GIT [258]. In 2013, a systematic review was conducted to evaluate the evidence for interventions for the management of GIM in patients with cancer [259]. The review concluded that the use of neomycin was not recommended for the management of GIM in patients with cancer due to conflicting evidence of its benefit [257, 259]. Another study on paediatric patients with acute myeloid leukaemia that were treated with a combination of chemotherapeutics displayed the highest incidence of GIM during the chemotherapy cycles, when microbiome diversity is at its lowest [260]. The patients were also administered prophylactic broad-spectrum of antibiotics during febrile neutropenia, a side-effect of chemotherapy [260]. It was observed that the antibiotics depleted the GI microbiome diversity which caused an influx of potential pathogenic bacteria, increasing the patient's risk to bacterial translocation and infection [260]. These studies show the important role the microbiome plays in GIM, providing justification for further research in this topic.

Studies using colonic tumour-bearing mouse models are necessary for future studies investigating the potential of TLR4 antagonism. Although the model used in **Chapter 5** is a mouse subcutaneous tumour-bearing model and relevant to the study **(Chapter 5)**, it cannot replicate the original site of CRC. Studies have shown that colonic epithelial cancer cells growing under the skin of the flank can change their phenotype due to the differences in microenvironment [261, 262]. In turn, this may also affect tumour response to CPT-11. An orthotopic tumour model involves seeding tumour cells into the corresponding tissue in the animal and may be more relevant [263]. The

orthotopic tumour model will mimic the disease process in humans with high fidelity. This method will allow the assessment of tumour development in the relevant environment [263]. However, there is a technical barrier to the orthotopic tumour model as implantation directly in the GIT requires either surgery or colonoscopic guided insertion to the GIT wall. Alternatively, transgenic mice that spontaneously develop CRC such as APC mice [264] or chemically inducing CRC into a mouse using AZO and DSS may be considered [265].

Another factor to consider is the route/method of treatment administration. The TLR4 antagonists, TAK-242 and IAXO-102, are also known as small-molecule inhibitors [55, 266]. This means that their small size allows them to cross cell membranes to act on specific intracellular proteins within TLR signalling pathways [267]. Recent advancements in nanotechnology have enabled the encapsulation and functionalisation of the therapeutic and nanoparticles, respectively; resulting in decreased off-target effects and increased efficacy [268]. The use of nanoparticles as an alternative method of targeted therapy delivery will be a significant improvement to the current delivery methods of therapeutics for CRC and GIM. As chemotherapeutics are often administered intravenously which is associated with increased infection risks and decreased patient comfort [269]. Nanoparticles could be specifically engineered to release the encapsulated payload in a topical manner in the GI tract. To achieve this, it is essential to fine-tune the physicochemical properties of the selected carrier. Surface properties and area as well as a controlled release profile are particularly beneficial since degradation and non-specific release of the encapsulated therapeutic can be decreased [270]. Further research into nanomedicines will also help advance the development of more effective strategies to treat patients with CRC and treatment-related side-effects such as GIM, respectively. However, a more fundamental understanding and further knowledge about the characteristics of both the physiology

of the intestine, in particular in the disease stage, and the structural requirement for optimised nanomedicines is crucial to facilitate efficient uptake of the nanoparticles by the GIT. This nanomedicine approach has the potential to discover new effective strategies for oral delivery of TLR4 antagonists that attenuate inflammatory responses in human diseases.

Conclusion

Research has demonstrated that TLR4 is pivotal in the development of both healing and toxicity in the GI tract. The research conducted in this thesis not only improves our understanding of the underlying mechanisms involved, but also reveals a promising opportunity to intervene in the complex pathophysiology of the side-effects of CPT-11. Research efforts must now be targeted at the effects of the GI microbiome with TLR4 as well as analyse alternative pathways of inflammation and apoptosis in the GI tract such as targeting IL-6R.

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Appendix 1: Publications arising from this thesis

Chapter 2, 4 and 5 have been published in a peer-reviewed journal. These chapters are presented in this thesis in the original format, except for spelling and table/figure number changes to ensure consistency and referencing style. Here, the chapters are included in the original published format.

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REVIEW ARTICLE

Toll-like receptor 4 (TLR4) antagonists as potential therapeutics for intestinal inflammation

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Abstract
Gastrointestinal inflammation is a hallmark of highly prevalent disorders, including cancer treatment–induced mucositis and ulcerative colitis. These disorders cause debilitating symptoms, have a significant impact on quality of life, and are poorly managed. The activation of toll-like receptor 4 (TLR4) has been proposed to have a major influence on the inflammatory signalling pathways of the intestinal tract. Inhibition of TLR4 has been postulated as an effective way to treat intestinal inflammation. However, there are a limited number of studies looking into the potential of TLR4 antagonism as a therapeutic approach for intestinal inflammation. This review surveyed available literature and reported on the *in vitro, ex vivo* and *in vivo* effects of TLR4 antagonism on different models of intestinal inflammation. Of the studies reviewed, evidence suggests that there is indeed potential for TLR4 antagonists to treat inflammation, although only a limited number of studies have investigated treating intestinal inflammation with TLR4 antagonists directly. These results warrant further research into the effect of TLR4 antagonists in the intestinal tract.

Keywords Acute inflammation · Chemotherapy · Chronic inflammation · Crohn's disease · Inflammatory bowel disease · Intestinal mucositis · Lipopolysaccharide · Radiation · TLR4 antagonists · Ulcerative colitis

Introduction

Inflammation of the intestinal tract can result in acute or chronic manifestations of intestinal diseases; it may cause irritation, exposure to bacteria, and a dysregulation of the homeostatic balance. This leads to a range of debilitating symptoms that may affect patients' quality of life. Current treatment modalities used for intestinal inflammation are associated with a range of disadvantages including poor efficacy and unwanted side effects. The incidence rates for intestinal inflammation have been steadily increasing around the world for the last 50 years with an increased prevalence most notable in newly industrialized nations [1, 2]. Factors such as cell types, immunological abnormalities, tissue specificity and genetic/environmental factors are involved in the pathogenesis of intestinal inflammation.

Toll-like receptors (TLRs) are type 1 transmembrane proteins belonging to the wider family of pattern recognition receptors, and are responsible for the recognition of a variety of molecular signals, including endogenous damage and pathogen-associated signals, damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), respectively. Both immune (dendritic cells, monocytes, mast cells, macrophages) and non-immune cells (fibroblasts, epithelial cells) express these pattern recognition receptors [3]. The pattern recognition receptor-ligand binding between DAMPs and PAMPs prompts a downstream signalling cascade, which results in the recruitment of leukocytes [3]. TLRs activate downstream signalling pathways, which originates from the toll-interleukin receptor (TIR) domain–containing adaptor proteins such as myeloid differentiation

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primary-response protein 88 (MyD88), TIR-domain–containing adaptor protein (TIRAP), TIR domain–containing adaptor protein inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) [4]. These adaptor molecules are essential to produce inflammatory cytokines, type 1 interferons, chemokines and co-stimulatory molecules. At present, 13 human TLRs have been identified and are located on various cellular compartments (including extracellular membrane, endosome and golgi apparatus) with each TLR responding to specific stimuli [4, 5].

In a healthy intestinal tract, enterocytes coexist with luminal and mucosa-associated commensal bacteria without the initiation of inflammatory responses. However, the exact mechanism behind bacterial tolerance within the intestinal tract is still largely unknown. TLRs play a pivotal role in immune tolerance to intestinal microbes [6]. These immune tolerance and responses are organized by Peyer's patches, mesenteric lymph nodes and the lamina propria [7]. These lymphoid organs are populated with dendritic cells, which produce interleukin 10 (IL-10) in turn, transforming T cells into transforming growth factor-β (TGF-β) [7]. The production of these cytokines leads to immune tolerance and homeostasis, as well as unnecessary inflammation [7]. This suggests that TLR4 signalling has an effect not only on immune responses but also on the balance of the intestinal microbial ecosystem [8, 9].

In contrast, during conditions of stress (such as disease) in the intestinal tract, inflammatory cytokines are released from enterocytes and mucosal immune cells responding to the stimulation of TLRs [10]. This leads to apoptosis and reduced proliferation of enterocytes, which in turn promotes translocation of bacteria into the lamina propria, exacerbating intestinal inflammation [10]. One of the most well-characterized TLRs is TLR4, which has been shown to be involved in homeostasis, apoptosis, intestinal inflammation and inflammatory bowel disease [11]. The focus of this review will therefore be based on the role of TLR4 antagonism in inflammatory conditions with the purpose of generating a hypothesis to support the use of TLR4 antagonists in intestinal inflammation.

Toll-like receptor 4 activation and signalling

In 1997, toll proteins in *Drosophila* were discovered to mediate protection against fungal infections [12]. Toll proteins in *Drosophila* were activated by fungi and Gram-positive bacteria, which do not contain lipopolysaccharide (LPS). They do, however, trigger a toxic shock response that is similarly induced by LPS [12]. This then led to research focusing on the now established TLR4-LPS signalling cascade. This early work also suggests a much broader role of TLR in homeostasis, tissue repair and immune defence [13].

TLR4 is an intra- and extracellular receptor expressed on endosomes and cytoplasmic membranes, which recognizes PAMPs (flagellin and LPS) and DAMPS (calprotectin, S100A8/9 HMGB1 and HSP70) through its co-receptors MD2 and CD14 [14, 15]. In addition, TLR4 has recently shown to be activated by certain pharmacological agents, including chemotherapeutic agents (paclitaxel). TLR4 is located on many different cell types (endothelial cells, lymphocytes, cardiac myocytes and glial cells) throughout the body [16–18]. In the intestine, TLR4 is expressed on antigen-presenting cells such as macrophages and dendritic cells, and on enterocytes and lymphocytes [19]. TLR4 consists of leucine-rich repeats (LRRs) with a horseshoe-like shape made up of 839 amino acids. The complex ligand specificity of the TLR4/MD2 complex is composed of two antiparallel β sheets, which form a large hydrophobic pocket in MD2 [20]. LPS is able to bind to this hydrophobic pocket through its lipid chains, which are completely buried in the MD2 hydrophobic pocket [20]. However, one of these lipid chains is partially exposed to the outer surface, which allows some interaction with TLR4 [20]. These hydrophilic and hydrophobic interactions between LPS and the TLR4/MD2 complex mediate the dimerization of extracellular domains in the TLR4, thus triggering a downstream signalling cascade leading to the release of pro-inflammatory cytokines [20]. A study by Abreu et al. [21] discovered that increases in TLR4 expression alone would not result in a reaction from LPS without the accompanying expression of MD2. In the study, they challenged different intestinal epithelial cell lines (Caco-2, T84, HT-29) with LPS and found that a decreased expression of TLR4 and MD2 correlated with intestinal epithelial protection against pro-inflammatory gene expression in response to bacterial LPS. It was concluded that careful regulation of both TLR4 and MD2 is necessary to maintain homeostasis in the intestinal tract due to it being continuously exposed to high concentrations of bacteria.

Upon stimulation, TLR4 will activate two signalling pathways, the TRIF-dependent pathway (Fig. 1) and the MyD88-dependent pathway (Fig. 2). In the TRIF-dependent pathway, TLR4 heterodimers recruit TRAM, which is needed to activate TRIF, resulting in the binding of TRIF with TNF receptor–associated factor 3 (TRAF3) and TRAF6 for binding with RIP, a receptor-interacting serine-threonine kinase 1 protein. Subsequently, this leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The TRIF-activated pathway leads to the activation of interferon regulatory transcription factor 3 (IRF3) by TANK-binding kinase 1 (TBK1) and inhibitor of NF-κB-kinase complex stimulation (IKK), which results in the production of type 1 interferons and anti-inflammatory cytokines (such as IL-10).

In the MyD88 signalling pathway, TLR4 heterodimers will bind to MyD88, which results in the formation of IRAK (interleukin 1 receptor–associated kinases) and TRAF6

complexes [14]. Formation of IRAK and TRAF6 complexes leads to a downstream signalling cascade. Various other complexes such as TAK1, TAB1/2/3, MAP kinases and IκB will be phosphorylated or activated to allow the translocation of NF-κB into the nucleus, ultimately driving the transcription of cytokine genes (such as TNFs, ILs and chemokines) to regulate pro-inflammatory responses [14, 22].

Dysregulation of TLR4 signalling has been linked to the development of a variety of inflammatory diseases. Studies have investigated functional genetic variants of TLR4 and their impact on LPS signalling response. A study by Hold et al. found that cells carrying TLR4 D299G and T399I variants, when stimulated with LPS, had a sixfold lower expression of NF-κB compared to wild-type TLR4 [23]. Ferwerda et al. demonstrated that patients carrying a variant at position 299 (Gly) but not at position 399 (Ile) had a stronger pro-inflammatory cytokine response with increased TNF-α levels in whole blood samples when stimulated with LPS compared to patients carrying wild-type TLR4 alleles at both positions [24]. Weinstein et al. showed that the same *TLR4* variants in patients with acute ischemic stroke are associated with worse neurological outcomes and alterations in systemic markers of inflammation [25]. This dramatic difference in cytokine expression caused by dysregulation of TLR4 signalling due to genetic polymorphisms will affect a person's ability to respond to LPS leading to a dysregulated immune response to infection. Considering that TLR4 downstream signalling plays a pathological role in inflammation, using antagonists or inhibitors to target TLR4 signalling may be beneficial in treating inflammatory disorders.

TLR4-mediated intestinal inflammation

An important component of immunity and host-microbial interactions in the intestinal tract is the recognition of DAMPs,

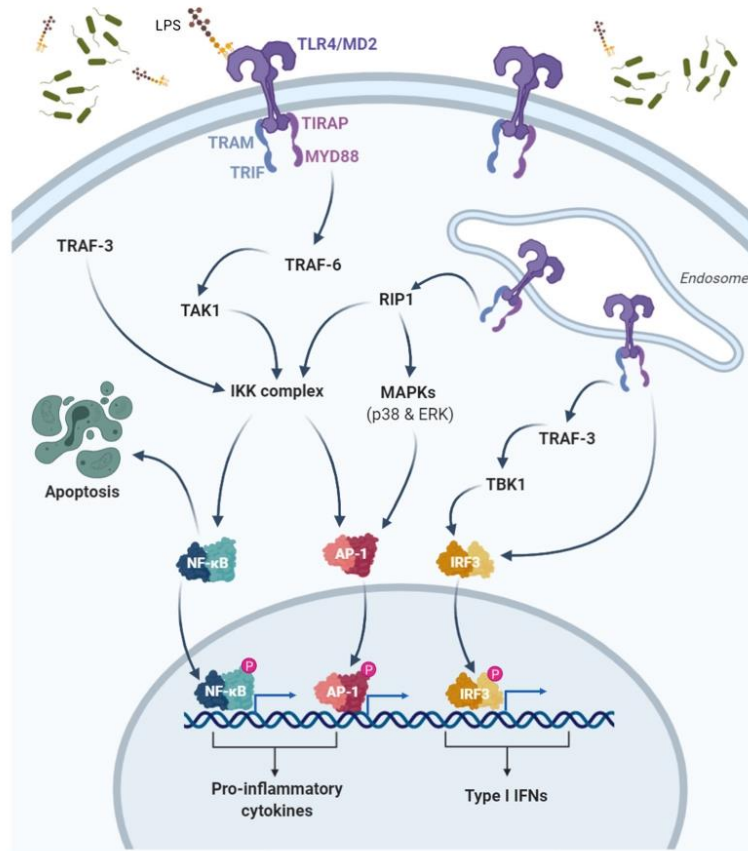

Fig. 1 Pathogen-associated molecular pattern toll-like receptor 4 signalling pathway in an enterocyte. *LPS* Lipopolysaccharide, *TLR* Toll-like receptor, *TIRAP* TIR domain–containing adaptor protein, *TRAM* TRIF-related adaptor molecule, *MyD88* Myeloid differentiation primary-response protein 88, *IKK* Inhibitor of NF-κB-kinase complex, *TRIF* TIR -domain–containing adaptor protein inducing interferon-β, *TBK1* TANK-binding kinase 1, *NF-κB* Nuclear factor-kappaB, *IRF3* Interferon regulatory transcription factor 3

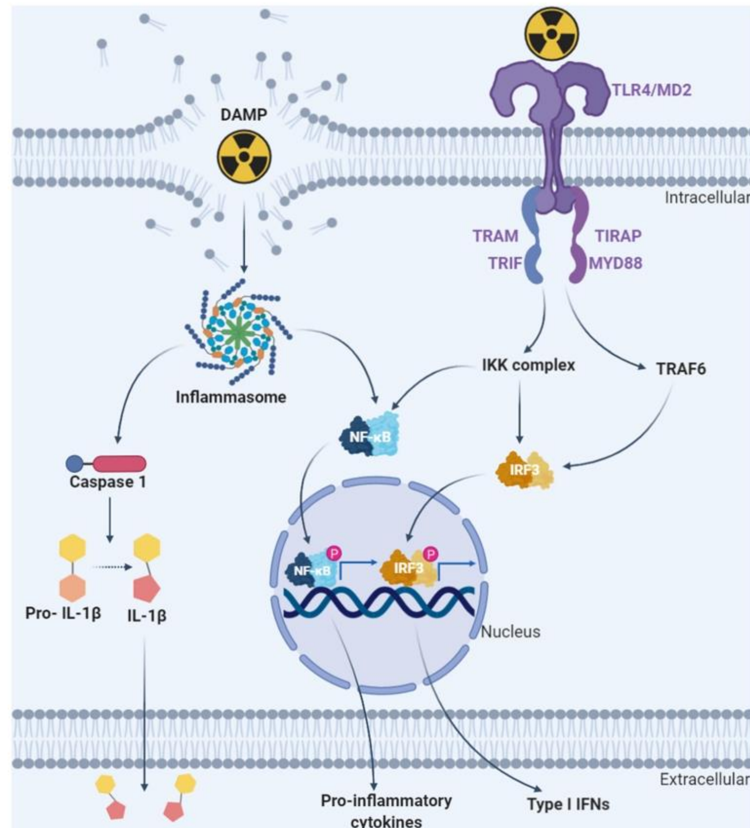

Fig. 2 Toll-like receptor 4 activation by damage-associated molecular patterns from tissue damage leads to a downstream signalling pathway, which induces inflammatory gene expression. *DAMP*s Damage-associated molecular patterns, *TLR* Toll-like receptor, *TRAM* TRIF-related adaptor molecule, *TRIF* TIR -domain–containing adaptor protein inducing interferon-β, *TIRAP* TIR domain–containing adaptor protein, *MyD88* Myeloid differentiation primary-response protein 88, *IKK* Inhibitor of NF-κB-kinase complex, *IRF3* Interferon regulatory transcription factor 3, *NF-κB* Nuclear factor-kappaB, *IL* Interleukin

PAMPs and endogenous ligands by TLR4 expressed on the enterocytes and antigen-presenting cells. Any imbalance from this interaction may contribute to the pathogenesis of inflammation within the intestinal tract [26]. There has also been substantial evidence indicating the involvement of TLR4 in intestinal inflammatory diseases such as ulcerative colitis (UC), Crohn's disease (CD) and intestinal mucositis (IM). It was reported that, in the colonic mucosa of patients with UC and CD, a significant increase in TLR4 mRNA and protein expression was observed compared to healthy controls [27, 28]. However, this may be due to the increased influx of TLR4 expressing innate immune cells. There is also mounting evidence that TLR4 polymorphism is associated with the development of UC and CD, whereby the allele frequencies of the TLR4 Asp299Gly polymorphism were discovered to be significantly higher in UC and CD patients [29].

There have also been several studies using animal models of acute intestinal inflammation. TLR4 expression is strongly upregulated in animal models induced with colitis [30]. Animal models with TLR4 knocked-out were observed to be protected from colitis or colon tumorigenesis by preventing the downstream signalling pathways that induce colitis [30]. For example, TLR4 knockout (KO) mice induced with acute colitis had a decrease in COX-2 expression, prostaglandin production and NF-κB signalling, which lead to a significant reduction of acute inflammatory cells and therefore significantly reduced acute inflammation in the intestinal tract [31]. Other studies have observed an increase in pathogenic *E. coli* and a decrease in beneficial intestinal microbiomes (*Bifidobacterium* spp. and *Lactobacillus* spp.) in a DSS-induced mouse model, which may be associated with an increase in the TLR4 expression observed in the colon of the animals [8], while another study using TLR4 KO mice showed a reduction in pathogenic *E. coli* compared to the DSS-induced wild-type mice, which showed a tenfold increase in pathogenic *E. coli* [9]. These TLR4-deficient mice

also displayed reduced disease activity index and histopathological scoring.

It therefore stands to reason that, by inhibiting TLR4, a protective effect from intestinal inflammation will be induced. Recent research has shown that, by inhibiting TLR4 using antagonists such as paeoniflorin, monoclonal antibodies and CRX-526, DSS-induced intestinal inflammation was attenuated with a significant reduction in disease activity and histopathological scoring [32–34]. However, other studies have discovered that there was no protective effect observed in the clinical symptoms and histology scores when blocking TLR4 during chronic intestinal inflammation [35] despite the opposite results observed during acute intestinal inflammation [31], most likely due to the low involvement of innate immune cells in chronic compared to acute inflammation [36].

TLR4 antagonists as a potential therapeutic alternative for treatment of intestinal inflammation

Inflammatory bowel disease (IBD) (consisting of UC and CD) is known as a non-specific, chronic gastrointestinal inflammatory disorder [37], with periods of disease activation and remission, and in some cases progressive disease [38]. It is considered an autoimmune disease due to the combined effect of genetic factors and abnormal immune responses to the intestinal bacteria and other foreign substances [37]. In addition, one of the most serious complications that IBD patients encounter is colorectal cancer, which accounts for increased mortality rates associated with UC [39]. The severity of inflammation in the intestinal tract also correlates with the risk of colorectal cancer in patients with IBD [39].

Severe intestinal inflammation leads to a range of debilitating symptoms that significantly affects patient quality of life. Current treatment modalities for intestinal inflammation are associated with a range of disadvantages including poor efficacy and unwanted side effects. The TLR4 signalling cascade also plays an important role in intestinal inflammation, with its extracellular and intracellular components being attractive therapeutic targets for the treatment of both acute and chronic intestinal inflammation. The link between intestinal inflammation and colon cancer also offers the possibility of identifying and developing novel ways to prevent cancer.

The incidence rates for IBD have been steadily increasing around the world for the last 50 years with the majority of cases occurring in westernized and industrialized countries [1, 40]. IBD is a chronic and lifelong condition that has no cure and requires a lifetime of care. It has a significant effect on patient quality of life [41, 42]. However, UC and CD are clinically distinct diseases and known to have different anatomical, clinical and histological features [43].

Presently, the etiology and pathogenesis for IBD remain largely unknown [43], with a plausible hypothesis for the etiology of IBD being the unregulated activation of both the body's innate and adaptive immune systems, potentially in response to resident gut microbes. This immune response may be mediated by the innate immune receptor TLR4 in response to luminal antigens (fungi, bacteria) in the intestinal tract [43]. Treatment approaches include aminosalicylates, corticosteroids and antibiotics [44, 45].

As previously stated, patients with IBD are at higher risk of developing colorectal cancer, and a common complication of cancer treatment is intestinal mucositis (IM), occurring in 40% of patients who receive a standard dose of chemotherapy and 100% of patients who are receiving high doses of chemotherapy [46, 47]. IM is the ulceration and inflammation of the mucosa in the intestinal tract caused by chemotherapy and radiation for cancer, and is an acute form of intestinal inflammation [48].

Unfortunately, the gastrointestinal tract is particularly susceptible to the devastating effects of chemotherapy and radiation. TLR4 is the main receptor that detects DAMPs and responds to tissue damage in the intestinal tract [49]. The cytotoxic effects of chemotherapy and radiation on both normal and malignant cells cause the release of DAMPs. This produces a sustained innate immune activation, which develops into the mucosal inflammation seen in patients with repeating cycles of chemotherapy and radiation treatments [49]. While mucositis has been recognized as a major dose-limiting toxicity for decades, there is yet to be an effective treatment to manage intestinal inflammation. Pre-clinical studies have focused on inhibition of inflammation via multiple mechanisms, or accelerating healing with growth factors [50].

IM has a significant impact on the quality of life of patients whereby the average number of days a patient suffering from IM needs to be hospitalized is 3 times more than the 4 days required by patients not suffering from IM [51]. This increased length of stay increases the strain on hospital resources [51]. Patients also suffer from symptoms such as vomiting, abdominal pain and severe diarrhea. In certain cases, the symptoms of IM will cause patients to require a dose reduction, delay or even discontinuation of their regimen, which will affect the patient's survival [52]. To date, studies have shown a link between TLR4/MD2 signalling and the development of IBD and IM [34, 53]. TLR4 antagonists show potential as therapeutic agents in both the settings. However, the majority of studies have focused mainly on sepsis models as well as diseases and infections unrelated to the development of intestinal inflammation, leaving a significant gap in the literature.

TLR4 is overexpressed in both UC (fold increase: 2.33) and CD (fold increase: 1.71) [27, 54, 55]. In IBD, abnormal signal transmission mediated by the upregulation of TLR4 promotes the sustained release of inflammatory cytokines (IL-6, TNF-α). This, in turn, develops and persists as intestinal inflammation. Only low levels of TLR4 and MD2 are expressed on the intestinal epithelium and very little was known about their regulation on intestinal epithelial cells. However, it was established that, during inflammation, expression of both TLR4 and MD2 is increased. It was later discovered by Abreu et al. [56] that the expression of TLR4 and MD2 in the intestinal tract is also regulated by immune-mediated signals. There was an increase in TLR4/MD2 expression when the intestinal epithelial cell lines (T84, HT-29) were exposed to pro-inflammatory cytokines (IFN-γ, TNF-α) highlighting the potential link between the innate and adaptive immune systems in intestinal epithelial cells only in response to pathogenic organisms. Another study by Ungaro et al. has also shown that the inflammation in IBD is decreased in TLR4-deficient mice [35]. However, the study also found that TLR4-deficient mice were unable to undergo mucosal healing and demonstrated decreased epithelial cell proliferation [35]. This shows that TLR4 serves as a mediator for both mucosal healing and inflammation in the intestinal tract.

A similar pattern can also be observed in IM. A study by Wardill et al. has shown that genetic deletion of TLR4 from mice was able to improve chemotherapy-induced gut toxicity and pain [53]. TLR4 KO mice had reduced diarrhea and weight loss compared to wild-type mice [53]. The TLR4 KO mice also exhibited a muted inflammatory response, with no significant increase in IL-1β, IL-6 or TNF-α, compared to their wild-type counterparts [53].

These studies point out the critical role of TLR4 in regulating inflammation in the intestinal tract, and by targeting and inhibiting TLR4, the outcome of intestinal inflammation and its consequence may be prevented. However, careful selection of TLR4 elimination vs. selective or temporary inhibition as a therapeutic is needed since TLR4 has beneficial effects for mucosal healing and homeostasis.

TLR4 antagonists

Targeting TLR4 could represent a potential approach to regulate immune responses and treat inflammation. However, any potential therapeutic agent must be able to block the harmful effects of TLR4 activation without negatively affecting the host's defence functions. Currently, many different antagonists are being investigated for their potential in managing inflammatory-based diseases and settings, summarized in Table 1.

Naturally occurring

The first naturally occurring TLR4 antagonist discovered was from a photosynthetic Gram-negative bacterium that was non-pathogenic, known as *Rhodobacter sphaeroides* [57]. The LPS produced from this bacterium, known as *Rhodobacter sphaeroides* lipid A (RsDPLA), was non-toxic towards murine and human cells and was able to compete with toxic LPS for binding sites. RsDPLA was also able to interact with the TLR4/MD2 complex found in rodents and humans with antagonistic effects [58]. Further in vitro and in vivo studies on the LPS produced by *Rhodobacter sphaeroides* and other bacteria/cyanobacteria have shown potent antagonistic activity of this type of LPS in murine and human cells as well as preventing endotoxic shock in mice.

Additionally, traditional Asian medicines produced from plants, including curcumin, turmeric and a variety of herbs, provide a rich and natural source of molecules which are being investigated for bio-activities that act as TLR4 antagonists [59, 60]. The modulation of TLR4 using herbal extracts promoted a large area of research to determine their pharmacological potential. It was found that certain bio-actives from bacteria or plants had a positive relationship against sepsis and septic shock [61–63]. These bio-actives were also discovered to have positive relationships against inflammatory diseases such as Alzheimer's, arthritis and inflammatory bowel diseases [35, 64, 65]. A summary of research conducted on some of these naturally sourced TLR4 antagonists can be found in Table 1. Although the main focus of this study is on the therapeutic potentials of TLR4 antagonists on intestinal inflammation, there are a limited number of studies, which used intestinal inflammation as a disease model.

Synthetic

Although there are many plant-based products capable of targeting and inhibiting TLR4 in vitro and in vivo in both rodent and human models, these do not possess the necessary stability and target specificity to be considered a potential therapeutic option compared to products and molecules extracted from microorganisms [59], which was why the molecules produced from microorganisms such as *Rhodobacter sphaeroides* have been used as a model to create synthetic antagonists. RsDPLA was used to design the synthetic TLR4 antagonists eritoran (E5564) and E5531 [80, 81]. E5531 was a first-generation lipid A analogue synthesized as part of a program to develop therapeutic agents for septic shock [82], while eritoran (E5564) is a second-generation lipid A analogue designed for the same purpose but was found to be more potent in its anti-endotoxin effects, longer lasting and easier to manufacture

Table 1 Summary of the effect of natural TLR4 antagonists in previous pre-clinical *in vitro, in vivo* and *in silico* studies

Study	Study model	TLR4 antagonist	Outcome
Qureshi et al. [61]	Bacterial sepsis *In vivo*: BDF1 mice injected with LPS (1 μg)	*Rhodobacter sphaeroides* lipid A (RsDPLA)	RsDPLA (100 μg, i.p.) pre-treatment was associated with 91% inhibition of LPS-induced response as measured by serum TNF-α concentration (246±95 pg/mL vs. 2653±286 pg/mL vehicle control).
Kirikae et al. [66]	*In vitro* inflammation model *In vitro*: mouse macrophage-like J774.1 cell line challenged with LPS		RsDPLA treatment decreased LPS response in a dose-dependent fashion as measured by TNF and IL-6 secretion (65% inhibition at 1:3 and 100% at 1:62 LPS:RsDPLA ratio). Mechanism proposed to be through binding of CD14 receptor.
Anwar et al. [58]	*In silico* Molecular dynamics simulation		Simulation predicted inhibitory behavior of RsDPLA on the TLR4/MD2 complex in rodents and humans.
Malgorzata-Miller et al. [62]	Septic shock *In vitro*: Human PBMC challenged with LPS *In vivo*: C57Bl/6 mice injected with LPS	Lipooligosaccharide (LOS) from *Bartonella quintana* (BqLOS)	Human PBMCs pre-incubated with BqLOS (100 ng/mL) was associated with inhibition of LPS-induced response measured by supernatant concentration of IL-1β, TNF-α, IL-6, IL-8 ($p<0.001$). Mice pre-treated with BqLOS (100 μg) had improved survival rates.
De Paola et al. [67]	Amyotrophic lateral sclerosis *In vitro*: Motor neuron/glia co-cultures *In vivo*: Wobbler mice	LPS from *Oscillatoria Planktothrix FP1* (Cyp/VB3323)	*In vitro:* Cells exposed to LPS (1 μg/mL) reduced viability by 30.8±11.9% ($p<0.001$ vs. control). This toxic effect was reduced by VB3323 (20 μg/mL) which almost completely restored motor neuron viability in the cells (91.3±9.9% with $p<0.001$ vs. LPS). *In vivo:* Wobbler mouse with spontaneous motor neuron degeneration chronically treated with VB3323 (5 mg/kg/d i.p., final concentration 0.5 mg/mL) displayed decreased microglial activation and morphological alterations of spinal cord neurons; and better performance in the paw abnormality and grip-strength tests.
Balducci et al. [64]	Alzheimer's disease *In vivo*: C57Bl/6 mice		Amyloid-β oligomers (AβO) injection (7.5 μL at 1 μM) rapidly activated glial cells and induced a memory establishment deficit. When treated with CyP (10 μg, ICV) before AβO, the memory deficit was prevented ($p=0.0055$).
Iori et al. [68]	Seizures *In vivo*: C57Bl/6 mice		Carbamazepine (CBZ) is an anticonvulsant to treat neuropsychiatric disorders. Mice treated with CyP (1 mg/mouse, i.p.) + CBZ (20 mg/mouse, in food) during disease onset. CBZ-treated mice displayed a three-fold higher seizure frequency compared to CyP-treated mice ($p<0.01$). TLR4 antagonism by CyP was effective in delaying seizure onset and reduced recurrence in the established murine model of acquired epilepsy.
Yao et al. [69]	Inflammatory bowel disease *In vitro*: Sprague-Dawley rats injected with 2,4,6-trinitro-benzene sulfonic acid	Probiotics, Golden bifid	Rats treated with the probiotics had a significantly lower disease activity ($p<0.05$), histopathological score ($p<0.05$) and

Table 1 (continued)

Study	Study model	TLR4 antagonist	Outcome
			inflammatory cytokine levels (TNF-α and IL-1β, $p<0.05$) compared to control groups.
Chu et al. [70]	*In silico* Docking analysis	Berberine, extracted from the herb Huang Lian (*Rhizoma coptidis*)	Docking analysis suggested that 3 berberine molecules were able to bind to MD2 and block TLR4/NF-κB downstream signalling. Binding free energies of the 3 berberine molecules was 7.70, −7.33 and −6.75 kcal/mol, respectively.
	Bacterial infection *In vivo*: Balb/c mice challenged with *Salmonella typhimurium* and bacterial endotoxin		Mice treated with 2 EU/mL endotoxin solution (i.p.) had a lethal rate of 80%. When treated with berberine at different doses (0.13, 0.16 and 0.20 g/kg) after endotoxin administration, mice had survival rate of 50%, 50% and 60% respectively. Average death time of each mouse group treated with berberine was significantly better compared to mice only exposed to LPS ($p<0.05$).
Liang et al. [71]	*In vitro* inflammation model *In vitro*: THP-1 human monocyte cells challenged with LPS Sepsis *In vivo*: C57Bl/6 mice administered with LPS	Sparstolonin B (SsnB) extracted from a Chinese herb (*Sparganium stoloniferum*)	*In vitro:* SsnB (100 μM) inhibited LPS-induced (50 ng/mL) response as measured by an 18-fold decrease in TNF-α and 10-fold decrease in IL-6 expression levels vs. LPS-treated cells only. Mechanism proposed to be through binding of the CD14/TLR4 receptor. *In vivo:* Mice co-treated with LPS (100 μg/mouse) and SsnB (100 μg/mouse) displayed lower expression of TNF-α ($p=0.0075$), IL-6 ($p=0.1077$) and IL-1β ($p<0.0001$) vs. LPS-treated mice. SsnB was able to suppress inflammation induced by LPS by attenuating the TLR4-mediated activation of NF-κB.
Li et al. [72]	Leukemia *In vitro*: THP-1 cells treated with LPS	Parthenolide (PTL), extracted from the plant feverfew (*Tanacetum parthenium*)	3 and 12 μM PTL significantly decreased pro-inflammatory cytokine expression and diminished LPS-induced (1 μg/mL) TLR4 expression compared to LPS-treated group ($p<0.01$). PTL was able to inhibit the expression of these cytokines by blocking the TLR4 which in turn blocks the subsequent downstream signalling cascade.
Saadane et al. [73]	Cystic fibrosis *In vitro*: 16 HBE (human bronchial epithelial cell line) transfected with AS oligonucleotide that inhibits expression of CFTR. Stimulated using IL-1β/TNF (100 ng/mL each). *In vivo*: Cystic fibrosis transmembrane conductance regulator (CFTR)–knockout mice challenged with LPS		*In vitro*: At 3 h and 6 h, AS cells pre-treated with PTL (40 μM) had decreased IL-8 secretion vs non-treated cells ($p=0.02$ and 0.03, respectively). *In vivo*: LPS (25 ng, intratracheally)–treated mice had increased polymorphonuclear leukocytes (PMN) (9±1.54% at 1 h, 38.8±7.23% at 3 h and 63±6.0% at 8 h). When co-treated with PTL (3 μg/g), a decrease in PMN % at 8 h was observed ($p=0.006$). Proposed mechanism of action for PTL was NF-κB-dependent inhibition of cellular responses.
Gradišar et al. [74]	*In vitro* inflammation model *In vitro*: Human embryonic kidney (HEK) 293 cells stimulated with LPS.	Curcumin, extracted from the turmeric plant (*Curcuma longa*)	40% inhibition of TLR4/MD2 complex observed at approximately equimolar concentration of curcumin and MD2 in presence of

Table 1 (continued)

Study	Study model	TLR4 antagonist	Outcome
			LPS. Cells co-treated with LPS (100 ng/mL) and higher doses of curcumin (0–20 μM) showed no difference in NF-κB activity.
Zhu et al. [75]	Traumatic brain injury (TBI) *In vivo*: Feeney weight-drop contusion model on C57Bl/6 mice		Injured brain tissue had a significant increase in TLR4 expression vs. sham control brains 24 h post-trauma ($p<0.01$). Curcumin (100 and 200 mg/kg) administered post-trauma reduced TLR4 expression and had lower neurological deficit scores and brain water content vs, vehicle-treated mice with $p<0.01$ and $p<0.05$, respectively. A decrease in concentrations of inflammatory mediators (IL-1β, IL-6, TNF-α, MCP-1) vs. vehicle-treated mice ($p<0.01$) was also observed.
Zhang et al. [76]	Acute lung injury *In vivo*: BALB/c mice with injury induced by LPS	Atractylenolide I (AO-I/ AT-I) extracted from the Chinese herb Cang Zhu (*Rhizoma Atractylodis macrocephalae*)	LPS (10 μg)–treated mice displayed pathological changes: inflammatory cells infiltration, interalveolar septal thickening and edema which were attenuated in co-treated mice (LPS + AO-I at 5, 10 and 20 mg/kg). MPO activity and inflammatory cell infiltrate were reduced in co-treated mice (5 mg/kg: $p<0.01$, 10 mg/kg: $p<0.01$ and 20 mg/kg: $p<0.01$) and (5 mg/kg: $p<0.05$, 10 mg/kg: $p<0.01$ and 20 mg/kg: $p<0.01$) vs. LPS-treated mice, respectively.
Wang et al. [63]	Sepsis *In vivo*: Cecal ligation and puncture (CLP) model of mice		Survival of mice increased with AT-I dose at 10, 20 and 40 mg/kg (i.p.) ($p<0.05$, $p<0.01$, $p<0.01$, respectively) vs. control, respectively. AT-I-treated mice took a shorter time to return to normal temperature ($p<0.05$) and displayed dose-dependent decrease in pro-inflammatory cytokines TNF-α and IL-6 ($p<0.05$, $p<0.01$, $p<0.01$, respectively). Decrease in white blood cells ($p<0.05$) and IL-1β ($p<0.05$ and $p<0.01$, respectively) was observed at 20 and 40 mg/kg doses.
Li et al. [77]	Acute respiratory distress syndrome *In vivo*: BALB/c mice with LPS administered intranasally to induce lung injury	Asiatic acid (AA) extracted from the plant Gotu Kola/Pennywort (*Centella asiatica*)	LPS-treated mice displayed increased lung wet/dry weight ratio, inflammatory cell infiltrate and MPO activity. Co-treated mice (LPS + AA at 25, 50 and 100 mg/kg) displayed decreased lung wet/dry weight ratio ($p<0.05$, $p<0.01$, $p<0.01$, respectively) inflammatory cell infiltrate ($p<0.05$, $p<0.01$, $p<0.01$, respectively) and MPO activity ($p<0.01$, $p<0.01$, $p<0.01$, respectively) vs. LPS group.
Lee et al. [78]	*In vitro* inflammation model *In vitro*: Bone marrow cells isolated from C57Bl/6 mice challenged with LPS	Celastrol extracted from the plant Thunder God Vine (*Tripterygium wilfordii*)	Celastrol (0.1, 0.5 and 1 μM) inhibited LPS-induced (10 ng/mL) responses measured by TNF-α, IL-6, IL-12 and IL-1β at mRNA and protein levels ($p<0.05$). Confocal imaging analysis of celastrol demonstrated decreased co-localisation of fluorescent LPS with MD2.
Yuan et al. [65]	Arthritis *In vivo*: C57Bl/6 mice with induced adjuvant arthritis		Celastrol (0.5 mg/kg) improved clinical outcome via clinical and histopathological scoring vs. non-treated mice ($p<0.01$). Decreased expression of TNF-α (1.9-fold)

Table 1 (continued)

Study	Study model	TLR4 antagonist	Outcome
			and IL-6 (3.1-fold) in celastrol treated mice vs. non-treated mice.
Cho et al. [79]	*In vitro* inflammation model *In vitro*: RAW264.7 cells challenged with LPS	Xanthohumol (XN) extracted from the plant Hops (*Humulus lupulus*)	Cells co-treated with LPS (0.1–0.5 μg/mL) and XN (0.5, 1, 2.5 and 5 μg/mL) displayed a dose-dependent decrease in NO levels (2.5 and 5 μg/mL, $p<0.01$ vs. LPS group), TNF-α (2.5 and 5 μg/mL, $p<0.01$ vs. LPS group) and IL-1β (1, 2.5 and 5 μg/mL, $p<0.01$ vs. LPS group).
Ungaro et al. [35]	Inflammatory bowel disease C57Bl/6J mice with dextran sulphate sodium (DSS) administered in drinking water.	IgG2b monoclonal antibody	Mice co-treated with DSS (2.5%) and IgG2b (20 mg/kg) displayed decrease in expression of TNF-α (141.5±16.3 pg/mL vs. 336±53.8 pg/mL, $p<0.01$), IL-6 (4816±145.5 pg/mL vs. 5850.4±144.4 pg/mL, $p<0.01$) and % dendritic cells in the lamina propria (3.4±0.7 vs. 8.7±0.4%, $p<0.05$) vs. control. No difference in DAI scoring vs. control (1.28±0.19 vs. 1.33±0.23, $p=0.42$, maximum score: 4).
Zhang et al. [32]	Inflammatory bowel disease C57Bl/6 mice with dextran sulphate sodium (DSS) administered in drinking water.	Paeoniflorin extracted from peony root	Mice pre- or co-treated with paeoniflorin (50 mg/kg) and DSS (4%) suppressed weight loss: Pre-paeoniflorin at day 7 ($p<0.05$), co-paeoniflorin at days 5–6 ($p<0.05$), day 7 ($p<0.001$); Diarrhea/bloody diarrhea: Pre-paeoniflorin at days 5 ($p<0.05$), 6 ($p<0.01$), 7 ($p<0.001$), co-paeoniflorin at days 6–7 ($p<0.05$); Shortening of colon length: Pre- and co-paeoniflorin ($p<0.05$); Histological score: Pre- and co-paeoniflorin ($p<0.01$) vs. vehicle control. Paeoniflorin-treated mice had lower expression of TLR4 protein and mRNA vs. DSS only mice ($p<0.001$).

RsDPLA Rhodobacter sphaeroides lipid A, *i.p.* intraperitoneal injection, *LPS* lipopolysaccharide, *TNF-α* tumor necrosis factor alpha, *IL-6* interleukin 6, *CD14* cluster of differentiation 14, *TLR4* toll-like receptor 4, *MD2* myeloid differentiation factor 2, *LOS* lipooligosaccharide, *BqLOS Bartonella quintana*, *PBMC* peripheral blood mononuclear cell, *IL-1β* interleukin 1 beta, *IL-8* interleukin 8, *AβO* amyloid β oligomers, *ICV* intracerebroventricular injection, *CBZ* carbamazepine, *NF-κB* nuclear factor kappaB, *SsnB* Sparstolonin B, *PTL* parthenolide, *HBE* human bronchial epithelial cell line, *AS* allele specific, *CFTR* cystic fibrosis transmembrane conductance regulator, *PMN* polymorphonuclear leukocytes, *HEK* human embryonic kidney, *MCP-1* monocyte chemoattractant protein 1, *AO-I/AT-I* atractylenolide I, *MPO* myeloperoxidase, *CLP* cecal ligation and puncture, *AA* asiatic acid, *mRNA* messenger RNA, *XN* xanthohumol, *NO* nitric oxide, *DSS* dextran sulphate sodium, *DAI* disease activity index

compared to E5531 [83]. Studies on eritoran have shown a positive effect against sepsis [83] and other inflammatory conditions [84, 85]. This led to the development of other synthetic analogues such as TAK-242 and FP7 with antagonistic effects on the TLR4/MD2 complex to treat various inflammatory diseases such as neuroinflammation and influenza infections. Although the main focus of this study is on the therapeutic potentials of TLR4 antagonists on intestinal inflammation, there are a limited number of studies, which used intestinal inflammation as a disease model. Therefore, studies which encompass different inflammatory diseases have been included and summarized in Table 2 in order to show the potential broader anti-inflammatory effects of TLR4 antagonism.

The most well-known TLR4 antagonist to enter the clinical phase was eritoran, followed by TAK-242; and although many synthetic TLR4 antagonists have been developed and studied, very few have actually made it into clinical trials, due to the limited evidence currently available. Table 3 summarises only the TLR4 antagonists that have been, or are, undergoing clinical trials in different inflammatory disease models. However, this will allow for a broader view of using TLR4

Table 2 Summary of the effect of synthetic TLR4 antagonists in pre-clinical *in vitro* and *in vivo* research studies

Study	Study model	TLR4 antagonist	Outcome
Mullarkey et al. [83]	Sepsis *In vivo*: C57Bl/6 mice, Hartley guinea pigs, Fischer rats challenged with i.v. LPS	Eritoran (E5564)	Mice: E5564 (100, 300, 1000 μg/kg) co-treatment was associated with 37%, 81% and 93% inhibition of LPS-induced (100 μg/kg) response as measured by serum TNF-α levels, respectively (p<0.05 vs. control). Guinea pigs: E5564 (30, 100, 300 μg/kg) co-treatment was associated with 29%, 57% and 94% inhibition of LPS-induced (1000 μg/kg) response as measured by serum TNF-α levels, respectively (p<0.05 vs. control). Rats: E5564 (10, 100, 1000 μg/kg) co-treatment was associated with 84%, 97% and 100% inhibition of LPS-induced (3 μg/kg) response as measured by serum TNF-α levels, respectively (p<0.05 vs. control).
Kitazawa et al. [84]	Acute liver failure (ALF) *In vivo*: Wistar rats challenged with D-galactosamine (GalN) and LPS		Rats treated with E5564 after ALF (500 mg/kg GalN + 50 μg/kg LPS) displayed a decrease in serum TNF-α levels and had an improved survival rate of 42.9% compared to untreated rats (p<0.05).
Liu et al. [85]	Inflammatory effects of ischemia-reperfusion in kidneys *In vivo*: Fisher rats with kidney nephrectomy and ischemia performed.		Rats treated with E5564 displayed a significant improvement in renal function as measured by serum creatinine levels (p<0.05) and higher survival rates (p< 0.05) vs. vehicle controls.
Sha et al. [86]	Endotoxin shock *In vivo*: BALB/c mice treated with LPS i.p.	TAK-242 (Resatorvid)	Pre-treatment of TAK-242 (0.1, 0.3, 1 and 3 mg/kg) was associated with a decrease in LPS-induced (10 mg/kg) responses as measured by IL-6, IL-10, MIP-2, IL-1β and NO serum levels vs. vehicle control (p<0.025). A 40% increase in survival rate of mice was also observed vs vehicle control (p≤0.05). Post-treatment of TAK-242 (1 mg/kg) was associated with a decrease in LPS-induced (5 mg/kg) response as measured by IL-6 and MIP-2 serum levels vs. vehicle control (p≤0.01). A survival rate of 45% was also observed vs. vehicle control (p≤0.01).
Kuno et al. [87]	Endotoxemia *In vivo*: Hartley guinea pigs treated with LPS i.v.		TAK-242 (3 and 10 mg/kg) pre-treatment was associated with a dose-dependent decline in colonic muscle tension (p=0.001 and p<0.001, respectively) and mean arterial pressure (p=0.036 and p=0.004, respectively) caused by LPS (10 mg/kg, i.v.). A 50% survival rate was observed when pre-treated with TAK-242 at 10 mg/kg vs. the 10% observed in the control group.
Garate et al. [88]	Neuroinflammation *In vivo*: Wistar Hannover rats restrained to induce stress.		Pre-treatment of TAK-242 (0.5 mg/kg, i.v.) decreased expression of the pro-inflammatory enzymes: IL-1β, COX-2 and iNOS expression levels, p<0.05 vs control, p<0.05 vs stress only group.
Hua et al. [89]	Cerebral ischemia *In vivo*: C57Bl/6 mice induced with focal cerebral ischemia/reperfusion		Treatment with TAK-242 (3 mg/kg) was associated with reduce levels of serum TNF receptor II, monocyte chemotactic protein-1, macrophage inflammatory protein-1γ and tissue inhibitor of metalloproteinases-1 (p<0.05 vs. untreated mice). An 8.8% reduction in brain infarct size and improved neurologic function score (6.73) were also observed (p<0.05 vs. untreated mice).
Perrin-Cocon et al. [90]	Lethal influenza infection *In vitro*: monocyte-derived dendritic cells (DCs) challenged with influenza virus, strain A/PR/8/34 *In vivo*: C57Bl/6 infected with mouse-adapted influenza virus, strain A/PR/8/34	FP7	*In vitro*: FP7 (1 and 10 μM) treatment was associated with decreased levels of LPS-induced (10 ng/mL) responses as measured by supernatant levels of IL-8, IL-6, MIP-1β, TNF-α, IL-12 and IL-10 in both monocytes and DCs (p<0.05 vs. LPS). *In vivo*: Mice treated with FP7 (200 μg/mouse, i.v.) after influenza infection displayed reduced gene production of TNF-α,

Table 2 (continued)

Study	Study model	TLR4 antagonist	Outcome
			IL-1β, IFN-β, murine IL-8 (p<0.01) and IL-6 (p<0.05) in the lungs. FP7-treated mice had decreased viral load (log FP7-treated titre=4.1±0.39) vs. vehicle-treated mice (log vehicle-treated titre=5.27±0.15) as measured by a virus titration assay (p=0.0225).
Palmer et al. [91]	Cardiovascular inflammatory-based diseases *In vitro*: Human umbilical vein endothelial cells (HUVEC), THP-1 and mouse RAW-264.7 macrophages challenged with LPS *In vivo*: Angiotensin II-infused apolipoprotein E-deficient mice		*In vitro*: FP7 (0–10 μM) negatively regulated LPS-induced production (100 ng/mL) of pro-inflammatory cytokines in a dose-dependent manner: THP-1: IL-8 (p<0.001), IL-6 (p<0.01), MIP-1α (p< 0.001) at 5 μM and IL-1β (p<0.001) at 0.1, 1, 5 μM vs. LPS. RAW-264.7: p65 NF-κB at 1, 5, 10 μM (p<0.001), IL-6 at 5 μM (p<0.05), 10 μM (p<0.001) and p38 MAPK at 0.1 (p<0.05), 1 (p<0.01), 5 and 10 μM (p<0.001) vs. LPS. HUVEC: p38 MAPK and p65 NF-κB at 0.1, 0.5 and 1 μM (p<0.01, p<0.05, p<0.01, respectively), MCP-1 at 1 μM (p<0.05) vs. LPS. *In vivo*: FP7 (3 mg/kg/day) inhibited angiotensin II-driven production of pro-inflammatory proteins, and MIP-1γ and JNK phosphorylation (p<0.05 vs. angiotensin II group).
Facchini et al. [92]	Inflammatory bowel disease *In vitro:* Peripheral blood mononuclear cells and lamina propria mononuclear cells collected from patients with IBD *In vivo*: BALB/c mice with DSS administered in their water.		*In vitro*: FP7 at 10 μM negatively regulated LPS-induced production (100 ng/mL) of pro-inflammatory cytokines: mRNA relative expression: TNF-α (p<0.001); IL-1β (p< 0.05); IL-6 (p<0.05). ELISA: TNF-α, IL-1β and IL-6 (p<0.05). *In vivo*: FP7 (250 μg/kg) treatment was associated with a lower histological score (p<0.01 vs. DSS) and significantly reduced the release of inflammatory cytokines TNF-α (p<0.05), IL-1β (p<0.001) and IL-6 (p<0.05).
Huggins et al. [93]	Abdominal aortic aneurysm (AAA) *In vitro*: HUVEC challenged with LPS *In vivo*: C57Bl/6 mice induced with AAA	IAXO-102	*In vitro*: IAXO-102 (10 μM) blocked LPS-stimulated (100 ng/mL) production of JNK, ERK, p65 NF-κB (p< 0.05) and p38, MCP-1, IL-8 (p<0.01) vs. LPS. *In vivo*: IAXO-102 (3 mg/kg/day) blocked angiotensin II-induced response as measured by protein expression of JNK, ERK, p65, NF-κB (p<0.05) vs. angiotensin II only group. IAXO-102 also downregulated expression of MIP-1γ and TLR4 (p<0.05 vs. angiotensin II group) and reduced incidence of AAA (30% IAXO-102-treated vs. 86% angiotensin II group).
Zhang et al. [94]	Acute lung injury (ALI) *In vitro*: Mouse RAW 264.7 macrophages challenged with LPS *In vivo*: Sprague-Dawley rats with ALI induced by intratracheal LPS instillation	Chalcone derivatives - Compound 20	*In vitro*: Fluorescent probe determined compound 20 is a specific inhibitor of MD2 (KD=189μM). Addition of compound 20 (10μM) inhibited LPS-induced (0.5 μg/mL) secretion of TNF-α, IL-1β, COX-2 (p<0.01) and IL-6 (p<0.05) vs. LPS. *In vivo*: Compound 20 (20 mg/kg) reduced LPS-induced (5 mg/-kg) pulmonary edema as measure by the decrease in lung wet/dry weight ratio (p<0.01) vs. LPS. Compound 20 also inhibited IL-1β secretion (p<0.01) and MPO activity (p<0.05) vs. LPS.

Table 2 (continued)

Study	Study model	TLR4 antagonist	Outcome
Wang et al. [95]	Septic shock and lung injury *In vitro*: Mouse primary peritoneal macrophages challenged with LPS *In vivo*: C57Bl/6 mice injected with LPS	Curcumin analogues - L48H37	*In vitro*: Fluorescent probe determined L48H37 is a specific inhibitor of MD2 (KD=11.3 μM). L48H37 (1, 2.5, 5 or 10 μM) inhibited LPS-induced (0.5 μg/mL) phosphorylation in a dose-dependent manner: ERK at 1, 2.5, 5 and 10 μM (p<0.01), p38 at 2.5 μM (p<0.05), 5 and 10 μM (p<0.01), and JNK at 5 and 10 μM (p<0.01) vs. LPS. L48H37 (10 μM) inhibited secretion of TNF-α, IL-6, IL-1β and iNOS (p<0.01 vs. LPS-treated group); IL-10 and COX-2 (p<0.05 vs. LPS-treated group). *In vivo*: L48H37-treated (10 mg/kg) mice had higher survival rates vs. LPS (20 mg/kg, i.v.) (p<0.01). Pulmonary damage and LPS-injured tissue structure of lungs was amended.
Hodgkinson and Ye. [96]	*In vitro* inflammation model *In vitro*: Human embryonic kidney (HEK) 293-CD14-MD2 cells challenged with LPS	Statins - Simvastatin - Pravastatin	Both simvastatin and pravastatin (2 μM) pre-treatment was associated with the inhibition of LPS-induced (5 ng/mL) response as measured by supernatant concentrations of NF-κB, IL-6 and TNF-α (p<0.05 vs. LPS).
Katsargyris et al. [97]	Carotid atherosclerotic plaques *Ex vivo*: atherosclerotic plaques from patients		Patients who used statins had lower TLR4 expression in their endothelial cells and atherosclerotic plaques vs. non-statin patients (p=0.02 and p=0.03, respectively). Prevalence cerebrovascular accident was 18.6% in statin group vs. 61.4% of non-statin group (odds ratio [95% CI] 0.14 [0.07–0.31] p<0.001).
Fort et al. [98]	Inflammatory bowel disease BALB/c mice with DSS administered in their water.	Lipid A-mimetic - CRX-526	CRX-526 (2, 10, 50 μg) treatment was associated with a lower DAI (p=0.421, 0.056, 0.016, respectively) and histological score (p=0.032, 0.008, 0.008, respectively) vs. DSS in a dose-dependent manner.

E5564 eritoran, *LPS* lipopolysaccharide, *IV* intravenous, *TNF-α* tumor necrosis factor alpha, *ALF* acute liver failure, *GalN* D-galactosamine, *i.p.* intraperitoneal, *Resatorvid* TAK-242, *IL-6* interleukin 6, *IL-10* interleukin 10, *MIP-2* macrophage inflammatory protein 2, *IL-1β* interleukin 1 beta, *NO* nitric oxide, *COX-2* cyclooxygenase 2, *iNOS* nitric oxide synthase, *DCs* dendritic cells, *IL-8* interleukin 8, *MIP-1β* macrophage inflammatory protein 1 beta, *IL-12* interleukin 12, *IFN-β* interferon beta, *HUVEC* human umbilical vein endothelial cells, *THP-1* human acute monocytic leukemia cell, *MAPK* mitogen-activated protein kinase, *MIP-1α* macrophage inflammatory protein 1 alpha, *MAPK* mitogen-activated protein kinase, *MIP-1γ* macrophage inflammatory protein 1 gamma, *JNK* c-Jun N-terminal kinase, *IBD* inflammatory bowel disease, *DSS* dextran sulphate sodium, *mRNA* messenger RNA, *AAA* abdominal aortic aneurysm, *HUVEC* human umbilical vein endothelial cell, *ERK* extracellular signal-regulated kinase, *ALI* acute lung injury, *MD2* myeloid differentiation factor 2, *KD* equilibrium dissociation constant, *HEK* human embryonic kidney, *CD14* cluster of differentiation 14, *DAI* disease activity index

antagonists in inflammatory diseases to support its use in intestinal inflammation.

Conclusions

Both IBD and IM have significant effects on a patient's quality of life as well as economic and social burdens [51, 99, 100]. While the pathophysiology for chronic intestinal inflammation remains unknown, previous research has identified that TLR4 signalling in the intestinal tract is a critical regulator of intestinal immune homeostasis. The use of a TLR4 antagonist has potential as a novel therapeutic for IBD and IM patients whose disease pathogenesis relies heavily on TLR4 signalling. Previous studies have shown that inhibiting LPS-induced TLR4 stimulation with antagonists can reduce intestinal inflammation in animal models [101]. Regardless of how promising TLR4 antagonists are in the treatment of intestinal inflammation, there are still challenges in bioavailability and delivery. Nonetheless, anti-TLR4 therapies present a promising alternative for future innovative treatments for both IBD and IM. In the future, there is a need for tissue specific studies looking into these anti-TLR4 therapies in order to mimic the therapeutic setting of IBD and IM.

Table 3 Summary of TLR4 antagonists used in clinical trials

TLR4 antagonist	Condition/disease	Mechanism of action	Clinical trial design and aim	Trial status and outcome	Reference/ clinical trial number
Eritoran (E5564)	Sepsis/severe sepsis/septic shock	Lipid A mimic, binds to MD2	Phase 2, a safety and efficacy study of intravenous E5564 in patients with severe sepsis	Completed. Eritoran appeared well tolerated and showed a lower mortality rate (105 mg dose) in patients with severe sepsis and high predicted risk of mortality.	NCT00046072
			Phase 3, a controlled comparison of eritoran tetrasodium and placebo in patients with severe sepsis	Completed. Patients with severe sepsis did not have reduced 28-day mortality when administered with eritoran, compared with placebo.	NCT00334828
Resatorvid (TAK-242)	Severe sepsis	Binds covalently to Cys747 of TLR4-TIR domain and blocks TLR4/TIRAP and TLR4/TRAM interactions	Phase 3, a pivotal, multicentre, multinational, randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of TAK-242 in adults with severe sepsis	Completed. TAK-242 did not suppress cytokine levels in patients with sepsis. TAK-242 was well tolerated but patients developed mild increases in serum methemoglobin levels.	NCT00143611
			Phase 3, randomized, double-blind, placebo-controlled study of the efficacy and safety of TAK-242 vs. placebo in subjects with sepsis-induced cardiovascular and respiratory failure	Terminated. Business decision; no safety or efficacy concerns.	NCT00633477
NI-0101	Healthy volunteers	Monoclonal antibody blocking TLR4 signalling	Phase 1, randomized double-blind study to determine the safety, tolerability and distribution and elimination of a novel therapeutic drug (NI-0101) when administered to healthy volunteers.	Completed. NI-0101 showed good tolerability, favorable safety and PK profile, and durable anti-inflammatory effect in healthy volunteers.	NCT01808469
	Rheumatoid arthritis		Phase 2, randomized, placebo-controlled, double-blind study to explore the effect of a new antibody to treat patients with rheumatoid arthritis.	Completed. Results unavailable.	NCT03241108

E5564 eritoran, *MD2* myeloid differentiation factor 2, *TAK-242* resatorvid, *TLR4* toll-like receptor 4, *TIR* toll-interleukin receptor domain, *TIRAP* TIR domain–containing adaptor protein, *TRAM* TRIF-related adaptor molecule, *PK* pharmacokinetics

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Compliance with ethical standards

Conflict of interest JSYT, JKC, PAH, CAP, and JMB declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Investigation of TLR4 Antagonists for Prevention of Intestinal Inflammation

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Abstract—Activation of toll-like receptor 4 (TLR4) has been shown to be a major influence on the inflammatory signalling pathways in intestinal mucositis (IM), as demonstrated by TLR4 knock-out mice. Pharmacological TLR4 inhibition has thus been postulated as a potential new therapeutic approach for the treatment of IM but specific TLR4 inhibitors have yet to be investigated. As such, we aimed to determine whether direct TLR4 antagonism prevents inflammation in pre-clinical experimental models of IM. The non-competitive and competitive TLR4 inhibitors, TAK-242 (10 µM) and IAXO-102 (10 µM), respectively, or vehicle were added to human T84, HT-29, and U937 cell lines and mouse colonic explants 1 h before the addition of lipopolysaccharide (LPS) (*in vitro*: 100 µg/mL; *ex vivo*: 10 µg/mL), SN-38 (*in vitro*: 1 µM or 1 nM; *ex vivo*: 2 µM), and/or tumour necrosis factor-alpha (TNF-α) (5 µg/mL). Supernatant was collected for human IL-8 and mouse IL-6 enzyme-linked immunosorbent assays (ELISAs), as a measure of inflammatory signalling. Cell viability was measured using XTT assays. Explant tissue was used in histopathological and RT-PCR analysis for genes of interest: TLR4, MD2, CD14, MyD88, IL-6, IL-6R, CXCL2, CXCR1, CXCR2. SN-38 increased cytostasis compared to vehicle ($P<0.0001$). However, this was not prevented by either antagonist ($P>0.05$) in any of the 3 cell lines. Quantitative histological assessment scores showed no differences between vehicle and treatment groups ($P>0.05$). There were no differences in *in vitro* IL-8 ($P>0.05$, in all 3 cells lines) and *ex vivo* IL-6 ($P>0.05$) concentrations between vehicle and treatment groups. Transcript expression of all genes was similar across vehicle and treatment groups ($P>0.05$). TLR4 antagonism using specific inhibitors TAK-242 and IAXO-102 was not effective at blocking IM in these pre-clinical models of mucositis. This work indicates that specific epithelial inhibition of TLR4 with these compounds is insufficient to manage mucositis-related inflammation. Rather, TLR4 signalling through immune cells may be a more important target to prevent IM.

KEY WORDS: toll-like receptor 4 (TLR4); TLR4 antagonist; intestinal inflammation; intestinal mucositis; SN-38

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INTRODUCTION

Toll-like receptors (TLRs) are an important class of pattern recognition receptors of the innate immune system and are expressed on a variety of both immune cells (macrophages, dendritic cells) and non-immune cells (epithelial cells) in the intestine [1–4]. Each TLR family member contains a ligand-specific extracellular domain and conserved intracellular domain, which allows highly selective responses to intestinal environmental stimuli, including homeostatic, pathogenic, and damage-associated signals [5, 6]. However, TLRs can also amplify immune responses under stress conditions which leads to chronic inflammation [7–11].

TLR4, the best studied TLR family member in the context of infection and inflammation is primarily beneficial to the intestine as it induces an inflammatory response to provide protection from invading bacteria and promotes mucosal integrity [12]. However, TLR4 can also be overexpressed in chronic inflammatory conditions such as inflammatory bowel disease (IBD), whereby people with ulcerative colitis (UC) have a 2.3-fold increase ($P=0.02$) and people with Crohn's disease (CD) have a 1.7-fold increase ($P=0.04$), compared to people who have normal colonic mucosal tissue [13]. Signal transmission mediated by the upregulation of TLR4 promote the sustained release of pro-inflammatory cytokines [14] (e.g. interleukin-1 beta (IL-1β), IL-6, and tumour necrosis factor-alpha (TNF-α)). This, in turn, develops and persists as intestinal inflammation, and has also been associated with risk of inflammation-associated colon cancer [15, 16].

The pathobiology of acute intestinal inflammation as seen in intestinal mucositis (IM) in people with cancer following chemotherapy with irinotecan has also been linked to the activation of TLR4. In IM, TLR4 activation upregulates pro-inflammatory cytokines TNF-α and IL-6 [17]. This occurs via a downstream signalling pathway whereby chemotherapeutic agents cause direct injury to the intestinal epithelial cells, allowing the luminal antigens to enter the lamina propria. Lipopolysaccharides (LPS), or endotoxins, are a product of luminal antigens which activate TLR4 expressed on the intestinal epithelial layer and mucosa-associated immune cells when the luminal antigens cross over the damaged epithelial layer [18]. Subsequently, causing inflammation and ulceration. Ulceration then leads to enhanced translocation of luminal contents and increases the risk of bacteraemia in immunocompromised patients [18]. Previous study has shown that the genetic deletion of TLR4 renders mice resistant to chemotherapy-induced mucositis [19]. However, due to limitations of genetically modified animals in research translation, research efforts are now targeted at tailoring methods of inhibiting TLR4 pharmacologically.

Currently, TLR4 antagonists are being investigated for their potential in managing inflammatory-based diseases such as sepsis and arthritis [20, 21]. TAK-242 is a small-molecule TLR4 inhibitor that interferes with the down-stream signalling mediated by the CD14–TLR4 complex without directly inhibiting the binding of LPS to TLR4 [22]. It had previously undergone clinical trials as a treatment for severe sepsis [23], while IAXO-102 is a synthetic glycolipid that modulates TLR4 activation and signalling by interfering selectively with the TLR4 co-receptors CD14 and MD-2 [24]. IAXO-102 has only been used in experimental studies in abdominal aortic aneurysms [25]. There is a significant lack in studies using these antagonists (such as TAK-242 and IAXO-102) in IBDs such as IM. This study therefore aimed to investigate the potential of the TLR4 antagonists, TAK-242 and IAXO-102, to attenuate intestinal inflammation using *in vitro* and *ex vivo* models.

MATERIALS AND METHODS

Chemicals

TLR4 antagonists TAK-242 (Sapphire Bioscience, Australia) and IAXO-102 (Innaxon, UK) and TLR4 agonists and inflammatory mediators, LPS O55:B5 (Sigma-Aldrich, USA), SN-38 (Tocris Bioscience, United Kingdom) and TNF-α (Research and Diagnostic Systems, United States), were reconstituted according to manufacturer's instructions for *in vitro* and *ex vivo* experiments: TAK-242: DMSO; IAXO-102: DMSO and ethanol; LPS: sterile MilliQ water; SN-38: DMSO; TNF-α: sterile PBS and 0.1% bovine serum albumin.

In Vitro Human Cell Culture

Human colorectal adenocarcinoma cell lines T84 and HT-29 were grown in DMEM (Thermo Fisher Scientific, United States) supplemented with 10% FBS (Scientifix Pty Ltd., Australia) and 1% penicillin–streptomycin (Sigma-Aldrich) to simulate intestinal colonocytes. In contrast, the pro-monocytic, human myeloid leukaemia cell line U937 which are innate monocyte-like immune cells were grown in RPMI-1640 (Thermo Fisher Scientific) supplemented with

10% FBS (Scientifix Pty Ltd.) and 1% penicillin–streptomycin (Sigma-Aldrich). All cells were grown in 75 cm^2 flasks in a 37 °C incubator with 5% CO_2. For all experiments, all cells were used between passages 3 and 20.

T84, HT-29, and U937 cell viability were evaluated using a Cell Proliferation Kit II (XTT) (Merck & Co., United States). One hundred microliters of suspension containing T84: 5×10^4; HT-29: 1×10^4; U937: 3×10^4 cells were seeded in 96-well plates (Corning, USA). The plates were then incubated for 24 h at 37 °C in 5% CO_2. After 24 h incubation, the medium was replaced, and the cells were treated with TAK-242 (10 µM) and IAXO-102 (10 µM) and incubated at 37 °C with CO_2 for 1 h. Cells were then treated with LPS (100 ng/mL), active metabolite of irinotecan SN-38 (T84 & HT-29: 1 µM; U937: 1 nM) and TNF-α (5 µg/mL) and incubated at 37 °C with CO_2 for 36 h. Concentrations of all treatments used as well as incubations times were from previous [25, 26] and extensive dose finding studies. DMSO (0.1%) was used as the vehicle treatment. After the 36-h incubation period, the media was replaced with 100 µL media and 50 µL of XTT solution (composed of 5 mL XTT labelling reagent and 100 µL of electron coupling reagent). The plate was then incubated for 4 h at 37 °C with 5% CO_2. Then, the cell viability was measured using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek, Vermont, United States) at 490 nm. Percentage (%) of cell cytostasis was calculated using the following equation:

$$\text{Cytostasis}(\%) = \left[\frac{\text{A490Vehicle} - \text{A490Treated}}{\text{A490Vehicle}}\right] \times 100$$

Ex Vivo Culture of Mouse Colonic Explants

The study was approved by the University of Adelaide Animal Ethics Committee and complied with the National Health and Research Council Australia Code of Practice for Animal Care in Research and Training (2020). Twelve wild-type C57BL/6 mice (Animal Resource Centre, Australia) were culled via CO_2 inhalation and cervical dislocation. The entire gastrointestinal tract was removed, and the colons were flushed with chilled 1 × phosphate buffered saline (PBS) (Thermo Fisher Scientific) to remove contents. The colon was then divided into 9 equal pieces and stored in chilled 1 × PBS. Each piece was cut longitudinally along the mesentery line, flattened onto a piece of manila paper and placed lumen side down in a 24-well plate (Corning) containing RPMI (400 µL) media and stored in an incubator at 37 °C at 5% CO_2 for 10 m to equilibrate. Tissue was pre-treated with TAK-242 (10 µM) and IAXO-102 (10 µM) for 1 h before the administration of LPS (100 ng/mL) and SN-38 (2 µM) for 3 h at 37 °C at 5% CO_2. After 3 h, the tissue and supernatant were collected and processed. Concentrations of all treatments used as well as incubation times were taken from the *in vitro* study. DMSO was used as the vehicle treatment. Following treatment, all explant supernatant was collected and stored at −20 °C for enzyme-linked immunosorbent assays (ELISAs). In addition, the explant tissue was either fixed in 10% neutral buffered formalin (ChemSupply Australia Pty Ltd., Australia) for 24 h, transferred to 70% ethanol (ChemSupply Australia Pty Ltd.) and embedded in paraffin wax (ChemSupply Australia Pty Ltd.) for histopathological analysis, or immediately snap-frozen in liquid nitrogen and stored at −80 °C for real-time polymerase chain reaction (RT-PCR) analysis.

Histopathological Analysis of Distal Colonic Explant Tissue

Haematoxylin and eosin (H&E) staining was performed using 5 µm sections of the embedded explant tissue, cut on a rotary microtome and mounted onto glass microscope slides (Thermo Fisher Scientific). Slides were scanned and assessed (× 100 magnification) using a NanoZoomer 2.0-HT slide scanner (Hamamatsu Photonics, Shizuoka Pref., Japan). All slides underwent qualitative histopathological assessment to generate an injury score. The histological criteria used in the assessment were as follows: epithelial disruption; crypt loss; crypt abscesses; goblet cell loss; oedema; submucosal thickening; muscularis externa thickening; and polynuclear cell infiltration [27]. Each parameter was scored as: 0 = absent; 1 = mild; 2 = moderate; or 3 = severe, with a possible maximum score of 24.

Immunohistochemistry Assessment of Cellular Markers of Apoptosis and Proliferation

Immunohistochemistry (IHC) was carried out on 5 µm sections of explant tissue, cut on a rotary microtome and mounted onto FLEX IHC microscope slides (Flex Plus Detection System, Dako; #K8020). IHC analysis was performed for caspase-3 (Abcam; #ab4051), a marker of apoptosis, and Ki67 (Abcam; #ab16667), a marker of

proliferation. Changes in both parameters are validated markers for altered tissue kinetics and an excellent way to assess the subclinical severity of toxicity [28]. IHC analysis was performed using Dako reagents on an automated machine (AutostainerPlus, Dako; #AS480) following standard protocols supplied by the manufacturer. Briefly, sections were deparaffinised in xylene and rehydrated through graded ethanols before undergoing heat-mediated antigen retrieval using an EDTA/Tris buffer (0.37 g/L EDTA, 1.21 g/L Tris; pH 9.0). Retrieval buffer was preheated to 65 °C using the Dako PT LINK (pretreatment module; Dako; #PT101). Slides were immersed in the buffer, and the temperature was raised to 97 °C for 20 min. After returning to 65 °C, slides were removed and placed in the Dako AutostainerPlus (Dako; #AS480) and stained following manufacturer's guidelines. Negative controls had the primary antibody omitted. Slides were scanned using the NanoZoomer (Hamamatsu Photonics) and assessed with NanoZoomer Digital Pathology software view.2 (Histalim). The criteria used in the assessment were as follows according to percentage (%) of area positively stained for either Ki67 or Caspase 3: $<25\% = 0$; $25\% = 1$; $50\% = 2$; $75\% > = 3$.

RT-PCR of Human Cell Culture and Colonic Explant Tissue

RNA was isolated from T84, HT-29, U937 and snap frozen colonic intestinal explant tissue using the NucleoSpin RNA Plus kit (Scientifix Pty Ltd., Victoria, Australia) following the manufacture's protocol. RNA was quantified using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek) and reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, California, United States) according to the manufacturer's protocol. cDNA was quantified using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek) and diluted to a working concentration of 100 ng/µL. Primers for genes of interest were designed using web-based primer design program, PRIMER 3 (v. 0.4.0) [29, 30] and manufactured by Sigma-Aldrich (Missouri, United states). A list of all the primers used is shown in Table 1. Amplified transcripts were detected by SYBR Green (Quantitect, Qiagen Pty Ltd., Victoria, Australia) in a Rotor-Gene Q Series Rotary Cycler (Qiagen Pty Ltd., Victoria, Australia). All reactions were completed in triplicate. Fold change in mRNA expression was calculated using the $2^{(\text{delta CT})}$ ($2^{\Delta Ct}$) method using GAPDH as the housekeeper gene [31].

ELISAs of Human Cell Culture and Mouse Colonic Explant Supernatants

Human IL-8 production was measured in cell culture supernatant using an ELISA kit (Abcam, Cambridge, United Kingdom) following the manufacturer's instructions. Mouse IL-6 production was measured in intestinal explant culture supernatant using an ELISA kit (Invitrogen, Massachusetts, United States) following the manufacturer's instructions. Absorbance was measured at 450 nm using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek). Absorbance output was calculated and converted into protein concentration using a standard curve from the ELISA kit (IL-8: 1000 – 31.25 pg/mL; IL-6: 500 – 4 pg/mL) and the GraphPad Prism Software version 9.0 (GraphPad® Software, USA).

Statistical Analysis

Data was graphed and analysed using the GrahPad Prism Software 9.0 (GraphPad® Software, San Diego, USA). A Kruskal–Wallis test with Dunn's multiple comparisons test was performed on non-parametric data to compare between the treatment groups. An ordinary one-way

Table 1 RT-PCR primer sequences designed by PRIMER 3, version 0.4.0

Gene	Primer sequences
Mouse TLR4	Forward: 5'-CTC TGC CTT CAC TAC AGA GAC-3' Reverse: 5'-TGG ATG ATG TTG GCA GCA ATG-3'
Mouse MD2	Forward: 5'- GTC CGA TGG TCT TCC TGG CGA GT-3' Reverse: 5' GCT TCT CAG ATT CAG TCA ATA TGG G-3'
Mouse CD14	Forward: 5'- GTC AGG AAC TCT GGC TTT GC-3' Reverse: 5' GGC TTT TAC CCA CTG AAC CA-3'
Mouse MyD88	Forward: 5'- GGA GCC AGA TTC TCT GAT GC-3' Reverse: 5' TGT CCC AAA GGA AAC ACA CA-3'
Mouse IL-6	Forward: 5'- AGT TGC CTT CTT GGG ACT GA-3' Reverse: 5' TCC ACG ATT TCC CAG AGA AC-3'
Mouse IL-6 Receptor	Forward: 5'- TGA ATG ATG ACC CCA GGC AC-3' Reverse: 5' ACA CCC ATC CGC TCT CTA CT-3'
Mouse CXCL2	Forward: 5'- AAG TTT GCC TTG ACC CTG AA-3' Reverse: 5' AGG CAC ATC AGG TAC GAT CC-3'
Mouse CXCR1	Forward: 5'- GGG TGA AGC CAC AAC AGA TT-3' Reverse: 5' GCA GAC CAG CAT AGT GAG CA-3'
Mouse CXCR2	Forward: 5'- GCA GAG GAT GGC CTA GTC AG-3' Reverse: 5' TCC ACC TAC TCC CAT TCC TG-3'
Mouse GAPDH (housekeeper)	Forward: 5'- CCT CGT CCC GTA GAC AAA ATG-3' Reverse: 5' TCT CCA CTT TGC CAC TGC AA-3'

ANOVA with Tukey's multiple comparisons test was performed on parametric data to compare between the treatment groups. Any data point that had a higher value than 3 times the standard deviation from the mean was excluded. P-values of <0.05 were considered statistically significant.

RESULTS

Effect of TAK-242 and IAXO-102 Treatment on Cell Viability (Cytostasis)

Since a hallmark feature of IM leading to inflammation is cell loss, we measured cytostasis in three different cell lines: T84, HT-29, and U937. The TLR4 antagonists, TAK-242 (10 μM) and IAXO-102 (10 μM), alone did not cause cytostasis ($P > 0.05$, Fig. 1). However, they also did not provide protection against cytostasis following treatments with LPS (100 μg/mL), TNF-α (5 μg/mL), and SN-38 treatment (T84 and HT-29: 1 μM; U937: 1 nM) in any of the cell lines ($P > 0.05$, Fig. 1).

Effect of IAXO-102 Treatment on IL-8 Production

In human models of inflammation, IL-8 is a key downstream cytokine released following TLR4 activation. As such, IL-8 secretion was tested in intestinal and immune cell lines. All 3 cell lines produced an IL-8 secretory response following treatment (Fig. 2). Due to the similarity observed in the cytostasis results of all 3 cell lines, we proceeded to focus only on IAXO-102 due to its novel aspect compared to TAK-242 which is already a popular research compound. We found the TLR4 antagonist IAXO-102 (10 μM), alone did not cause

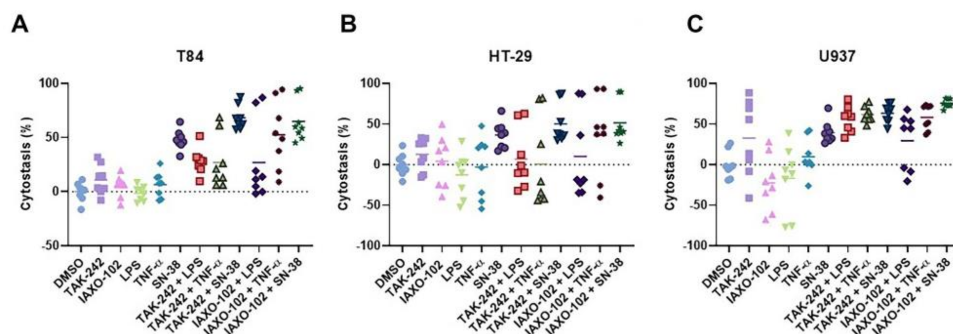


Fig. 1 Effect of TLR4 antagonism on cell cytostasis following LPS (100 μg/mL), TNF-α (5 μg/mL), and SN-38 treatment (T84 and HT-29: 1 μM; U937: 1 nM) in (A) T84, (B) HT-29, and (C) U937 cell lines. 0.1% DMSO was used as the vehicle. **A** T84: DMSO *vs.* SN-38 ($P < 0.0001$), DMSO *vs.* TAK-242 + SN-38 ($P < 0.0001$), DMSO *vs.* IAXO-102 + TNF-α ($P < 0.0001$), DMSO *vs.* IAXO-102 + SN-38 ($P < 0.0001$), TAK-242 *vs.* SN-38 ($P < 0.01$), TAK-242 *vs.* TAK-242 + SN-38 ($P < 0.0001$), TAK-242 *vs.* IAXO-102 + TNF-α ($P < 0.01$), TAK-242 *vs.* IAXO-102 + SN-38 ($P < 0.0001$), IAXO-102 *vs.* SN-38 ($P < 0.001$), IAXO-102 *vs.* TAK-242 + SN-38 ($P < 0.0001$), IAXO-102 *vs.* IAXO-102 + TNF-α ($P < 0.001$), IAXO-102 *vs.* IAXO-102 + SN-38 ($P < 0.0001$), LPS *vs.* SN-38 ($P < 0.0001$), LPS *vs.* TAK-242 + SN-38 ($P < 0.0001$), LPS *vs.* IAXO-102 + TNF-α ($P < 0.0001$), LPS *vs.* IAXO-102 + SN-38 ($P < 0.0001$), TNF-α *vs.* SN-38 ($P < 0.01$), TNF-α *vs.* TAK-242 + SN-38 ($P < 0.0001$), TNF-α *vs.* IAXO-102 + TNF-α ($P < 0.001$), TNF-α *vs.* IAXO-102 + SN-38 ($P < 0.0001$), TAK-242 + LPS *vs.* TAK-242 + SN-38 ($P < 0.01$), TAK-242 + LPS *vs.* IAXO-102 + SN-38 ($P < 0.0001$), TAK-242 + TNF-α *vs.* TAK-242 + SN-38 ($P < 0.01$), TAK-242 + TNF-α *vs.* IAXO-102 + SN-38 ($P < 0.0001$), TAK-242 + SN-38 *vs.* IAXO-102 + LPS ($P < 0.01$), IAXO-102 + LPS *vs.* IAXO-102 + SN-38 ($P < 0.0001$). **B** HT-29: LPS *vs.* TAK-242 + SN-38 ($P < 0.05$), LPS *vs.* IAXO-102 + SN-38 ($P < 0.05$). **C** U937: DMSO *vs.* TAK-242 + LPS ($P < 0.01$), DMSO *vs.* TAK-242 + TNF-α ($P < 0.001$), DMSO *vs.* TAK-242 + SN-38 ($P < 0.001$), DMSO *vs.* IAXO-102 + TNF-α ($P < 0.001$), DMSO *vs.* IAXO-102 + SN-38 ($P < 0.0001$), TAK-242 *vs.* IAXO-102 ($P < 0.01$), TAK-242 *vs.* LPS ($P < 0.05$), IAXO-102 *vs.* SN-38 ($P < 0.001$), IAXO-102 *vs.* TAK-242 + LPS ($P < 0.0001$), IAXO-102 *vs.* TAK-242 + TNF-α ($P < 0.0001$), IAXO-102 *vs.* TAK-242 + SN-38 ($P < 0.0001$), IAXO-102 *vs.* IAXO-102 + LPS ($P < 0.01$), IAXO-102 *vs.* IAXO-102 + TNF-α ($P < 0.0001$), IAXO-102 *vs.* IAXO-102 + SN-38 ($P < 0.0001$), LPS *vs.* SN-38 ($P < 0.01$), LPS *vs.* TAK-242 + LPS ($P < 0.0001$), LPS *vs.* TAK-242 + TNF-α ($P < 0.0001$), LPS *vs.* TAK-242 + SN-38 ($P < 0.0001$), LPS *vs.* IAXO-102 + LPS ($P < 0.05$), LPS *vs.* IAXO-102 + TNF-α ($P < 0.0001$), LPS *vs.* IAXO-102 + SN-38 ($P < 0.0001$), TNF-α *vs.* TAK-242 + LPS ($P < 0.05$), TNF-α *vs.* TAK-242 + TNF-α ($P < 0.01$), TNF-α *vs.* TAK-242 + SN-38 ($P < 0.01$), TNF-α *vs.* IAXO-102 + TNF-α ($P < 0.05$), TNF-α *vs.* IAXO-102 + SN-38 ($P < 0.001$), IAXO-102 + LPS *vs.* IAXO-102 + SN-38 ($P < 0.05$). Data are presented as mean ($n = 8$ per group).

an IL-8 secretory response ($P > 0.05$, Fig. 2). However, they also did not suppress any IL-8 secretory responses following treatments with LPS (100 µg/mL), TNF-α (5 µg/mL), and SN-38 treatment (T84 and HT-29: 1 µM; U937: 1 nM) in any of the cell lines ($P > 0.05$, Fig. 2).

Histopathological Analysis and Immunohistochemistry (IHC) Assessment of Cellular Markers of Apoptosis and Proliferation of Mouse Colonic Explants

The ability to model inflammation in single cell lines is limited, thus we adapted a colonic explant model to further examine TLR4 signalling in mucositis development. No histological changes were observed in the mouse colon explants after treatment with DMSO (0.2%), TAK-242 (10 µM), IAXO-102 (10 µM), LPS (100 µg/mL), and SN-38 (2 µM) (Fig. 3A). All sections showed infiltration of neutrophils and disruption of the epithelial layer with no distinguishable differences observed between colon tissue treated with the TLR4 antagonists, TAK-242 and IAXO-102, or pro-inflammatory mediators (Fig. 3A). This is supported by no differences in the quantitative histopathological scores in the colonic explants following any treatments ($P > 0.05$, Fig. 3D).

To follow up on the cell viability assay, Ki67 IHC staining was used to determine cell proliferation while Caspase 3 IHC staining was used to determine presence of apoptotic cells in the explants treated with DMSO (0.2%), TAK-242 (10 µM), IAXO-102 (10 µM), LPS (100 µg/mL), and SN-38 (2 µM) (Fig. 3B, C, respectively). All sections displayed widely distributed staining of Ki67 with no distinguishable differences observed between colon tissue treated with the TLR4 antagonists, TAK-242 and IAXO-102, or pro-inflammatory mediators (Fig. 3B). This is supported by no differences in the quantitative scores in the colonic explants following any treatments ($P > 0.05$, Fig. 3E). As for caspase 3, apoptosis was observed to be decreased in the explant tissues treated with both the antagonist and inflammatory mediator compared to explants tissues treated with either DMSO only, antagonist only, or inflammatory mediator only (Fig. 3F).

Secretion of Pro-inflammatory Cytokine IL-6 from Mouse Colonic Explants

Histological visualisation is not sufficient to evaluate release of pro-inflammatory signals that may contribute to mucositis development. As such, we measured secretion of the key inflammatory cytokine linked to intestinal tissue inflammation in mucositis, IL-6. Inflammatory mediators and TLR4 agonists LPS (100 µg/mL) and SN-38 (2 µM) did not significantly increase the IL-6 secretion in the explant media ($P > 0.05$, Fig. 4). In addition, the TLR4 antagonists TAK-242 (10 µM) and IAXO-102 (10 µM) did not cause IL-6 secretion alone or alter the secretion of IL-6 following treatments ($P > 0.05$, Fig. 4).

Effect of TAK-242 and IAXO-102 Treatment on Gene Expression in Colonic Mouse Explants

We decided to look at the levels of expression of genes associated with the TLR4/MD2 downstream signalling pathway. LPS (100 µg/mL) and SN-38 (2 µM) stimulation did not result in higher transcription levels of the associated genes TLR4, MD2, MyD88, CD14, IL-6, IL-6R, CXCL2, CXCR1, and CXCR2 ($P > 0.05$, Fig. 5). In addition, there was no change observed in gene expression when the explants had been pre-treated with the TLR4 antagonists TAK-242 (10 µM) and IAXO-102 (10 µM) ($P > 0.05$, Fig. 5).

DISCUSSION

IM is defined as the inflammation of the mucosa of the intestinal tract and is a side-effect of high dose chemotherapy. Patients who develop IM will often suffer from its side-effects such as pain, nausea, and diarrhoea. These side-effects can be so debilitating that the life-saving treatments patients with cancer undergo need to be stopped, which considerably effects their survival. However, TLR4 signalling has been strongly implicated in the development of intestinal mucositis. Evidence from *in vitro* studies using human cells and *in vivo* studies using animal models supports the hypothesis that the activation of TLR4 is related to the pathogenesis of intestinal inflammation [19, 32]. Studies have also shown evidence that when TLR4 is inhibited, there was a decrease in inflammatory infiltrate and protection against damage. For example, a study by Ungaro et al. showed that mice with intestinal inflammation that were pre-treated with TAK-242 had a decreased disease activity index (DAI) score and IL-6 secretion ($P < 0.05$ *vs.* disease only) [12]. While a study by Fort et al. found that the DAI and histological score was significantly decreased ($P < 0.05$ *vs.*

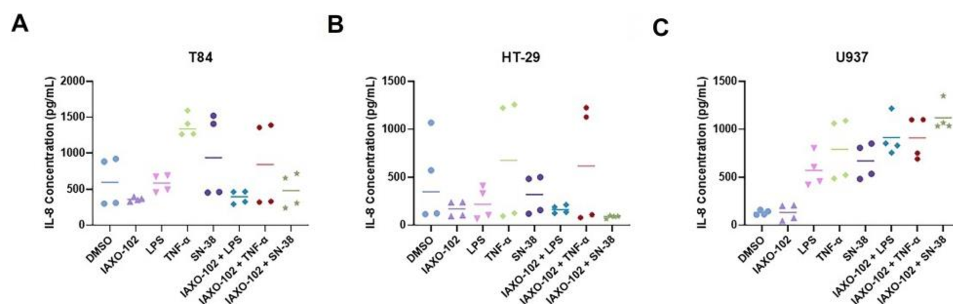


Fig. 2 Effect of the TLR4 antagonist IAXO-102 (10 μM) on suppression of IL-8 secretion following LPS (100 μg/mL), TNF-α (5 μg/mL), and SN-38 treatment (T84 and HT-29: 1 μM; U937: 1 nM) in **A** T84, **B** HT-29, and **C** U937 cell lines. 0.1% DMSO was used as the vehicle. There was no significant difference in IL-8 secretion between the treated and vehicle groups in the T84 and HT-29 cell lines ($P > 0.05$). **C** U937: DMSO *vs.* TNF-α ($P < 0.01$), DMSO *vs.* SN-38 ($P < 0.05$), DMSO *vs.* IAXO-102+LPS ($P < 0.001$), DMSO *vs.* IAXO-102+TNF-α ($P < 0.001$), DMSO *vs.* IAXO-102+SN-38 ($P < 0.0001$), IAXO-102 *vs.* TNF-α ($P < 0.01$), IAXO-102 *vs.* SN-38 ($P < 0.05$), IAXO-102 *vs.* IAXO-102+LPS ($P < 0.001$), IAXO-102 *vs.* IAXO-102+TNF-α ($P < 0.001$), IAXO-102 *vs.* IAXO-102+SN-38 ($P < 0.0001$), LPS *vs.* IAXO-102+SN-38 ($P < 0.01$), SN-38 *vs.* IAXO-102+SN-38 ($P < 0.05$). Data are presented as median (T84 and HT-29) and mean (U937) ($n = 4$ per group).

vehicle) in mice pre-treated with a synthetic TLR4 antagonist CRX-526 (50 μg) [33].

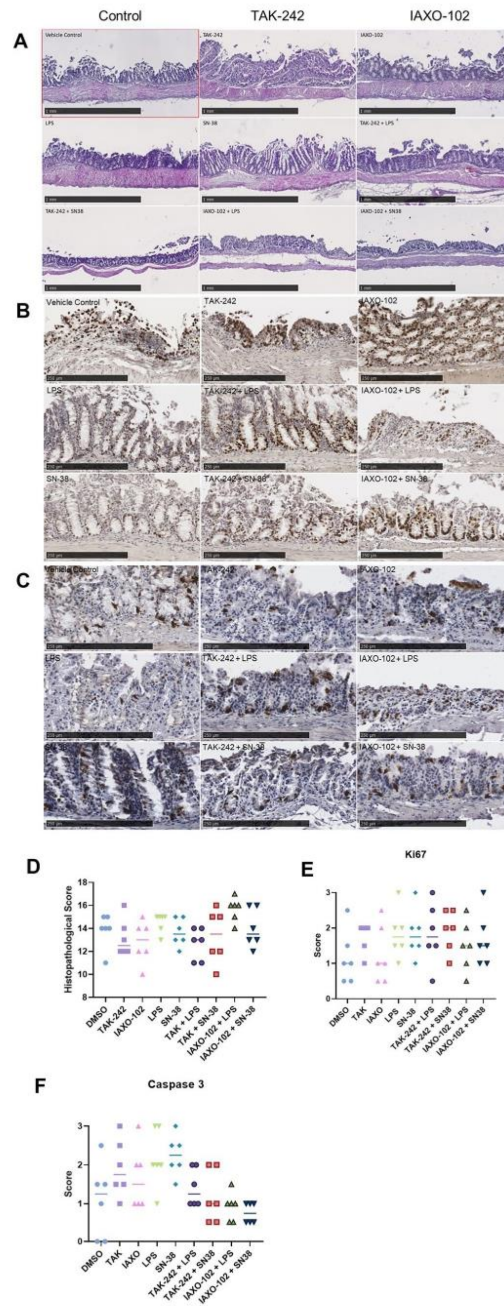
Therefore, the TLR4 activation and signalling provides a strong target for pharmacological intervention. This study therefore aimed to determine how TLR4 inhibition protects against intestinal mucositis using preclinical models of inflammation. It was the first time the specific TLR4 antagonists, TAK-242 and IAXO-102, have been investigated in such models. We observed that these TLR4 antagonists did not display any toxic side effects in the preclinical models of inflammation.

In our *in vitro* model, the cell lines T84, HT-29, and U937 had different relative expression levels of TLR4, but this did not impact their response to TLR4 antagonists. The concentration of TLR4 antagonists used in this study was determined by previous studies using the same compounds [25, 34]. All three cell lines displayed a relatively similar reaction as seen in our results of cell cytostasis and secretion of inflammatory mediator IL-8. However, the TLR4 antagonists were not able to prevent any inflammation or cell death induced by LPS, TNF-α, and SN-38. Although it was observed that the TLR4 antagonists did not protect against SN-38 in our cell cytostasis results, this is consistent with studies showing that TLR4 is required for healing in colitis [12].

When we observed the results from the human IL-8 ELISA, it was observed that all 3 cell lines secreted the inflammatory cytokine after being treated with LPS, TNF-α, and SN-38. However, when the cell lines with LPS, TNF-α, and SN-38 were pre-treated with IAXO-102, there was no decrease in IL-8 observed. Compared to a study by Huggins et al. [25] stimulated human umbilical cord vein endothelial cells (HUVEC) with LPS (100 ng/mL) with and without pre-treatment of IAXO-102 (1 and 10 μM), with IAXO-102 (10 μM) inhibiting the secretion of IL-8 ($P < 0.01$ against LPS only) [25].

One possible reason for the contradictory *in vitro* results is that the intestinal epithelium, i.e. the T84 and HT-29 cells, must remain mute to the presence of commensal flora and bacterial pathogens to avoid a constant need to defend the intestinal environment against invading pathogens. The colonic epithelial cell types would also be exposed heavily to LPS as they are the main protective barrier between the lumen and the lamina propria. Due to the constant exposure to LPS, these cell types may limit their response to LPS and bacterial pathogens by downregulating the TLR4/MD2 expression [32]. Another limitation is that immortalised cells do not mimic a human intestinal tract as they are derived from human tumour cells. As such, TLR4 signalling may not reflect the healthy intestine and primary cell lines may need to be considered for future work.

This limitation was why an explant model was used in the present study, to better mimic a healthy intestinal tract system. However, similar results were observed in the explant model of intestinal inflammation as were observed in the *in vitro* cell lines;


Control
TAK-242
IAXO-102
A
B
C
D
Histopathological Score
E
Ki67
Score
F
Caspase 3
Score
DMSO
TAK-242
IAXO-102
LPS
SN-38
TAK + LPS
TAK + SN-38
IAXO-102 + LPS
IAXO-102 + SN-38

◂**Fig. 3** **A** Representative H&E stained colonic explants following treatments. 0.2% DMSO was used as the vehicle. **D** Histopathological analysis of the H&E images from multiple mice. 0.2% DMSO was used as the vehicle. No significant differences in histopathological scores in the colonic explants between the treated and vehicle tissue was observed ($P > 0.05$). Data are presented as median ($n = 6$ per group). **B** Representative images of colonic explants following treatments stained with Ki67 (brown staining). **C** Representative images of colonic explants following treatments stained with Caspase 3 (brown staining). **E** Analysis and scoring of the colonic explants stained with Ki67. No significant differences in scores in the colonic explants between the treated and vehicle tissue was observed ($P > 0.05$). **F** Analysis and scoring of the colonic explants stained with Caspase 3. DMSO *vs.* TAK-242 ($P < 0.01$), DMSO *vs.* LPS ($P < 0.0001$), DMSO *vs.* SN-38 ($P < 0.0001$), TAK-242 *vs.* TAK-242 + SN-38 ($P < 0.01$), TAK-242 *vs.* IAXO-102 + LPS ($P < 0.0001$), TAK-242 *vs.* IAXO-102 + SN-38 ($P < 0.0001$), IAXO-102 *vs.* IAXO-102 *vs.* LPS ($P < 0.01$), IAXO-102 *vs.* IAXO-102 *vs.* SN-38 ($P < 0.001$), LPS *vs.* TAK-242 + LPS ($P < 0.01$), LPS *vs.* TAK-242 + SN38 ($P < 0.0001$), LPS *vs.* IAXO-102 + LPS ($P < 0.0001$), LPS *vs.* IAXO-102 + SN38 ($P < 0.0001$), SN-38 *vs.* TAK-242 + LPS ($P < 0.01$), SN-38 *vs.* TAK-242 + SN38 ($P < 0.0001$), SN-38 *vs.* IAXO-102 + LPS ($P < 0.0001$), SN-38 *vs.* IAXO-102 + SN38 ($P < 0.0001$). 0.2% DMSO was used as the vehicle. Data presented as median (Ki67) and mean (Caspase 3), $n = 6$ per group.

no significant protection against inflammation was observed in colonic explants treated with TLR4 antagonists and the inflammatory mediators or TLR4 agonists. This indicates that inhibition of TLR4 was not able to suppress the inflammatory response. The unexpected lack of response highlights the limitations of *ex vivo* explant models in cytotoxic studies and that IL secretion did not reflect the damage in colon architecture and morphology caused by cytotoxics in the colon.

Possible explanations for these results maybe that it was too late to inhibit TLR4 to see a significant reaction. Due to the nature of explants, inflammatory signals would have been released and cells would undergo apoptosis when the intestine was removed from the mouse. When the tissue was divided into the wells, the media would have been saturated with pro-inflammatory mediators as seen in the increase in IL-6 secretion in our colonic explant model. When the TLR4 antagonist was finally added to the tissue, a difference in cytokine secretions would be unidentifiable. Which may be why TLR4 knockout models of mice are so effective at preventing mucositis. A similar reason can be used to explain the variability seen in the gene expression of the explant tissue. Whereby due to the degradation of the colonic explants, the DNA extracted from the tissue may have been compromised.

However, previous studies using intestinal explants have been quite successful. For example, a study by Guabiraba et al. [26] reported no increase in

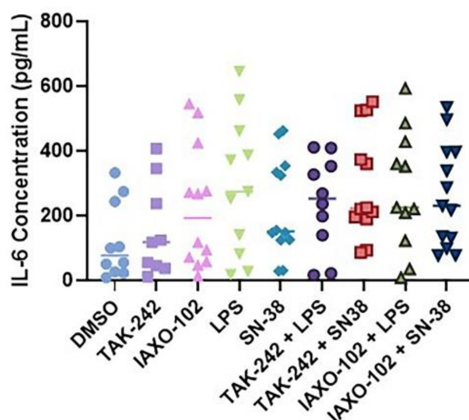


Fig. 4 IL-6 secretion from mouse colonic explant supernatant following various treatments: DMSO (0.2%), TAK-242 (10 µM), IAXO-102 (10 µM), LPS (100 µg/mL), and SN-38 (2 µM). TAK-242 and IAXO-102 did not significantly inhibit IL-6 concentration after treatment with inflammatory mediators and TLR4 agonists LPS and SN-38 ($P > 0.05$). Data are presented as median ($n = 13$ per group).

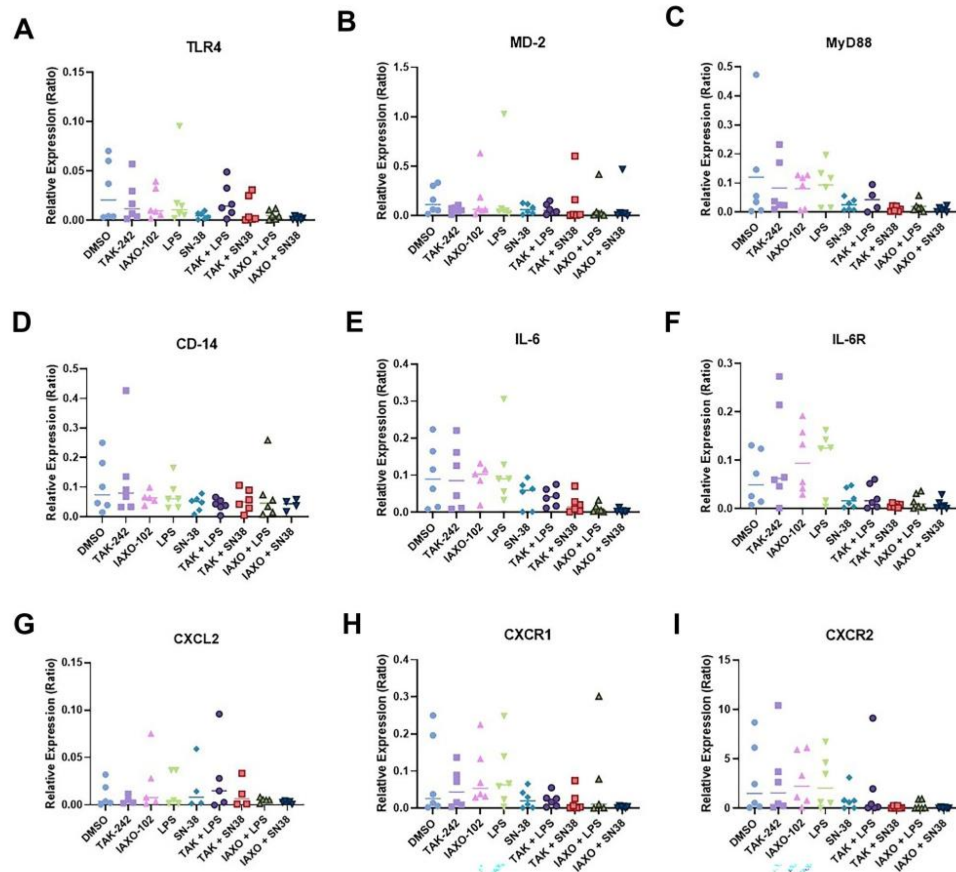


Fig. 5 Relative gene expression of **A** TLR4, **B** MD2, **C** MyD88, **D** CD14, **E** IL-6, **F** IL-6R, **G** CXCL2, **H** CXCR1, and **I** CXCR2 from mouse colonic explants following various treatments: TAK-242 (10 μM), IAXO-102 (10 μM), LPS (100 μg/mL), and SN-38 (2 μM). 0.2% DMSO was used as the vehicle. No significant upregulation of the genes was observed in the tissue treated with LPS and SN-38 ($P > 0.05$). No significant downregulation of the genes was observed in tissue pre-treated with TAK-242 and IAXO-102 ($P > 0.05$). Data are presented as median ($n = 6$ per group).

the pro-inflammatory cytokine IL-33 in their colonic explants, but when SN-38 was added, there was a significant increase in the IL-33 levels ($P < 0.001$ *vs.* vehicle) [26]. Mouse intestinal explants are a popular model to use in a variety of studies [35, 36]. However, none of these studies has provided any histology on the tissue. As such, our study extends knowledge in the field and limitation of explant tissue models. As such, whole animal studies are preferable.

In conclusion, the results demonstrated no protective capabilities of TLR4 antagonism against the *in vitro* and *ex vivo* intestinal inflammation models. Although T84 and HT-29 cells mimic the colonic epithelial cell phenotype in many regards, these cell lines

cannot replicate all stages of colonic epithelial cell differentiation and lack key microbiomes that may be important for TLR4 signalling [37]. The explant models would also need to be refined to prevent the tissue from degrading rapidly in order to provide more consistent results or more viable, long-term models such as organoids, or other co-culture systems considered. Further investigation on the specific binding sites of these TLR4 antagonists should also be considered. Therefore, our data must be interpreted with the considerations of the inherent limitations of these systems. However, we were able to fill a knowledge gap in the explant model with our histology findings.

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AUTHOR CONTRIBUTION

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Shu Yie Janine Tam. The first draft of the manuscript was written by Shu Yie Janine Tam and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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DECLARATIONS

Ethics Approval The study was approved by the University of Adelaide Animal Ethics Committee and complied with the National Health and Research Council Australia Code of Practice for Animal Care in Research and Training (2020).

Competing Interests The authors declare no competing interests.

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ORIGINAL ARTICLE

Effects of a novel toll-like receptor 4 antagonist IAXO-102 in a murine model of chemotherapy-induced gastrointestinal toxicity

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Abstract

Introduction Gastrointestinal mucositis (GIM) is a side effect of high-dose irinotecan (CPT-11), causing debilitating symptoms that are often poorly managed. The role of TLR4 in the development of GIM has been clearly demonstrated. We, therefore, aimed to investigate the potential of the TLR4 antagonist, IAXO-102, to attenuate gastrointestinal inflammation as well as supress tumour activity in a colorectal-tumour-bearing mouse model of GIM induced by CPT-11.

Methods 24 C57BL/6 mice received a vehicle, daily i.p. IAXO-102 (3 mg/kg), i.p. CPT-11 (270 mg/kg) or a combination of CPT-11 and IAXO-102. GIM was assessed using validated toxicity markers. At 72 h, colon and tumour tissue were collected and examined for histopathological changes and RT-PCR for genes of interest; TLR4, MD-2, CD-14, MyD88, IL-6, IL-6R, CXCL2, CXCR1, and CXCR2.

Results IAXO-102 prevented diarrhoea in mice treated with CPT-11. Tumour volume in IAXO-102-treated mice was lower compared to vehicle at 48 h ($P<0.05$). There were no differences observed in colon and tumour weights between the treatment groups. Mice who received the combination treatment had improved tissue injury score ($P<0.05$) in the colon but did not show any improvements in cell proliferation or apoptotic rate. Expression of all genes was similar across all treatment groups in the tumour ($P>0.05$). In the colon, there was a difference in transcript expression in vehicle vs. IAXO-102 ($P<0.05$) and CPT-11 vs. combination ($P<0.01$) in MD-2 and IL-6R, respectively.

Conclusion IAXO-102 was able to attenuate symptomatic parameters of GIM induced by CPT-11 as well as reduce tissue injury in the colon. However, there was no effect on cell proliferation and apoptosis. As such, TLR4 activation plays a partial role in GIM development but further research is required to understand the specific inflammatory signals underpinning tissue-level changes.

Keywords Toll-like receptor 4 (TLR4) · TLR4 antagonist · Gastrointestinal mucositis · CPT-11 · Tumour · MC-38 cells

Introduction

Gastrointestinal mucositis (GIM) is a difficult to manage complication of cancer treatment characterised by inflammation of the mucosa of the intestinal tract that leads to immunological, functional, and structural changes [1]. It is a common side effect of high-dose irinotecan (CPT-11) and remains one of the most debilitating side effects of cancer treatment despite decades of research. GIM has also been known to cause other symptoms such as pain, nausea, vomiting and diarrhoea [2]. These symptoms significantly reduce patient quality of life, as well as survival, as GIM can negatively impact tolerance of chemotherapy which leads to discontinuation or de-escalation of treatment.

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The pathobiology of acute intestinal inflammation as seen in GIM following CPT-11 has been linked to the activation of innate immune receptor TLR4. In GIM, TLR4 activation upregulates pro-inflammatory cytokines TNF-α and IL-6 [1]. This occurs via a downstream signalling pathway whereby CPT-11 causes direct injury to the intestinal epithelial cells, allowing the luminal antigens to enter the lamina propria. Antigen-derived lipopolysaccharides (LPS), or endotoxins, then activate TLR4 expressed on the basal membrane of epithelial cells and mucosa-associated immune cells [3]. Subsequently, these interactions lead to inflammation and eventual ulceration. Ulceration then leads to enhanced translocation of luminal contents and increases the risk of bacteraemia in immunocompromised patients [3]. There has been previous research examining the role of TLR4 on the development of CPT-11-induced mucositis. However, to date there has been no consistency in the role of TLR4 in the development of CPT-11-induced GIM [4, 5]. A study by Boeing et al. reported that the colon of wild-type mice treated with CPT-11 displayed an increase in histoarchitecture loss, inflammatory infiltrate and the presence of cryptitis compared to the colon of vehicle treated mice [6]. Mice that are germ-free, thus lacking LPS signals are also protected from CPT-11 GI injury [7]. However, due to limitations of genetically modified animals in research translation, research efforts are now targeted at tailoring methods of inhibiting TLR4 pharmacologically to confirm its role in GIM.

Previous experiments have also shown that TLR4 expression by tumour cells can be a contributing factor that promotes tumour cell proliferation, survival, migration, and metastasis [8]. Research has shown that tumours activated the suppression of T-cell and natural killer cell activity, but when TLR4 was inhibited, this tumour-mediated suppression of T-cell and natural killer cells was prevented, which delayed tumour growth and increased survival of the tumour-bearing mice [9]. Another study showed LPS stimulation of the TLR4/MD-2 complex can activate downstream signalling pathways that promotes the adhesiveness and metastatic capacity of colorectal cancer (CRC) cells [10]. These findings have shown the impact TLR4 has in CRC progression. While TLR4 activation can increase tumour growth and immunosuppression, it can also promote anti-tumour activity. For example, a study has shown that TLR4 expressed on dendritic cells plays an important role in promoting anti-tumour immune responses following chemotherapy [11].

Any treatment that modifies TLR4 signalling may have protective effects for the intestine while also increasing anti-tumour activity during chemotherapy. However, there has yet to be a specific TLR4 antagonist used in a tumour-bearing preclinical model to investigate the impact on GIM and tumour growth simultaneously. IAXO is a highly specific ligand that interferes selectively with the TLR4 and its co-receptors MD-2 and CD-14. IAXO-102 has been investigated in experimental studies of abdominal aortic aneurysms to date displaying its ability to inhibit TLR4 and subsequent downstream effects in an inflammatory disease [12]. This study therefore aimed to investigate the potential of IAXO-102 to attenuate gastrointestinal inflammation as well as suppress tumour activity in a colorectal tumour-bearing mouse model of GIM induced by CPT-11.

Methods

Animal model and ethics

The study was approved by the University of Adelaide Animal Ethics Committee (M-2021–033) and complied with the National Health and Research Council Australia (Australia) Code of Practice for Animal Care in Research and Training (2013) [13]. Mice were group housed in ventilated cages ($n = 3$–6 mice/cage) with a 12 h light/dark cycle, while food and water were provided ad libitum.

Experimental design

All mice were on a C57BL/6 background. Female and male mice ($n_{\text{total}} = 24$) weighing between 15 and 25 g (6–13 weeks of age) were bred in the University of Adelaide Laboratory Animal Service (SA, Australia). Mice were subcutaneously transplanted in the right flank with 2×10^6 cells/mL MC-38 cells, a murine colon adenocarcinoma cell line derived from C57BL/6 mice as previously published in Secombe et al. [14]. MC-38 cells were kindly provided by Associate Professor Michele Teng of the Cancer Immunoregulation and Immunotherapy Laboratory, QIMR Berghofer Medical Research Institute, Australia. When tumour growth reached approximately 0.2 cm^3 the mice were treated with either of the following: 3 days of daily 3 mg/kg intraperitoneal (i.p.) dose of the TLR4 antagonist IAXO-102 (MedChemExpress, USA) in a diluent of 10% EtOH, 40% PEG400, 5% Tween-80 and 45% saline; a single 270 mg/kg i.p. dose of CPT-11 (kindly provided by Pharmacia/Pfizer, USA) prepared in a sorbitol/lactic acid buffer (45 mg/mL sorbitol/0.9 mg/mL lactic acid; pH 3.4; Sigma-Aldrich, USA); the combination of CPT-11 and IAXO-102; or sorbitol/lactic acid buffer only (vehicle mice) which acted as the control group. Equivalence in body weight and tumour size on the day of treatment was confirmed by ANOVA. Mice were randomly assigned to treatment groups and culled by cervical dislocation at 72 h after being anaesthetised using inhalation isoflurane (1 L/min O_2 with 4% isofluorane). The study timeline is shown in Fig. 1A.

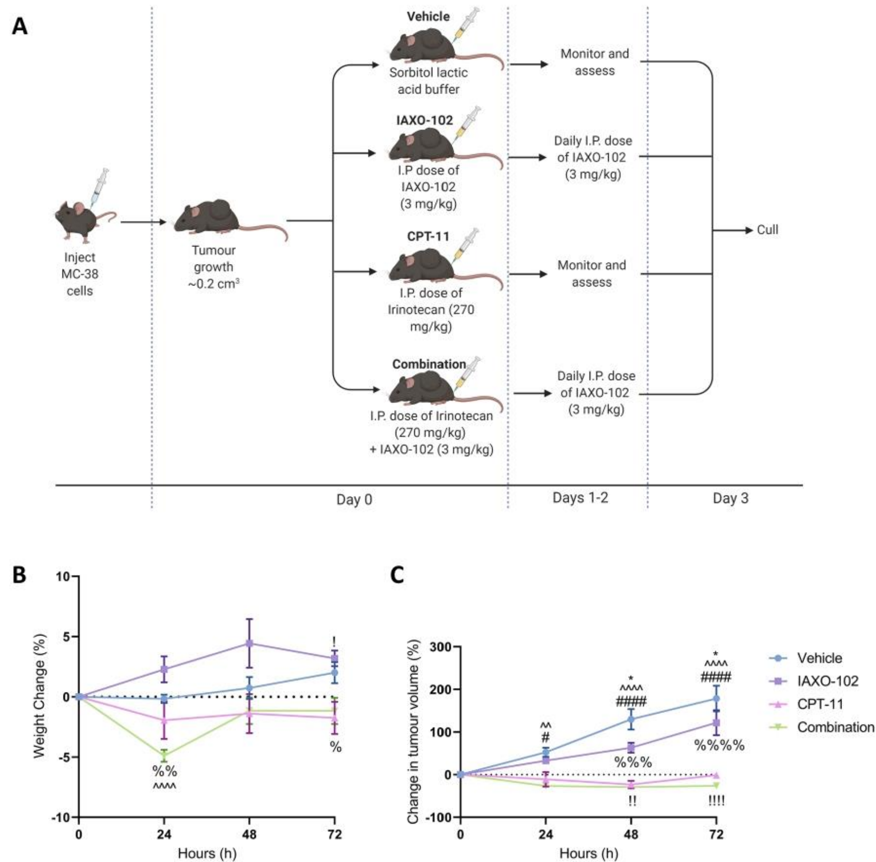

Fig. 1 Experimental timeline and toxicity assessment. **A** Experimental timeline showing the sequence of events and treatment timepoints. **B** Percentage change in weight over 72 h. Data displayed as a mean ± standard error of the mean (SEM) percentage weight change from baseline (0 h), $n=6$ per group. **C** Tumour volume over 72 h. Data displayed as a mean ± SEM percentage change in tumour volume from baseline (0 h), $n=6$ per group. Symbols indicate statistical significance: vehicle group vs. IAXO-102 group: * $P<0.05$; vehicle group vs. CPT-11 group: # $P<0.05$, #### $P<0.0001$; vehicle group vs. combination group: ^^ $P<0.01$, ^^^^ $P<0.0001$; IAXO-102 group vs. CPT-11 group: ! $P<0.05$, !! $P<0.01$, !!!! $P<0.0001$; IAXO-102 group vs. combination group: % $P<0.05$, %% $P<0.01$, %%% $P<0.001$, %%%% $P<0.0001$

Assessment of intestinal toxicity

Mice were weighed daily to track weight loss/gain. All mice were monitored twice daily for the presence of diarrhoea (scored as present or absent) and other toxicity parameters: ruffled coat, dehydration, hunched posture, rectal bleeding, and reluctance to move. Mice were killed if they displayed ≥ 15% weight loss or significant distress and deterioration, in compliance with animal study ethical requirements.

Tissue preparation

The entire gastrointestinal tract from pyloric sphincter to rectum was dissected and flushed with chilled 1 × phosphate buffered saline (PBS, pH 7.4, ThermoFisher Scientific, USA) to remove contents. The large intestine was weighed immediately after resection. Tumours were removed and weighed after skin and fat were dissected off. All weights were then represented as relative to body weight on the day of cull. Samples of colon (1 cm in length) and tumour were

collected and (i) drop-fixed using 10% neutral buffered saline for processing and embedding into paraffin wax, or (ii) snap frozen in liquid nitrogen and stored at − 80 °C for molecular analyses.

Histopathologic analysis

Haematoxylin and eosin (H&E) staining was performed on 5 µm sections of colon cut on a rotary microtome and mounted onto glass Menzel-Gläser Superfrost microscope slides (ThermoFisher Scientific). Slides were scanned using the NanoZoomer (Hamamatsu Photonics, Japan) and assessed with NanoZoomer Digital Pathology software. view2 (NDP.view2, Version 2.7.39) (Hamamatsu Photonics). The occurrence of eight histological criteria in the colon was examined to generate a total tissue injury score [15]. These criteria were disruption of brush border, architectural disruption, disruption of crypt cells, and infiltration of polymorphonuclear leukocytes cells, dilation of lymphatics and capillaries, oedema, reduction in goblet cell number and thickening of muscularis externa. Each parameter was scored as present = 1 or absent = 0 in a blinded fashion by two independent assessors (J.S.Y. Tam/A. Wignall). Concordance on all scores was confirmed between assessors.

Immunohistochemistry assessment of cellular markers of apoptosis and proliferation

Immunohistochemistry (IHC) was carried out on 5 µm sections of colon and tumour, cut on a rotary microtome and mounted onto FLEX IHC microscope slides (Agilent, USA). IHC analysis was performed for Ki67 (Abcam; #ab16667), a marker of proliferation and caspase-3 (Abcam; #ab4051), a marker of apoptosis. Changes in both parameters are validated markers for altered tissue kinetics and an excellent way to assess the subclinical severity of toxicity [16]. IHC analysis was performed using Agilent reagents on an automated machine (AutostainerPlus, Agilent) following standard protocols supplied by the manufacturer. Briefly, sections were deparaffinised in xylene and rehydrated through graded ethanols before undergoing heat-mediated antigen retrieval using an EDTA/Tris buffer (0.37 g/L EDTA, 1.21 g/L Tris; pH 9.0). Retrieval buffer was preheated to 65 °C using the Dako PT LINK (pretreatment module; Agilent; #PT101). Slides were immersed in the buffer, and the temperature was raised to 97 °C for 20 min. After returning to 65 °C, slides were removed and placed in the Agilent AutostainerPlus (Agilent; #AS480) and stained following manufacturer's guidelines. Negative controls had the primary antibodies omitted. Slides were scanned using the NanoZoomer (Hamamatsu Photonics) and assessed with NDP.view2 software (Hamamatsu Photonics). Cell proliferation data were represented as the percentage of positively stained cells relative to total cells in the intestinal crypts. Apoptosis was quantified by counting the number of positively stained cells for 15 crypts and the data were presented as average positively stained cells per crypt. Only well-oriented, non-oblique crypts were included for analysis. A scoring system of percentage area of cells stained brown in the tissue: 0—25% = 0; 26–50% = 1; 51—75% = 2; 76–100% = 3 was used to analyse the tumour tissue stained with Ki67 and caspase-3. Two blinded investigators (J.S.Y Tam/A. Wignall) independently scored each stained section and mean score from both investigators were calculated. Concordance was confirmed between investigators on all scoring results.

RT-PCR for markers of TLR4 signalling

RNA was isolated from snap frozen tumour and colonic tissue using the NucleoSpin® RNA Plus kit (Macherey–Nagel, Germany) following the manufacturer′s protocol. RNA was quantified using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek, USA) and reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA) according to the manufacturer′s protocol. cDNA was quantified using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek) and diluted to a working concentration of 100 ng/µL in nuclease-free water. Expression of key markers of TLR4/MD-2 downstream signalling pathway were investigated. Primers for genes of interest were designed using web-based primer design programme, PRIMER 3 (v. 0.4.0) and manufactured by Sigma-Aldrich (Table 1). Amplified transcripts were detected by SYBR Green (Qiagen Pty Ltd., Australia) in a Rotor-Gene Q Series Rotary Cycler (Qiagen Pty Ltd.).

Table 1 Mouse RT-PCR primer sequences designed by PRIMER 3 (v. 0.4.0)

TLR4	Forward: 5 '-CTC TGC CTT CAC TAC AGA GAC-3' Reverse: 5'-TGG ATG ATG TTG GCA GCA ATG-3'
MD-2	Forward: 5 '-GTC CGA TGG TCT TCC TGG CGA GT-3' Reverse: 5'-GCT TCT CAG ATT CAG TCA ATA TGG G-3'
CD-14	Forward: 5 '-GTC AGG AAC TCT GGC TTT GC-3' Reverse: 5'-GGC TTT TAC CCA CTG AAC CA-3'
IL-6	Forward: 5 '-AGT TGC CTT CTT GGG ACT GA-3' Reverse: 5'-TCC ACG ATT TCC CAG AGA AC-3'
IL-6R	Forward: 5 '-TGA ATG ATG ACC CCA GGC AC-3' Reverse: 5'-ACA CCC ATC CGC TCT CTA CT-3'
CXCR2	Forward: 5 '-GCA GAG GAT GGC CTA GTC AG-3' Reverse: 5'-TCC ACC TAC TCC CAT TCC TG-3'
CXCL1	Forward: 5 '-GGG TGA AGC CAC AAC AGA TT-3' Reverse: 5'-GCA GAC CAG CAT AGT GAG CA-3'
CXCL2	Forward: 5 '-GCA GAG GAT GGC CTA GTC AG-3' Reverse: 5'-TCC ACC TAC TCC CAT TCC TG-3'
β-actin	Forward: 5 '-CTC TTC CAG CCT TCC TTC CT-3' Reverse: 5'-AGC ACT GTG TTG GCG TAC AG-3'

All reactions were completed in triplicate including a non-template control to determine presence of contamination. The relative ratio of mRNA expression was calculated using $2^{\Delta Ct}$ method using β-actin as the normalising housekeeper gene [17]. β-actin has been shown to have stable expression levels across cell types and treatments [18].

Statistical analysis

Data was graphed and analysed using GraphPad Prism Software 9.0 (GraphPad® Software, San Diego, USA). A D'Agostino & Pearson normality tests were conducted to determine if data was parametric or non-parametric. A Kruskal–Wallis test with Dunn's multiple comparisons test was performed on non-parametric data to compare between the treatment groups. A two-way ANOVA with Tukey's multiple comparisons test was performed on parametric data to compare between the treatment groups. Any data point that had a value more than 3 times the standard deviation from the mean was excluded as an outlier. P values of <0.05 were considered statistically significant.

Results

Mice treated with IAXO-102 were protected from CPT-11-induced GIM symptom of diarrhoea

Weight loss following CPT-11 treatment was most severe at 24 h in the combination group (− 4.90% ± 1.22% vs baseline) (Fig. 1B). While the IAXO-102 group gained the most weight at 24 h (2.28% ± 2.65% vs baseline) and 48 h (4.43% ± 4.95 vs baseline). The weight loss in the combination group was different compared to the vehicle ($P<0.0001$) at 24 h, and the IAXO-102 group at 24 h ($P<0.01$) and 72 h ($P<0.05$). While the CPT-11 group had a difference in weight compared to the IAXO-102 group at 72 h ($P<0.05$).

CPT-11 caused diarrhoea in 50% of mice within 6 h of CPT-11 administration and 100% at 24 h (Table 2). However, IAXO-102 treatment attenuated diarrhoea of CPT-11 GIM as diarrhoea was prevented in mice in the combination group (Table 2). No diarrhoea was seen in any vehicle or IAXO-102 treated mice (data not presented).

IAXO slowed colorectal tumour growth

Tumours were measured daily and expressed as a change in volume from the day of CPT-11 injection. From 24 to 72 h, tumour volume of the vehicle group was higher compared to the CPT-11 group (24 h: $P<0.05$; 48 and 72 h: $P<0.0001$) and combination group (24 h: $P<0.01$; 48 and 72 h: $P<0.0001$) (Fig. 1C). Tumour volume of the IAXO-102 group was different at 48 h and 72 h compared to the vehicle group (48 h and 72 h: $P<0.05$), the CPT-11 group (48 h: $P<0.05$; 72 h: $P<0.05$) and the combination group (48 h: $P<0.001$; 72 h: $P<0.0001$) (Fig. 1C). There were no differences in tumour volume of the CPT-11 and combination group from 24 to 72 h (Fig. 1C).

There were no differences in colon and tumour weights of mice between treatment groups

There was no difference in colon wet weights between the treatment groups (Fig. 2A). There were also no differences observed in tumour weights between the treatment groups (Fig. 2B).

IAXO–102 protects against CPT-11-induced colonic histopathology independent of cell death and turnover

Representative H & E images (Fig. 3A) show minimal damage in vehicle, IAXO-102 and combination groups. CPT-11 treatment caused epithelial disruption (black arrow) and inflammatory infiltrate (black circle). Histopathological analysis (Fig. 3D) showed that combination treated mice were protected against CPT-11-induced mucosal tissue injury in the colon, with a lower histopathological score compared to the CPT-11 group ($P<0.05$). The IAXO-102 group also had a difference in tissue injury score compared to the CPT-11 group ($P<0.01$). There were no other differences observed between the groups.

Representative images show Ki67 positive cells (stained brown, Fig. 3B). Analysis of the Ki67 images (Fig. 3E) showed that the CPT-11 group had a decrease in proliferating cells compared to the vehicle and IAXO-102 groups ($P<0.01$). The combination group also had a lower number of proliferating cells compared to the vehicle and IAXO-102 groups

Table 2 Toxicity symptoms over 72 h. Data presented as total number of animals (per time point). Toxicity parameters includes: ruffled coat, dehydration, hunched posture, rectal bleeding and reluctance to move

Toxicity symptoms	CPT-11 (number of animals)				Combination (number of animals)			
	6 h	24 h	48 h	72 h	6 h	24 h	48 h	72 h
Diarrhoea	5/6	6/6	0/6	0/6	0/6	0/6	0/6	0/6
Toxicity parameters	3/6	3/6	1/6	0/6	5/6	2/6	1/6	0/6

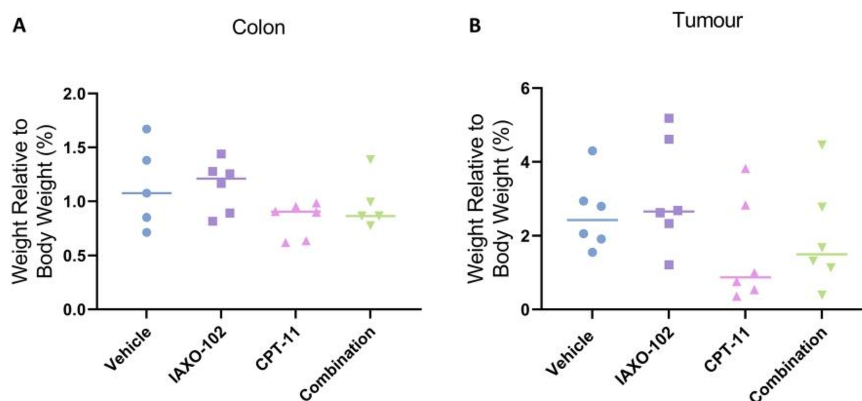

Fig. 2 Organ wet weight of all treatment groups. **A** Colon wet weights. **B** Tumour wet weights. All data displayed as a percentage of weight relative to body weight and lines represent group median, $n = 5–6$ per group. Symbols indicate statistical significance: IAXO-102 vs. CPT-11: ! $P < 0.05$

($P < 0.05$). There were no differences observed between the other groups.

Representative images of caspase-3 positive cells in the colonic crypts are shown (red arrow, Fig. 3C). Analysis of the caspase-3 images (Fig. 3F) showed that the CPT-11 ($P < 0.05$) and combination ($P < 0.01$) groups had a higher apoptotic rate compared to the vehicle group. There were no other differences observed between the groups.

Tumours in mice treated with CPT-11 had a higher apoptotic score compared to mice treated with vehicle

A scoring system of pertcentage area of cells stained brown in the tissue: 0 = ≤25%; 1 = 26–50%; 2 = 51–75%; 3 ≥ 76% was used to analyse the tumour tissue stained for Ki67 and caspase-3.

Representative immunostaining images of proliferating cells (Ki67 positive cells stained brown) in tumour tissue (Fig. 4A) revealed no differences in scores for positively stained proliferating cells between the groups (Fig. 4C).

Representative immunostaining images of apoptotic cells (caspase-3 positive cells stained brown) in tumour tissue (Fig. 4B) revealed that the CPT-11 group had a higher score for positively stained apoptotic cells compared to the vehicle group ($P < 0.01$) (Fig. 4D). There were no other differences observed between the other groups (Fig. 4D).

Effect of IAXO-102 treatment on gene expression in mouse colonic tissue

There was no change in transcript levels between treatment groups for **TLR4** (Fig. 5A), **CD-14** (Fig. 5C), and **CXCR2** (Fig. 5E). A difference was observed in **MD-2** transcript levels between vehicle and IAXO-102 groups ($P < 0.05$); no other differences were observed between the groups (Fig. 5B). A difference was also observed in **IL-6R** transcript levels between CPT-11 and combination groups ($P < 0.01$); no other differences were observed between the groups (Fig. 5D). The transcript expression of IL-6, CXCL1 and CXCR1 were investigated in the colon but there was no expression in any of the treatment groups.

Effect of IAXO-102 treatment on gene expression in mouse tumour tissue

Levels of transcript expression in the tumour tissue of all the groups were also analysed. There was no change in transcript levels across any groups in any of the genes of interest; **TLR4** (Fig. 6A); **MD-2** (Fig. 6B); **CD-14** (Fig. 6C); **IL-6** (Fig. 6D); **IL-6R** (Fig. 6E); **CXCL2** (Fig. 6F); **CXCR1** (Fig. 6G); **CXCR2** (Fig. 6H).

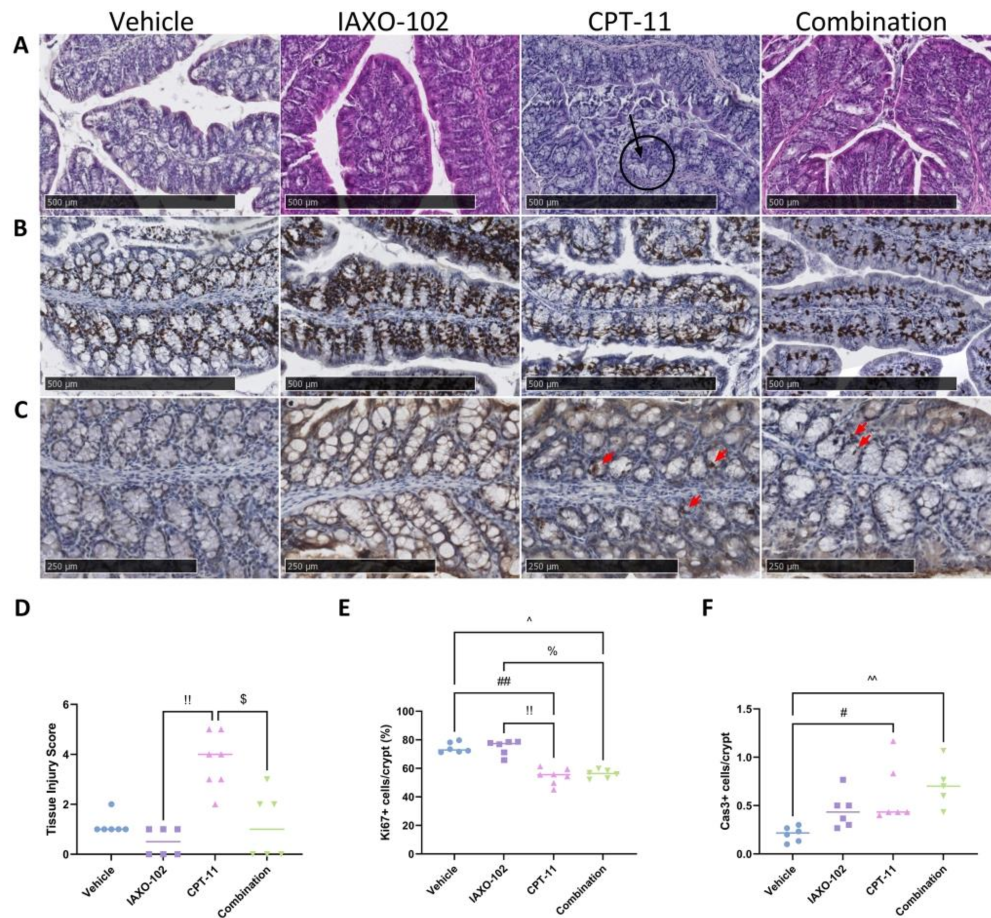

Fig. 3 H&E and IHC staining results in the colon. **A** Representative H & E images showing epithelial disruption (black arrow) and inflammatory infiltrate (black circle). Scale bars, 500 µm. 40×original magnification. **B** Representative immunostaining of Ki67 cells in colonic crypts. Proliferating cells are stained brown. Scale bars, 500 µm. 40×original magnification. **C** Representative immunostaining of caspase-3 cells in colonic crypts. Apoptotic cells are stained brown (red arrow). Scale bars, 250 µm. 40×original magnification. **D** Histopathological tissue injury scores in the colon of mice. Data presented as median, $n=6$ per group. **E** Percentage of Ki67 positively stained cells in the colonic crypts. Data presented as median, $n=6$ per group. **F** Number of caspase-3 positively stained cells in the colonic crypts. Data presented as median, $n=5$–6 per group. Symbols indicate statistical significance: vehicle vs. CPT-11: # $P<0.05$, ## $P<0.01$; vehicle vs. combination: ^ $P<0.05$, ^^ $P<0.01$; IAXO-102 vs. CPT-11: !! $P<0.01$; IAXO-102 vs. combination: % $P<0.05$; CPT-11 vs. combination: $ $P<0.05$

Discussion

Inflammation of the mucosa of the intestinal tract during cancer treatment is known as GIM and is most severe during high-dose chemotherapy. TLR4 signalling has been strongly implicated in the development and treatment of CRC and GIM through its regulation of inflammation. This study explored how interruption of TLR4 signalling using a pharmacological intervention modulates these outcomes. To exclude any influence of sex on the results, we also did

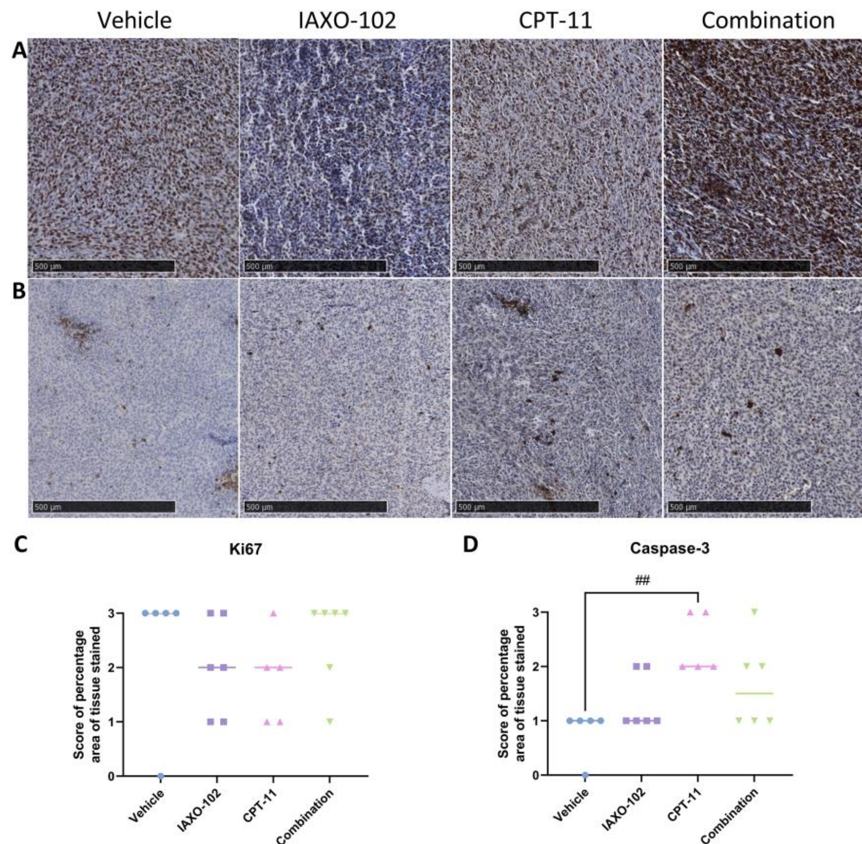

Fig. 4 IHC staining results in the tumour. **A** Representative immunostaining of Ki67 cells in tumour tissue. Proliferating cells are stained brown. Scale bars, 500 µm. 40×original magnification. **B** Representative immunostaining of caspase-3 cells in tumour tissue. Apoptotic cells are stained brown. Scale bars, 500 µm. 40×original magnification. **C** Analysis and scoring of tumour tissue stained with Ki67. Data presented as median, $n=5$–6 per group. **D** Analysis and scoring of tumour tissue stained with Caspase-3. Data presented as median, $n=5$–6 per group. Symbols indicate statistical significance: vehicle vs. CPT-11: ## $P<0.01$

a sub-analysis and found that male and female mice had equivalent body weight change, diarrhoea incidence and tumour growth.

It was found that the TLR4 antagonist IAXO-102 was able to prevent diarrhoea in mice treated with CPT-11. The dose and schedule of IAXO-102 was equivalent to previous work that found protection against inflammation was associated with downregulation of TLR4 protein expression [12]. Diarrhoea reduction was associated with improved histopathological scores, indicating retention of colonic morphology and subsequent function. Work by others have shown similar protective effects using non-specific TLR4 antagonists. For example, a study by Fakiha et al. reported that amitriptyline was able to prevent CPT-11-induced diarrhoea and colonic apoptosis in rats but did not see any protective effects in histological architecture in the intestinal tract [19]. Although not using a TLR4 antagonist, a study by Wardill et al. found that TLR4 knock-out mice were protected against CPT-11-induced mucosal tissue injury in the small intestine and also displayed a reduction in CPT-11-induced diarrhoea [5]. Another study has also shown that pharmacological inhibition of TLR4 was able to reduce disease activity and prevent morphological damage in an inflamed colon [20]. In contrast, a study using tumour-bearing rats reported that naloxone did not improve GIM following CPT-11 treatment. The naloxone also did not improve any weight loss and even

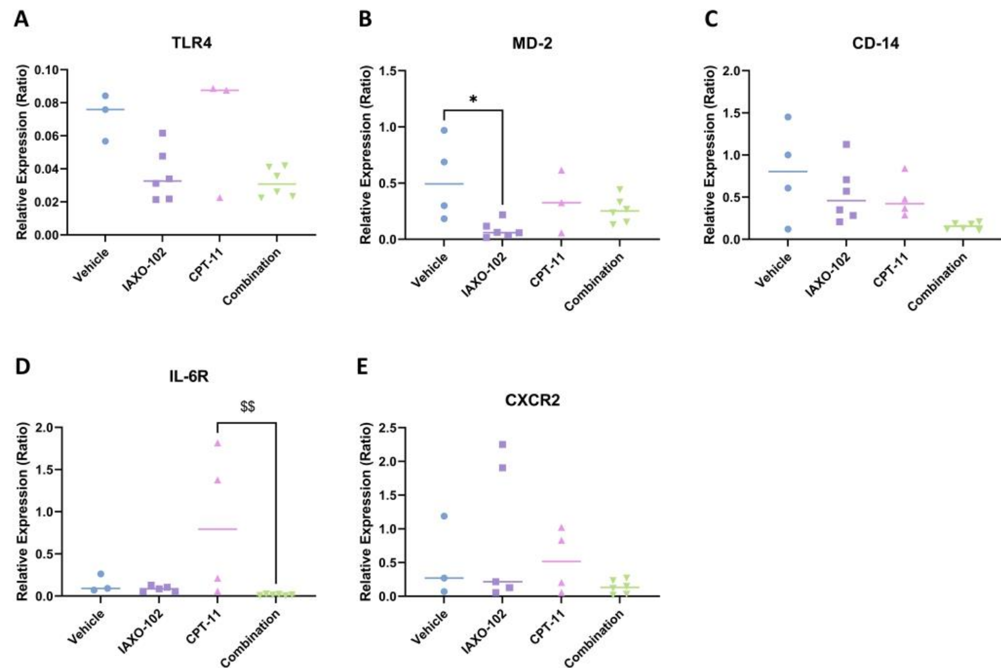

Fig. 5 Transcript expression in the colon. **A** TLR4, **B** MD-2, **C** CD-14, **D** IL-6R, and **E** CXCR2 from colonic tissue relative to the housekeeper β-actin. Data are presented as median, $n = 3$–6 per group. Symbols indicate statistical significance: vehicle vs. IAXO-102: * $P < 0.05$; CPT-11 vs. combination: $$ $P < 0.01$

increased tumour growth in the rats [21]. Collectively this provides evidence that targeting TLR4 signalling interferes with development of GIM and warrants further investigation.

The mechanisms by which TLR4 inhibition protects colonic tissue and prevents diarrhoea was then further investigated using well-established tissue markers. The typical markers of CPT-11-induced injury, apoptosis and reduced proliferation of crypt epithelial cells [16], were not significantly affected by IAXO-102. The lack of measurable changes may be due to the kinetics of cell death and halting of the cell cycle following chemotherapy exposure. Previous studies have shown that apoptosis may be an early indicator of intestinal damage with rates peaking at 6 h after administration of CPT-11 [5, 19]. Although slightly slower than apoptosis, halting of the cell cycle and reduced proliferation is known to peak between 24 and 48 h after exposure to chemotherapy [19, 22, 23]. Collectively, this may account for the lack of difference between the CPT-11 and combination groups where tissue was collected at 72 h. Conversely, this lack in difference may also suggest that TLR4 downstream signalling may not play a major role in apoptosis seen in GIM. However, early time points coinciding with maximal protection from diarrhoea such as 24 h would need to be investigated to confirm both possibilities as it was observed in previous studies that apoptosis was decreased after 6 h in the colon [5, 19].

TLR4 signalling in the colon has been long associated with inflammatory conditions. As such, we next investigated TLR4-related transcripts known to play key roles in inflammatory responses. There were no differences in transcript expression of TLR4 and CD-14 in the colon between the groups. However, there was a decrease in expression of the co-receptor MD-2 in the colon of the IAXO-102 group compared to the vehicle group. These results are unexpected as previous studies have found increased TLR4 expression in the colon following chemotherapy [24, 25]. IL-6R and CXCR2 are both receptors associated with pro-inflammatory cytokines which are upregulated during inflammation [26, 27]. There was no difference between the groups in levels of CXCR2 expression, but a more interesting observation was the effect of the combination treatment on the levels of IL-6R transcript expression in the colon. A decrease in

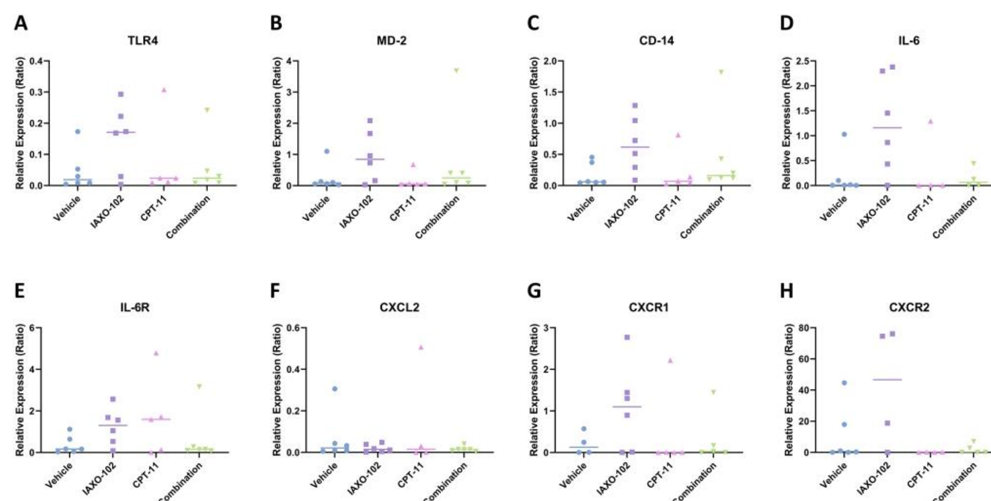

Fig. 6 Transcript expression in the tumour. **A** TLR4, **B** MD-2, **C** CD-14, **D** IL-6, **E** IL-6R, **F** CXCL2, **G** CXCR1 and **H** CXCR2 from tumour tissue relative to the housekeeper β-actin. Data are presented as median, $n=4$–6 per group

IL-6R levels was observed in the colon of the combination group compared to the CPT-11 group, which may indicate a mechanism by which IAXO is protective. IL-6 has been extensively studied in chemotherapy-induced GIM [27, 28] and in TLR4 knock-out mice lack an IL-6 response [5, 29]. Collectively, this eludes to IAXO–102 protecting against GIM through TLR4-depedent IL-6 regulation.

We also wanted to test whether TLR4 antagonism modulated CRC tumour growth and response to CPT-11 in our model. In the current study, IAXO-102 treatment alone led to a lower tumour volume compared to the vehicle group. A study by Pastille et al. has reported similar findings. They observed that by inhibiting TLR4 with an antagonist during intestinal inflammation, the development and progression of colonic tumours was significantly reduced compared to control mice [30]. They also observed a decrease in infiltration of pro-inflammatory cells and cytokines compared to control mice [30]. CPT-11 prevented tumour growth equally well in both groups. Based on the findings in the IAXO–102-alone group, it was expected that the combination group would have significant reduction in tumours compared to CPT-11 alone, but this was not observed. As such, there are clearly different roles for TLR4 during the development of tumours, compared to response to chemotherapy in our model. This is supported by other work showing conflicting roles of TLR4 in tumour response to cancer treatment [19, 21].

To explore the effect of TLR4 antagonsim on CRC tumours further, markers of cell proliferation and cell death were examined in all tumours at 72 h. Regarding levels of proliferation in the tumour, there were no differences between any of the groups, as such, the ability of IAXO–102 to decrease tumour growth is not attributable to increased cell turnover. As for levels of apoptosis, only the CPT-11 group had an increase in apoptosis levels compared to vehicle group. However, the results observed in both the proliferation and apoptosis scores were quite variable, which may be due to the heterogeneity of the tumour itself [31]. Consistent with the lack of significant effect of TLR4 antagonism on cell turnover, we were also unable to confirm any changes in inflammatory targets between the groups. Depending on where the tumour was examined, there may be differences in cellular morphology, gene expression, metabolism, and proliferation. This may be what caused the variability observed in the results and may have also affected the targeted treatments on these tumours.

While this is the first study to explore the specific TLR4 antagonist, IAXO–102, for its ability to protect against GIM in a CRC mouse model, there were limitations to the final interpretation of our findings. Statistical significance was difficult to establish in the RT-PCR analysis due to issues with the quality of cDNA which did not amplify the target genes as well as the housekeeper. Therefore, these results and numbers were not included in the analysis causing a decrease in sample size which led to difficulty in determining significance in the results. Whilst the advantages of using a tumour-bearing model means potentially more rapid translation into the clinical context, we need to also be

mindful that tumours do create a systemic effect on the mice. So future studies could be undertaken in non-tumour-bearing mice with this compound to explore any other impacts.

Given IAXO-102 is a novel compound with inhibitory actions on TLR4, it would be important to look at any late side effects of IAXO-102 on the immune system that could be explored in future studies. Future work to confirm these findings will also need to include additional time points of tissue collection to look for changes coinciding with peak diarrhoea and weight change, as well as allowing longer growth trajectory of the tumours. Another limitation that needs to be noted in this study is that the diluent for IAXO-102 was not used as a vehicle. Components of the diluent included PEG and ethanol which may have impacted the results. However, the diluent for CPT-11 was prioritised as it was determined to be the more toxic diluent compared to the IAXO-102 diluent. Although the diluents used to reconstitute IAXO-102 can cause toxicity, it is only at high concentrations for extended periods of time [32–34]. These diluents have also been diluted with 45% saline solution which would decrease the concentration and, therefore, toxicity.

Conclusion

In conclusion, the results demonstrated that IAXO-102 was able to attenuate CPT-11-induced diarrhoea as well as reduce tissue injury in the colon without impacting tumour response. However, given that there was no measurable impact on apoptosis or proliferation in either the colon or tumour, alternative mechanisms must account for these observations. Our work points to a downstream role for IL-6 in mediating the protective effects of IAXO-102, whereas other inflammatory markers were not significantly altered. Research efforts can therefore be shifted towards targeting IL-6R to understand its relationship with inflammation and apoptosis within the GIT.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval The study was approved by the University of Adelaide Animal Ethics Committee and complied with the National Health and Research Council Australia Code of Practice for Animal Care in Research and Training (2013).

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