Dark Agouti rat model of chemotherapy-induced mucositis: Establishment and current state of the art

Barbara Vanhoecke[1,2], Emma Bateman[1], Bronwen Mayo[1,3], Eline Vanlancker[2], Andrea Stringer[3], Daniel Thorpe[3] and Dorothy Keefe[1,4]

[1]Mucositis Research Group, Centre for Personalised Cancer Medicine (CPCM), Centre for Clinical Research Excellence (CCRE) in Oral Health, Faculty of Health Sciences, University of Adelaide, Adelaide, 5005 South Australia, Australia; [2]Laboratory of Microbial Ecology and Technology, University of Ghent, 9000 Ghent, Belgium; [3]Sansom Institute for Health Research, University of South Australia, Adelaide, 5001 South Australia, Australia; [4]Director, SA Cancer Service, Royal Adelaide Hospital, Adelaide, 5005 South Australia, Australia
Corresponding author: Andrea Stringer. Email: andrea.stringer@unisa.edu.au

Abstract

Mucositis is a major oncological problem. The entire gastrointestinal and genitourinary tract and also other mucosal surfaces can be affected in recipients of radiotherapy, and/or chemotherapy. Major progress has been made in recent years in understanding the mechanisms of oral and small intestinal mucositis, which appears to be more prominent than colonic damage. This progress is largely due to the development of representative laboratory animal models of mucositis. This review focuses on the development and establishment of the Dark Agouti rat mammary adenocarcinoma model by the Mucositis Research Group of the University of Adelaide over the past 20 years to characterize the mechanisms underlying methotrexate-, 5-fluorouracil-, and irinotecan-induced mucositis. It also aims to summarize the results from studies using different animal model systems to identify new molecular and cellular markers of mucositis.

Keywords: Rat, mucositis, methotrexate, 5-fluorouracil, irinotecan, chemotherapy

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Introduction

The major problem with anticancer therapies (radiation and chemotherapy) is the unwanted toxicity to normal tissues. Chemotoxic drugs are known to act by inducing apoptosis or cell cycle arrest in cancer cells but unfortunately also in normal cells.[1,2] Granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and platelet-derived growth factor can ameliorate common bone marrow side effects such as anaemia, thrombocytopenia and neutropenia, and since their development, the doses of chemotherapeutic drugs have been able to be increased, leading to toxicity of other organ systems, particularly the gastrointestinal (GI) tract, the genitourinary system, the nervous system, and the heart.

Mucositis is a clinical term used to describe damage to mucous membranes after anticancer therapies.[3] It occurs throughout the entire gastrointestinal tract (GT) (including the mouth) and genitourinary tract, and to a lesser extent in other mucosal surfaces. Its severity and duration varies with the dose and the type of drug used. The importance of mucositis is that it limits the dose of chemotherapy. Cancer therapy-associated mucosal injury of the alimentary tract (mouth to anus) (AT) is referred to as alimentary mucositis.[4] The GI crypt epithelium is particularly vulnerable to chemotherapeutic toxicity, with symptoms including nausea and vomiting, abdominal pain, distension, and diarrhoea due to direct effects of the cytotoxics on the mucosa. Abdominal pain is caused by the extensive damage occurring in the abdominal region. Diarrhoea and constipation are thought to be caused by the alteration in absorptive functions of cells, mucin distribution and composition, and microbial interactions with these cells and metabolites of the drugs themselves.[5,6] Direct effects of the cytotoxics result in massive apoptosis at the crypt base,[7] whereas indirect effects are due to lack of enteral intake, attenuation in secretion of GI hormones secondary to the bowel injury, and acute and chronic infections,[8,9] the latter due to the bacterial translocation (BT) and the systemic spread of microbiota from the gut to systemic organs. BT is a significant cause of sepsis in critically ill patients and is linked to microbial overgrowth, immunosuppression and loss of physical barrier.[10] Both microbiota and endotoxins cause systemic infection and multi-organ failure.

The exact mechanisms of oral and GI mucositis are not fully understood, yet a lot of progress has been made over the last several years. This is largely due to the development of representative preclinical animal models of mucositis (mouse, hamster, rat). Since AT damage is most visibly expressed in the mouth of patients, researchers developed models for mucosal injury focusing on oral damage following irradiation and chemotherapy and to which they applied a visual scoring system of the oral damage. The models include the mouse lip (irradiation),[11,12] the mouse ventral tongue mucosa (irradiation),[13] and the hamster cheek pouch (5-fluorouracil (5FU)),[14] and are now widely used to investigate various aspects of oral mucositis including the pathobiology and treatment as well as the mucotoxicity of the drugs used. GI mucositis models using histological evaluation of the small intestine and/or colon as the gold standard for the detection of GI inflammation and damage have also been developed, using mice and rats. Mouse GI mucositis models have been developed for melphalan,[15,16] cytosine arabinoside, vincristine, doxorubicin,[17,18] cyclophosphamide,[19] 5FU,[20,21] and methotrexate (MTX).[22] Rats have also become popular experimental animals in the establishment of GI mucositis models and are now used widely in mucositis studies. This review focuses on three rat models of cytotoxic drug-induced GI mucositis that have been developed and optimized by the Mucositis Research Group of Professor Dorothy Keefe over the past two decades. These models are based on work that has been done with Dark Agouti rats and the cytotoxic drugs MTX, 5FU, and irinotecan. The group also developed the rat model of lapatinib-induced diarrhoea which simulates the patients' diarrhoea following treatment with epidermal growth factor receptor tyrosine kinase inhibitors. However, since the establishment of this model has recently been reviewed, we decided to exclude it from this overview.[23] Moreover, the group is working on the development of a rat model of radiotherapy-induced GI mucositis. The models have allowed to unravel at least in part the complexity of the pathobiology of mucositis and its underlying mechanisms, and to develop preventive strategies and/or therapeutic drugs to treat mucositis without reducing the efficacy of chemotherapy.

Dark Agouti rat mammary adenocarcinoma model

In 2002, Keefe and co-workers established the Dark Agouti rat mammary adenocarcinoma model (DAMA) model for the simultaneous assessment of the effects of chemotherapy and chemoprotectants on both the entire AT and the tumor[24] and it is now one of the most extensively used models to investigate chemotherapy-induced mucositis in rats.[2,25–28] This model has the benefit that the rats are bearing breast cancer tumors and thus allows us to evaluate the additional effect of tumor burden on the overall chemotherapy-induced gut toxicity,[29] reflecting more accurately the patient setting of chemotherapy-induced mucositis.

The model is based on the injection of breast cancer cells subcutaneously into both flanks of the female rats, which are grown over approximately two weeks to represent up to 15% of bodyweight (BW). Although the model has previously been used for studies of malnutrition following chemotherapy[30,31] and for studies of neuroprotection by glutamate,[32] it had not been applied to assess GI injury nor protection after chemotherapy. Initially, Keefe and co-workers developed the model to assess small intestinal toxicity; however, it is now routinely used to investigate toxicities arising at different sites along the AT (oral mucosa, stomach, and intestines).

General treatment protocol

All studies start with the approval by the Animal Ethics Committees of The Institute of Medical and Veterinary Sciences (now SA Pathology) and of The University of Adelaide and comply with the latest National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training. All animals are monitored four times daily for clinical signs of stress; these scores are tallied, and if any animal exceeds the maximum acceptable total score (as defined by the Animal Ethics Committee) they are euthanized. These criteria include a dull ruffled coat with accompanying dull and sunken eyes, coolness to touch with no spontaneous movement, hunched appearance, diarrhoea, reduced food and water intake, and tumour burden >10% of body weight.

In each experiment, rats are randomly assigned to either a control or experimental group according to a specific time point. All rats in the experimental groups receive a single intramuscular dose in case of MTX, or a single intraperitoneal dose in the case of 5FU or irinotecan. Rats in the control groups receive treatment with the solvent vehicle (saline for MTX, dimethylsulphoxide for 5FU, sorbitol/lactic acid buffer for irinotecan). In the case of irinotecan administration, animals receive a subcutaneous injection of atropine immediately prior to irinotecan injection to prevent a severe cholinergic reaction. Subsequent to administration of the chemotherapy drugs, end points such as mortality, diarrhoea, and general clinical condition are assessed four times per 24 h period. Rats are killed by exsanguination and cervical dislocation usually at 6, 72, and 120 h following administration of the test drug. A section of the AT extending from the pyloric sphincter to the rectum is dissected out and flushed with chilled isotonic saline (0.9% w/v) to remove contents. Samples (1 cm in length) of the small intestine (taken at 25% of the length of the small intestine from the pylorus) and the colon (taken at midcolon position) are dissected and removed for further analysis. In addition, samples of oral mucosa (tongue and buccal mucosa) are taken. All tissue samples are immediately fixed in 10% neutral buffered formalin before processing and embedding in paraffin wax.

The DAMA model of MTX-induced mucositis

MTX is a chemotherapeutic agent that is structurally similar to folic acid which is essential for the *de novo* synthesis of all purine bases. MTX is a potent inhibitor of dihydrofolate reductase[33] which catalyses the conversion of dihydrofolate to the active tetrahydrofolate. DNA synthesis is inhibited if

tetrahydrofolate is not available for the production of a coenzyme required for thymidylate formation. Essentially, all purine synthesis and, therefore, also the synthesis of DNA, RNA, thymidylates, and proteins, will be blocked by MTX. As MTX acts specifically during DNA and RNA synthesis, it is cytotoxic during the S-phase of the cell cycle. Moreover, the anticancer effect of MTX has been linked to a decrease in tissue polyamine levels, since tumors are highly dependent on polyamines (putrescine, spermidine, and spermine) for growth.[34] MTX is effective for the treatment of a number of cancers including breast, head and neck, leukemia, lymphoma, lung, osteosarcoma, bladder, and trophoblastic neoplasms.[35] The most common adverse effects linked to MTX include ulcerative mucositis, low white blood cell count and thus predisposition to infection, nausea, abdominal pain, fatigue, fever, dizziness, acute pneumonitis, and rarely, pulmonary fibrosis. MTX is metabolized by the intestinal microbiota to the inactive metabolite 4-amino-4-deoxy-N-methylpteroic acid; however, this accounts for less than 5% loss of the oral dose.[36]

Development and characteristics of the model. In the first set of studies, the optimal dose of MTX was calculated to administer intramuscularly to the DA rats in order to produce significant, but non-lethal, small intestinal mucositis.[37] Since initial tests with doses <1.5 mg MTX/kg BW given at two consecutive days showed only mild symptoms of mucositis (diarrhoea), the following studies were performed using higher doses. An intramuscular injection of 1.5 or 2 mg MTX/kg BW for two consecutive days reliably caused severe, non-lethal mucositis, clinically manifest by abdominal pain, bloating, and diarrhoea.[2,28] No clinical signs of health deterioration or diarrhoea were observed with a single injection of 1.5 mg MTX/kg BW although histology revealed significant signs of GI injury.[38] Moreover, no clinical signs of oral mucositis could be observed, indicating that oral mucositis is not a good indicator of changes in the GI of rats.

Histopathological changes subsequent to MTX administration were observed at different sites along the AT and at different time points. Oral thickness decreased between 90 min and 6 h post-MTX treatment, although no sign of typical ulcerations could be observed at the level of the tongue, buccal mucosa, or gingiva. An initial increase in colon crypt length occured between 6 and 48 h, which coincided with a decrease in jejunal crypt length and was followed by a decrease in colon crypt length starting from 48 h post-treatment. Other signs of mucosal damage in the jejunum included blunting and fusion of the villi and obliteration of the crypts.[38]

Epithelium tissue levels of nuclear factor-kappaB (NF-kappaB) showed a significant elevation in the jejunum (peaking at 6 h) and colon (peaking at 12 h) but not within the oral mucosa. Significantly elevated levels of tumor necrosis factor-alpha (TNF-alpha) were observed in the oral mucosa, jejunum, and colon peaking between 2 and 6 h in the oral mucosa, at 6 h in the jejunum, whilst in the colon the elevation of TNF-alpha peaked at 24 h.[38] Conversely, significantly elevated levels of interleukin-1 beta (IL-1beta) were observed in the oral mucosa, jejunum, and colon. Within each of the three AT sites, two peaks of IL-1beta occurred at 6 and 48 h in the oral mucosa and at 2 and 24 h in the jejunum and colon. MTX further showed a systemic effect, as serum levels of NF-kappaB, TNF-alpha, and IL-6 were elevated, with peaks at 2 h, 90 min and 2 h, and 2 h, respectively, and decreased IL-1beta levels at 6 h, all preceding histological changes in tissues.[39]

Application of the model. The model has been extensively used by Keefe's group to characterize the mechanisms underlying MTX toxicities recorded in different locations along the AT, and to assess cytoprotective agents. However, in this review we will focus on the group's effort to better unravel the pathobiology of MTX-induced mucositis and discuss it in light of what other groups have found using similar animal models.

The Keefe group found that the small intestine seemed to be the predominant site of damage after high dose MTX treatment of tumor-bearing rats and that MTX-induced mucositis occurs through p53-dependent apoptosis.[2,28,37] A time-course study showed that MTX increased apoptosis by 28-fold in the crypts of the small intestine and by 3-fold in the tumor, both peaking at 6 h after the second dose of 1.5 mg MTX/kg BW. At this dose, the major tissue damage was found at the level of the jejunum and coincided with apoptosis and elevated p53 levels. Upregulation of p53 can either induce apoptosis or block cell proliferation. It inhibits progression of the cell cycle through p21, a cyclin-dependent kinase inhibitor necessary to induce cell cycle arrest that is p53-dependent in response to DNA damage following chemotherapy.[40] However, p21 levels seem to remain constant following high doses of MTX, suggesting a pro-apoptotic role of p53 following MTX.[37] Unlike the small intestine, apoptosis in the colon was recorded to be 10-fold lower, and did not induce overt damage.[37] Others have shown that Paneth cells and Peyers' patches (PP) seem to be spared from MTX-induced damage and that also the function of goblet and Paneth cells remains intact during MTX treatment.[39,41]

Other groups have used similar animal models (although often in absence of tumors) to identify important mediators and signalling pathways that are involved during MTX-induced toxicity, and can be summarized as follows (Table 1).

- The maintenance of intestinal barrier function is highly dependent on epithelial cell-to-cell adhesion, which is indispensable for intestinal architecture. Tight-junctions (TJs) are intercellular junctional complexes that act as a primary barrier to the diffusion of molecules. They are located at the most apical part of the lateral membranes of epithelial cells and comprise components such as transmembrane protein claudins, occludin, and peripheral membrane proteins zonula occludens (ZOs). ZOs bind to the cytoplasmic tail of claudins, which determine the properties of the cell barrier[43] and regulate the localization of the claudins in the TJs ensuring their

Table 1 Biomarkers for MTX-induced AT toxicity and injury

Animal	Protocol	Observations	Ref
Rats	15 mg MTX/kg BW; orally; daily for 3–5 days	Increased claudin-2 and decreased claudin-4 immunostaining. Deminished occludin mRNA levels; compromised interaction between ZO-1 and claudin-4. Increased mRNA levels of TNF-alpha, IL-1beta, MIP-2, and TLR4 in the small intestine	42
Rats	15 mg MTX/kg BW; orally; daily for 3–5 days	Increased tyrosine dephosphorylation of ZO-1 and a reduction of ZO-1 immunostaining along the apical membrane of intestinal villi	43
Mice	Three subcutaneous MTX injections (2.5 mg/kg BW)	No significant inflammation in MTX-treated iNOS −/− mice	44
Rats	20 mg MTX/kg BW; iv; single dose	ROS production preceeds the increase of paracellular permeability in MTX-treated rats. Treatment with NAC prevents MTX-induced ROS production and paracellular permeability	45
Rats	20 mg MTX/kg BW; iv; single dose	ROS production preceeds an increase of myeloperoxidase activity, suggestive of neutrophil infiltration in MTX-treated rats. Both treatments of NAC and tungsten prevent MTX-induced ROS production and neutrophil infiltration	46
Rats	7 mg MTX/kg BW; ip; daily for 3 days	Nitrotyrosine in all the parts of the small intestine (duodenum > ileum > jejunum) of MTX-treated rats	47
Rats	30 mg MTX/kg BW; iv; single dose	Coinciding with inflammatory features in the jejunum, in muscle/serosa and mucosa layers, the levels of anandamide, 2-AG, and PEA peak 3 days after MTX-treatment and return to basal levels at remission (7 days after treatment)	48
Rats	1.25, 2.5, and 5.0 mg MTX/kg BW per day; orally; daily for 6 days	Higher plasma GLP-2 levels in 2.5 mg/kg/day and 5.0 mg/kg/day group. Plasma GLP-2 levels seem to affect the degree of intestinal injury	49
Rats	3.5 mg MTX/kg BW; 3 days followed by gavage with *Escherichia coli* for 2 days and 10 ug/kg G-CSF for 4 days to prevent intestinal barrier dysfunction and BT	BT in MTX-treated rats. G-CSF significantly increases mucosal thickness and villous height of the ileum and decreases intestinal permeability and BT	50
Mice	60 and 120 mg MTX/kg BW; iv; daily for 2 days	Intestinal damage is associated with decreased expression of HNF-1alpha, Cdx2 and GATA-4. This correlates with decreased expression of SI, and seems inversely correlated with enhanced proliferation of epithelial crypt cells	51
Rats	20 mg MTX/kg BW; iv; single dose followed by a second injection of 10 mg/kg/BW after 1 day	mRNA expression of enterocyte and goblet cell markers significantly decreased during damage: SI (−62%) and CPS (−82%) are correlated; also correlations between lactase (−76%) and SGLT1 (−77%) and between I-FABP (−52%) and L-FABP (−45%). Decreases in GLUT5 (−53%), MUC2 (−43%), and TFF3 (−54%) mRNAs occurring independently. Increased lysozyme mRNA present in Paneth cells (+76%). During regeneration, expression of each marker returns to control levels	52
Rats	15 mg MTX/kg BW; orally; daily for 5 days	Increased contents of DNA, RNA, proteins and polyamines (spermine and spermidine) in the jejunal mucosa of MTX-treated rats	53
Rats	2.5 mg MTX/kg BW; sc; daily for 3 days	Upregulation of HGF and c-met coincides with crypt hyperproliferation and mucosal recovery, suggesting a role for HGF in intestinal repair following acute injury	54
Rats	2.5 mg MTX/kg BW; sc; daily for 3 days	Increased TFF3 mRNA but depleted protein levels during histological damage. Cell population expressing TFF3 mRNA expand from usual goblet cells to some non-goblet epithelial cells before goblet cell repopulation	55

(continued)

Table 1 Continued

Animal	Protocol	Observations	Ref
Mice	3 mg MTX/kg BW; ip; single dose	An increase in the adherence of *C. albicans* to murine GI mucosa in MTX-treated mice, concurrently with the maximal decrease in the total number of white blood cells	56
Rats	30 mg/kg BW; iv; single dose	Four days after MTX exposure, the gut enters a proliferative phase with defects in enterocyte maturation (villi of enterocytes show the enzyme profile of crypt cells) namely decreased disaccharidase, alkaline phosphatase, and Na^+/K^{+-}ATPase activities and increased thymidine kinase activity	57

maintenance. A variety of inflammatory mediators, such as reactive oxygen species (ROS), TNF-alpha and interferon(INF)-gamma, disrupt the TJ barrier and alter the localization and phosphorylation status of ZO-1, and therefore alterations of ZO-1 may be associated with disruption of the TJ barrier, leading to enhanced intestinal permeability. MTX-mediated alterations of ZO-1 were confirmed by Hamada et al. showing that the phosphorylation status and expression of ZO-1 was significantly decreased along the apical side of the villi after MTX administration and coincided with increased intestinal permeability.[42] The group further showed that other components of the TJs are altered following MTX.[58] Claudin-2 levels were significantly increased in the crypts and villus tips of the small intestine in MTX-treated rats, while claudin-4 levels were decreased in the villus tips. Furthermore, ZO-1/claudin-4 binding was inhibited by MTX, indicating that MTX impairs cell–cell adhesion and hence normal epithelial intestinal barrier function. Hamada's data further suggested that intestinal barrier dysfunction following MTX administration occurs via a pathway in which MTX-induced ROS elevates macrophage inflammatory protein-2 (MIP-2), TNF-alpha, and/or IL-1beta levels through a signaling cascade downstream of toll-like receptor (TLR4).[58] Paralleling the expression pattern of TLR4,[53] it is not surprising that MTX-induced mucositis occurs more readily in the small intestine than in the colon.[37]

- According to the Sonis model of GI mucositis, ROS generation during chemotherapy is the first step leading to intestinal inflammation, further activating NF-kappaB and upregulating the production of inflammatory cytokines. Different studies have reported that MTX administration in rats indeed enhances the production of ROS in small intestinal mucosa.[45,46,59] Phagocytes, such as macrophages and neutrophils, infiltrate into the inflamed region, and are thought to be responsible for the formation of ROS,[45] which can subsequently alter the localization of TJ components such as ZO-1 and occluding.[47,60]
- ROS probably act together with reactive nitrogen species (RNS) in damaging small intestinal cells, causing nitrosative stress. The role of nitrosative stress in MTX-induced intestinal damage was first studied by Kolli et al.[61] Following treatment with MTX, they found increased staining of nitrotyrosine in the intestinal samples, which was accompanied by neutrophil infiltration and tissue degeneration. RNS are derived from nitrogen oxide (NO) and superoxide produced by inducible nitrogen oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate oxidase, respectively. Cytokines are able to induce nitrosative stress through the expression of iNOS with consequent production of NO.[44] Leitão et al. evaluated the specific role of the iNOS in mice and showed that MTX injections induced intestinal epithelial damage in wild-type mice but not in iNOS −/− mice. The intestinal damage could be prevented by NOS inhibitors, suggesting a critical role of NO, via the inducible iNOS, in MTX-induced intestinal mucositis.[62]
- The endocannabinoid system has been shown to be active in the GI system playing important roles in the control of both gastric and intestinal motility and secretion.[63] Evidence exists that an overactive endocannabinoid system in the gut might intervene to reduce intestinal inflammation.[48] In 2007, D'Argenio et al. showed elevated endocannabinoid levels peaking with atrophy in the jejunum of rats injected with MTX.[49] In the muscle/serosa samples, a peak of anandamide, 2-arachidonoyl-glycerol (2-AG), and palmitoylethanolamide (PEA) levels was found three days after treatment, while in the mucosa samples, 2-AG and PEA were significantly elevated after three days in correspondence with the strongest damage.[49] The authors speculated that putative protective effects of endocannabinoids

are exerted at both the muscle level and the epithelial level to combat acute inflammation.

- Glucagon-like peptide-2 (GLP-2), a peptide member of a family of proglucagon-derived peptides, is secreted from L-type enteroendocrine cells of the distal small intestine and colon, stimulates crypt cell proliferation, and decreases apoptosis in small intestinal epithelium. Plasma GLP-2 levels appear to vary with intestinal mucosal condition and injury. Hirotani et al. demonstrated a relationship between plasma GLP-2 levels and changes in proliferative markers (mucosal weight, DNA and protein content in duodenum, jejunum, and ileum) resulting from MTX-induced intestinal mucositis.[64] Therefore, circulating levels of GLP-2 may be a potential marker for MTX-induced intestinal mucositis.
- BT has been defined as the passage of viable microbiota from the gut lumen to extra-intestinal organs.[65] When the mucosa is injured and the intestinal barrier is compromised, translocation of intestinal microorganisms can occur.[50] MTX may cause intestinal BT as confirmed by Song et al. by the use of a green fluorescent protein-labelled *Escherichia coli* strain fed to rats.[66] Mesenteric lymph nodes, spleen, liver, spleen, and kidney were all positive for the presence of *E. coli* after rats were treated with MTX. They further showed that G-CSF was effective in prevention of BT. Before microbiota can translocate to other tissues, they have to adhere to the intestinal mucosa. Sandovsky-Losica and Segal were the first to report that adherence of *Candida albicans* was significantly enhanced after total body irradiation of mice and after treatment with MTX and 5FU. The maximal increase was observed at 3–4 days post-treatment, concurrently with the maximal decrease in white blood cells and spleen weight of the treated animals.[51] Unfortunately, no mechanistic work or follow-up studies have been reported.
- It is well known that intestinal differentiation is compromised during MTX treatment. De Koning et al. suggested that this may be explained by the fact that intestine-specific transcription factors caudal type homeobox 2 (Cdx2), GATA binding protein 4 (GATA-4), and hepatocyte-nuclear factor-alpha (HNF-1alpha), important for intestinal development, differentiation, and gene expression, are downregulated during the phase of intestinal damage caused by MTX.[67] In combination, Cdx2, GATA-4, and HNF-1alpha have been shown to act as promoting factors of the sucrose-isomaltase (SI) gene[68] as well as several other enterocyte-specific markers.[69] SI is a brush border enzyme with an important function in degradation of disaccharides[70] and is specifically expressed by enterocytes in a differentiation-specific pattern and therefore is a widely used marker for intestinal differentiation. Its expression is characterized by a strong abundance at the crypt-villus junction and mid-villus, and a decreased intensity towards the tips of the villi[52] and is affected by MTX.[39,71] De Koning et al. showed that intestinal damage in mice following MTX was associated with decreased expression of HNF-1alpha, Cdx2, and GATA-4, which correlated with decreased expression of SI, and seemed inversely correlated with enhanced proliferation of epithelial crypt cells.[67] The clinical consequence of SI downregulation is dysfunction of degradation and uptake of sugars by the intestine during villus atrophy. In the rat, other enterocyte markers like lactase, fatty acid binding proteins (I-FABP and L-FABP), SGLT1, and the fructose transporter GLUT5 were also downregulated after MTX treatment similarly to SI.[71]
- Evidence from *in vitro* and *in vivo* experiments suggests that trefoil peptides may play a key role in protecting the GI mucosa from various insults,[72–74] probably by preserving the integrity of the epithelial barrier and promoting the formation of a continuous gel with mucin glycoproteins on the mucosal surface.[75] In mammals, trefoil peptides are produced predominantly in the GI tract and concentrated within the mucus layer.[76] Two studies have shown that the expression of trefoil factor 3 (TFF3) which is predominantly synthesized by the goblet cells in the small and large intestine[55,77] is altered after MTX administration. The number of TFF3-positive cells was decreased along the crypt-villous axis during the increasing villous atrophy and was followed by a depletion of TFF3-positive goblet cells in the lower villous region and an accumulation of TFF3-positive cells in the villous tip region.[41,71] In contrast, Xian et al. reported only significant changes in TFF3 protein expression in the jejunum of MTX-treated rats during damage, which might be due to a different treatment regime.[78]
- In relation to markers associated with intestinal repair following MTX, it is important to mention the hepatocyte growth factor (HGF). HGF has been shown to be a potent mitogen for a variety of epithelial cells,[79] and stimulates mucosal regeneration and functional recovery after massive small bowel resection.[80] It plays a role in gastric ulcer repair, intestinal cell proliferation and restitution.[54,81,82] Xian et al. demonstrated upregulation of both HGF and c-met during the repair of the small intestinal mucosa following MTX-induced damage in the rat. HGF and c-met upregulation coincided with the timing of hyperproliferation during the repair of the intestine, suggesting an involvement of HGF and c-met in the stimulation of crypt cell proliferation initiating mucosal regeneration.[83] This hyperproliferation in the recovery

phase has already been described by Taminiau et al. (1980).[57] Gut epithelial renewal was altered after MTX exposure showing a proliferative phase with defects in enterocyte maturation and villi of enterocytes that exhibited an enzyme profile similar to that of crypt cells (decreased disaccharidase, alkaline phosphatase, and Na^+/K^+-ATPase activities and increased thymidine kinase activity.

The DAMA model of 5FU-induced mucositis

5FU is a potent agent against solid tumors which was introduced in 1957 for the treatment of colorectal cancer, pancreatic cancer, and inflammatory breast cancer. The primary mechanism of action is to inhibit thymidylate synthase,[84] which affects pyrimidine synthesis and leads to depletion of intracellular thymidine triphosphate pools.[85] 5FU has also been proposed to interfere with the activity of ribosomal RNA binding protein (RRBP), at the level of pre-ribosomal RNA processing.[86] 5FU treatment leads to an accumulation of cancer cells in the S-phase.[87–89] Side effects include myelosuppression, mucositis, dermatitis, and diarrhoea.

Development and characteristics of the model. Based on the successful development and application of the DAMA model of MTX-induced mucositis, the Keefe group adapted the model for the study of GI toxicities associated with 5FU. The model is based on studies with large groups of rats that were treated intraperitoneally with a single dose of 150 mg 5FU/kg BW and showed clear clinical and histological signs of GI mucositis.[38]

Morphological changes were observed at different sites along the AT and at different time points. Oral epithelial thickness decreased between 2 and 12 h post-5FU treatment. In parallel with MTX, no sign of ulcerations were observed at the level of the tongue, buccal mucosa or gingiva.[38] In the jejunum, 5FU caused blunting and fusion of the villi as well as enterocyte hyperplasia. A reduction in jejunal crypt length occurred from 12 h extending over the remainder of the 72-h time period.[38] Clear signs of apoptotis were observed at 12 h. Histological examination revealed minimal changes in the colon with increased numbers of apoptotic bodies within the deep aspects of the crypts. Crypt length was increased at 90 min, followed by a brief reduction at 6 h and a further reduction occurred by 72 h.[38]

In contrast to MTX, 5FU administration did not cause elevated tissue staining for NF-kappaB within the oral mucosa, jejunum, or the colon. However, TNF-alpha levels were significantly elevated in the oral mucosa, jejunum, and colon and peaked earlier in the oral mucosa and jejunum than in the colon. With respect to IL-1beta levels, only the oral mucosa demonstrated a significant peak at 6 h. With respect to the blood serum levels of NF-kappaB and pro-inflammatory cytokines following 5FU, NF-kappaB, TNF-alpha, IL-1beta, and IL-6 all peaked before histological evidence of tissue damage, namely at 12 h, 90 min, 60 min and 6 h, and 30 and 60 min, respectively.[38] NF-kappaB is the key molecule involved in 5FU-induced mucositis, as demonstrated by Chang et al. using transgenic mice carrying the luciferase gene driven by a promoter with NF-kappaB responsive elements.[90]

Applications of the model. The model was used by the Keefe group to show that the GI microbiota and mucins play a role in the development of 5FU-induced alimentary mucositis.[91] The microflora found within the duodenum and jejunum mainly consists of anaerobes (*Peptostreptococcus* spp., *Fusobacterium* spp., and *Bacteroides* spp.), *Streptococcus* spp., *Lactobacillus* spp., *Veillonellae*, *Actinomyces* spp., and a variety of fungi.[92] Following 5FU, a decrease in *Clostridium* spp., *Lactobacillus* spp., and *Streptococcus* spp., and an increase in *Escherichia* spp. could be observed in the jejunum of rats.[91] In contrast to the small intestine, the colonic microflora is more diverse, consisting of over 400 different species[92] and includes large numbers of anaerobes (*Bifidobacterium* spp., *Bacteroides* spp., *Eubacterium* spp., *Peptotrecoccus* spp., and *Clostridium* spp.), enterococci and Enterobacteriaeceae.[92,93] In the colon, 5FU caused decreases in *Enterococcus* spp., *Lactobacillus* spp., and *Streptococcus* spp. Faecal samples showed decreasing trends in *Lactobacillus* spp. and *Bacteroides* spp., an increasing trend in *E. coli*, and significant increases in *Clostridium* spp. and *Staphylococcus* spp. at 24 h.[91]

Other groups have used similar animal models (although often in absence of tumors) to identify important mediators and signaling pathways that are involved during 5FU-induced toxicity, and can be summarized as follows (Table 2).

- In response to DNA damage, cells can either undergo apoptosis or G1 cell cycle arrest. Early studies[109–111] have shown that 5FU induces both apoptosis and cell cycle arrest in murine intestinal epithelial cells and tends to target cells at specific positions within the crypt. Later, it has been shown that 5FU induces apoptosis via a p53-dependent mechanism in murine intestinal crypt epithelium.[103,107,112,113] Also, cell cycle progression was significantly suppressed following 5FU administration and correlated with a prolonged, p53-dependent expression of p21waf-1/cip1.[112] Also, p53 −/− mice showed significant reduction in apoptosis and inhibition of cell cycle progression following 5FU compared to wild-type mice.[113] Inomata et al. detected p21WAF/Cip1 positivity in the nuclei of the first appearing apoptotic cells and proposed that cells in the stem cell zone respond to 5FU in inducing p53-dependent apoptosis or cell cycle arrest.[114] Apoptotic cells are eliminated while the p21WAF/Cip1-positive arrested cells appear to move up to the apex of the crypt.
- The mismatch repair (MMR) proteins have been shown to be essential for the normal response to a wide spectrum of agents including oxidative damage, ionizing radiation, cisplatin, UV, and 5FU damage.[102] They signal apoptosis either directly or

Table 2 Biomarkers for 5FU-induced AT toxicity and injury

Animal	Protocol	Observations	Ref
mice	450 mg 4FU/kg BW; ip; single dose	Significant damage, MPO activity and elevated levels of pro-inflammatory cytokines (IL-4, TNF-alpha, IL-1beta and CXCL-8) in the intestinal epithelium of 5FU-treated IL-4 wild-type mice but not in −/− mice	94
mice	600 mg 5FU/kg BW; ip; single dose	Hg signaling consistently downregulated following 5FU during the injury phase followed by upregulation during the repair phase. Hg signaling inhibition augments apoptosis and suppresses mitotic activity in intestinal crypts	95
mice	100 mg 5FU/kg BW; ip; single dose	Network analysis of 5FU-affected genes by transcriptomic tool shows that NF-kappaB is key molecule. 5-ASA inhibits 5FU-induced NF-kappaB activation and proinflammatory cytokine production	88
mice	450 mg 5FU/kg BW; ip; single dose	In PAF-R −/− mice and PAF-R antagonist-treated mice, 5FU-induced intestinal damage is less than in wild-type mice	96
mice	40 mg 5FU/kg BW; ip; single dose	IL-6 −/− mice exhibit increased apoptosis after 5FU relative to wild-type controls	97
rats	50 mg 5FU/kg BW; iv; daily for 6 days	Increased plasma endotoxin,TNF-alpha and IL-6 levels in the 5FU animals. BT at liver, spleen, mesenteric lymph node, and portal blood	98
mice	10 mg 5FU/kg BW; continuous iv infusion for 5 days	Reduced CD4/CD8 ratio in the lamina propria, GALT cell number and mucosal IgA levels after 5FU-exposure	99
rats	30 or 60 mg 5FU/kg BW; orally; daily for 4 days	Higher Na^+/K^+-ATPase activity and D-glucose absorption in 5FU-treated rats	100
hamsters	60 mg 5FU/kg BW; ip; on days 0, 5, and 10	Decreases in cheek pouch and jejunum epithelium proliferation rates, increases in MMP-2 and -9, and plasmin, and decreases in TIMP-1 and -2 following 5FU	101
mice	400 mg 5FU/kg BW; ip; 6 h between 2 injections	Reduced apoptosis in MBD4 −/− mice following treatment with gamma-irradiation, cisplatin, temozolomide and 5FU	102
mice	[^{14}C] 5FU for 1 h at a flow rate of 20 ml/kg BW	5FU induces apoptosis and/or cell cycle arrest: p21(WAF/Cip1)-positive nuclei migrate up the crypt, while bax-positive cytoplasm are observed throughout the crypt epithelial cells	103
mice	40 mg 5FU/kg BW; ip; single dose	bcl-w −/− mice exhibit more apoptosis in the small intestine than the wild-type mice after 5FU-exposure	104
mice	40 mg 5FU/kg BW; ip; single dose	Elevated apoptosis at base of crypts in colonic epithelium of 5FU-treated bcl-2 −/− mice. Bcl-2 seems to play a key role in determining the sensitivity of colonic stem cells to 5FU-damage	105
rats	15 or 30 mg 5FU/kg BW, orally; daily for 3-4 days	BW loss and epithelial barrier dysfunction of the small intestine in 5FU-treated rats as shown by increased permeation of fluorescein isothiocyanate-labelled dextran	106
mice	40 and 400 mg 5FU/kg; ip; single dose	Less 5FU-induced apoptosis and cell cycle inhibition in p53 −/− mice.	107
rats	300 mg 5FU/kg BW; intragastrically; single dose	Reduced levels of glucose, phosphate transporter, sucrase and SGLT1 after 5FU-exposure	108
mice	200 mg 5FU/kg BW; iv; single dose	Increase in adherence of *C. albicans* to murine GI mucosa after 5FU	56

through cycles of repair.[115] The thymine DNA glycosylase methyl-CpG binding domain protein 4 (MBD4) which interacts with MMR protein MutL homolog 1 has been shown to play a role in mediating the apoptotic response within the small intestine. MBD4 −/− mice showed significantly reduced apoptotic responses following treatment with irradiation, cisplatin, temozolomide, and 5FU.[105] This means that a significant proportion of the apoptotic response to 5FU and other chemotherapeutics are dependent on functional MBD4.

- The Bcl-2 gene family of proteins control apoptosis in response to a wide variety of stimuli, including chemotherapy.[104] They contain both pro- and anti-apoptotic members, which are mainly involved in the intrinsic apoptotic pathway through regulation of mitochondrial membrane permeability and caspase activation. Interestingly, the bcl-2 expression level is maximal in the colonic crypt and this seems to play a role in hindering apoptosis following 5FU. In the small intestine, however, bcl-2 did not appear to play a significant role in the control of

apoptosis. Overall, bcl-2 −/− mice display higher levels of spontaneous apoptosis following 5FU.[98,107] Experiments with bax −/− mice suggest that bax has only minor roles in the apoptotic response to gamma-radiation or 5FU in both the small intestine and the colon,[56,98] whereas bcl-w −/− mice exhibited more apoptosis after 5FU treatment than their wild-type counterparts in both colon and small intestine. Therefore, it seems likely that the final outcome is dependent on the regional and/or temporal balance between all members of the Bcl-2 family of regulator proteins.

- Similar to MTX, 5FU causes significant increase in the frequencies of BT in liver, spleen, mesenteric lymph nodes (MLNs), and portal blood which is counteracted by GM-CSF. Microbial isolates are mostly Gram-negative microbiota.[101] An increase in the adherence of *C. albicans* to murine GI mucosa was found following 5FU treatment.[116]
- A balance in expression between destructive matrix metalloproteinase (MMPs) and their inhibitors, tissue inhibitor of metalloproteinase (TIMPs), is essential to ensure that tissue homeostasis as has been shown in the hamster model for oral mucositis, where 5FU induced an increase in MMP-2 and -9 with concurrent decreases in TIMP-1 and -2 in the damaged cheek pouches.[117]
- Gut-associated lymphoid tissue (GALT) in the intestine has been considered a centre of mucosal immunity[99] and reduction of GALT mass and function may impair mucosal defense.[106] Nagayoshi et al. showed that even a small dose of 5FU reduced lymphocyte numbers at GALT effector sites, associated with a decrease in intestinal and respiratory tract immunoglobulin A (IgA) levels.[108] Therefore, GI symptoms and the systemic inflammatory response accompanying 5FU treatment may be associated at least in part with these GALT changes.
- 5FU administration to rats causes epithelial barrier dysfunction of the small intestine, resulting in the increase of small intestinal permeation.[100,103,118] For example, absorption of D-glucose which is actively transported across the cell membrane by Na+-dependent glucose cotransporter (SGLT1) on brush-border membrane of the small intestine[119] is increased following repeated administration of 5FU to rats.[120] Surprisingly, the SGLT1 protein expression was decreased, meaning that increased D-glucose absorption is probably related to the activation of the Na+/K+-ATPase activity which is a driving force of SGLT1-mediated transport. Interesting to note in this context, is that Sadoff reported that the administration of 5FU to patients with diabetes enhanced the glucose concentration in plasma and caused severe diarrhoea and blood poisoning.[94] Hence, the enhancement of glucose absorption in the small intestine by the 5FU treatment would worsen the condition of diabetics.
- IL-4 is a critical mediator of intestinal inflammation and functions as either an anti- or pro-inflammatory molecule depending on the model of intestinal inflammation.[121] Soares et al. observed that 5FU-induced intestinal mucositis with a concomitant increase in IL-4 concentration in wild-type mice compared to untreated wild-type mice.[97] Knockouts of IL-4 did not show the pathological alterations of 5FU-induced mucositis, hence demonstrating the pro-inflammatory role of this cytokine.
- IL-6 is recognized as an important mediator of gut dysfunction in inflammatory bowel disease.[122] The role of IL-6 in 5FU-induced small intestinal damage was investigated by Jin et al. who showed that IL-6 −/− mice exhibited increased caspase-3-dependent apoptosis in the ileum and colon after 5FU, relative to wild-type controls.[123] Therefore, endogenous IL-6 most likely plays an important role in limiting intestinal injury and cell death following 5FU treatment.
- Platelet-activating factor (PAF) is produced endogenously during gut inflammation.[124] It is a potent mediator of many inflammatory processes, important for prostaglandin and eicosanoid synthesis, the induction of apoptosis, and NF-kappaB activation.[96,125,126] Soares et al. demonstrated that PAFR −/− mice were protected against intestinal damage caused by 5FU treatment. They further demonstrated that PAFR −/− mice had significantly less pro-inflammatory cytokine production in duodenal tissue, suggesting that PAF is involved in the activation of the cytokine cascade that leads to the intestinal damage.[127] PAF has indeed been shown to increase the proteolytic processing of NF-kappaB and expression of pro-inflammatory cytokines, thereby mediating acute bowel injury in mice.[128]
- There is some evidence to suggest that developmental pathways play a role in adult tissue regenerative processes.[129] The Hedgehog (Hh) pathway is known to be essential for GI development. Hh signaling occurs from the epithelial cell layer to stimulate mesenchymal growth in the developing digestive tract[95] and is vital for patterning the intestinal crypt-villus axis in mice.[130] Recent data have shown that Hh ligand expression displays a biphasic expression pattern of downregulation during injury phase and upregulation during the repair phase of the gut mucosa damage following 5FU.[131] This finding is consistent with the results of a previous report which shows that Hh signaling is involved in gastric ulcer repair.[132]

Irinotecan-induced DAMA model of mucositis

Irinotecan is a relatively new cytotoxic agent used to treat a variety of solid tumors. The primary mechanism of action is to inhibit DNA topoisomerase I.[5,6] DNA damage is induced by trapping topoisomerase I during its normal action in regulating DNA structure.[133] During DNA replication, topoisomerase I produces reversible single-strand breaks by cutting and reattaching double chain DNA. These breaks relieve the torsional strain generated by the advancing replication forks.[133]

Irinotecan causes severe diarrhoea in 60–80% of patients.[134] Cholinergic, secretory diarrhoea occurs early, and can be prevented by the administration of atropine prior irinotecan. A delayed diarrhoea also occurs, compounded by the action of β-glucuronidase making irinotecan toxicity worse.[6,135] Leukopenia is another dose-limiting side effect of irinotecan, compromising the patient and resulting in opportunistic infection.[5,6] Irinotecan is converted by hepatic and GI carboxylesterases to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38) which is responsible for irreversible DNA damage.[6,133] SN-38 has a stronger anti-carcinogenic activity but is further processed by glucuronyltransferase to become SN-38 glucuronide, a less toxic form of SN-38.[6] However, the glucuronide is transported to the intestine during bile excretion and is able to be hydrolyzed by glucuronidase to return to the toxic form. Both bind the topoisomerase I-DNA complex, preventing relegation of the DNA strand. This leads to double-strand DNA breakage and cell death. A number of intestinal microbiota have β-glucoronidase activity and may be responsible in part for the intestinal cytotoxicity of irinotecan.[6] Microbial β-glucoronidase is produced primarily by *Enterobacteriaeceae* (*E.coli*, *Salmonella* spp., *Shigella* spp., *Yersina* spp., *Citrobacter* spp., *Hafnia* spp., and *Edwardia* spp.) and has been reported to be produced by *Flavobacterium* spp., and *Bacteriodes* spp.[136]

Development and characterisation of the model. A single-dose irinotecan model of GI mucositis was established in 2003 using different doses of 100, 150, and 200 mg irinotecan/kg BW in the rat with implanted breast cancer tumors.[25] Animals receiving 100 and 150 mg irinotecan/kg BW showed mild to moderate diarrhoea by 96 h; however, 50% of the animals receiving 150 mg irinotecan/kg BW had died at that time point. At 200 mg irinotecan/kg BW, all animals had severe diarrhoea and a 100% mortality rate at 96 h.

Histopathology showed that irinotecan caused increased apoptosis, crypt hypoplasia, crypt dilation, and mucus secretion in the colon. In the small intestine, the jejunum showed increased apoptosis, villous atrophy, and crypt hypoplasia. Moreover, irinotecan decreased the liver weight significantly.[25] Irinotecan caused a peak of jejunal apoptosis at 6 h (100 mg/kg BW and 200 mg/kg BW) which decreased at later time intervals, but remained elevated. A similar peak of apoptosis occurred in the colon at 6 h (100 mg/kg BW and 200 mg/kg BW) which decreased more rapidly than in the small intestine. In contrast to MTX and 5FU, goblet cell numbers and mucus secretion in the small intestine did not alter after irinotecan treatment, whereas goblet cells in the colon showed mucus hypersecretion and at 96 h, goblet cell number had significantly decreased as previously reported by other studies.[5,137]

A more detailed analysis of the mucosal changes in the AT following a single intraperitoneal dose of 200 mg irinotecan/kg BW in non-tumor bearing rats showed that rats began to demonstrate diarrhoea and symptoms of GI mucositis already after 2 h which was more prevalent at 24 h.[138] Tissue damage was most pronounced in the jejunum, where cell death was already obvious after 6 h following treatment. In general, the stomach showed the lowest severity of tissue damage, followed by the colon where cell death was significantly increased at 24 h. In the oral mucosa, changes were noted in the thickness of the epithelium over a 72 h time period, however, no obvious areas of ulceration could be identified. In the jejunum, changes included the presence of degenerative enterocytes within the crypts at 6 h followed by villus blunting, epithelial atrophy, and increased infiltration of inflammatory cells. A decrease in villus and crypt length could be observed already between 30 and 60 min and was followed by a resolution at 2 h and another significant reduction at 12 h before returning to lengths comparable to controls by 24 h. In the colon, initial histological changes included the presence of individual degenerative enterocytes within the crypts, followed by complete ablation of the crypts at later time points. Crypt length in the colon increased at 30 and 60 min, returned to normal levels at 2 h, and then increased again at 24 h.

Irinotecan administration did cause significantly elevated tissue staining for NF-kappaB within the oral mucosa, jejunum and the colon.[138] Levels peaked in the oral mucosa at 2 h following irinotecan administration and were consistently higher throughout studies. In the jejunum, tissue NF-kappaB levels peaked at 12 h before subsiding and then slowly increased over the later time points. In the colon, NF-kappaB levels were significantly elevated at all time points. Oral mucosa and jejunal TNF-alpha levels peaked at 6 h, while significantly elevated levels were observed in the colon peaking early at 2 h. With regards to IL-1beta, an elevated (non-significant) trend was observed in the oral mucosa at the later time points, whilst in the jejunum, significantly elevated levels peaked at 6 h. In the colon, the tissue levels of IL-1beta peaked at 12 h. As for IL-6, tissue levels in the oral mucosa were significantly elevated at the later time points, whereas a (non-significant) peak could be observed in the jejunum and colon at 6 h.[138] A peak in serum concentration of NF-kappaB was detected at 6 h following irinotecan, remaining significantly elevated at 12 h. Two peaks in serum levels of TNF-alpha at 60 min and 24 h were observed. Serum IL-1beta levels were decreased at 60 min and were then increased at 2 h following irinotecan administration. IL-6 demonstrated a steady increase in concentration peaking at 12 h and then rapidly reduced to baseline levels.[139]

Application of the model. The model has been used to investigate irinotecan-induced alterations in extracellular matrix (ECM) components.[140] A dose of 200 mg/kg BW

Table 3 Biomarkers for irinotecan-induced AT toxicity and injury

Animal	Protocol	Observations	Ref
Rats	250 mg irinotecan/kg BW; ip; daily for 2 days	Bacteria detected in the mesenteric lymph node or spleen. Large intestinal resistance of the rats is decreased, while small intestinal resistance is increased. Decreased claudin-1 expression in small and large intestine, decreased occludin expression in small and large intestine (tendency) in IR-treated rats	[147]
Mice	100 mg irinotecan/kg BW; ip; daily for 3 days (3 times)	Increased morbidity, mortality and BT in GLP-2 R −/− mice in response to IR	[148]
Mice	60, 80, 100, and 150 mg irinotecan/kg BW; ip; for 4 days	Germ-free mice are more resistant to IR than the holoxenic group	[146]

resulted in a substantial increase in the total collagen deposits around crypts from 24 h in both the jejunum and colon, although collagen IV expression decreased significantly in the crypt region in a delayed fashion. Likewise, fibronectin expression decreased significantly in jejunum and colon from 6 to 24 h following treatment. These changes in the ECM seemed to correlate with the altered epithelial cell kinetics in both the jejunum and colon as cellular proliferation was halved in the jejunum and colon at 48 and 24 h, respectively, while apoptosis peaked at 6 h.[141] This peak in apoptosis was further confirmed by gene array analysis experiments showing that genes involved in apoptosis, mitogen-activated protein kinase (MAPK) signaling, and inflammation were upregulated in the small intestine following irinotecan treatment.[26]

The mechanism behind irinotecan-induced mucosal injury was further explored using tumor-bearing rats that underwent irinotecan treatment with and without the p53 inhibitor, pifithrin-alpha.[142] Although pifithrin-alpha reduced severity and duration of intestinal apoptosis, it did not significantly affect p53 expression, intestinal cell death, or mucositis following irinotecan, suggesting that irinotecan may act through upregulation of pro-apoptotic proteins or the activation of caspases to induce cell death. This was shown in tumor-bearing rats treated with two consecutive doses of 150 mg irinotecan/kg BW, where an increase in caspase-1 and -3 expression and downregulated levels of bcl-xL, bcl-2 like 1 and cyclin D2 and G were noticed in the small intestine.[27] A more detailed kinetic study using microarray and RT-PCR analysis of intestinal tissues was done with non-tumor-bearing rats treated with a single dose of 200 mg irinotecan/kg BW. At 1 h, many of the genes induced were known p53 targets, some of which are also negative regulators of the MAPK signaling pathway. These early response genes are probably increased in response to cellular stress, particularly DNA damage, and help control apoptosis and the cell cycle. These results suggest that irinotecan-induced damage signaling is p53-dependent to some degree and that early interference with p53 and MAPK activation could affect downstream development of tissue damage. At 6 h, the predominant inhibitory gene changes were related to the cell cycle, including cyclins and cyclin dependent kinases and their regulators which play a major role in mediating the antiproliferative effects of irinotecan. A number of genes involved in the response to DNA double-strand breaks were also identified. Genes that were upregulated at 6 h included transcription factors and cytokine receptors (like TNF and INF receptors) that are known to be activated by inflammatory signals and act through MAPK signaling to induce apoptotic and inflammatory responses in tissue injury. At 24 h, apart from the genes that reflect an antiproliferative and metabolically inactive environment, also the ones involved in the carbohydrate metabolism were altered. At 72 h, genes involved in regulation of cell proliferation, wound healing, and blood vessel formation were upregulated, reflecting the repair phase following tissue insult.[141]

Also, tissue levels of MMPs and TIMPs have been shown to be significantly altered following treatment with irinotecan.[143] MMPs are endopeptidases with a predominant role in ECM though regulation of its components. Recently, they have been shown to act as mediators of damage in mucositis development,[143] which is not surprising given the fact that MMPs are known to play a key role in tissue injury and inflammation in many GI disorders.[144,145] A dose of 200 mg irinotecan/kg BW resulted in an increase in MMP-2, -3, -9, and -12 levels at various time points depending on the tissue. These changes were associated with inflammatory infiltrate and maximum tissue damage. In contrast, MMP-1 expression correlated with tissue restitution. TIMP-1 and -2 levels were only significantly altered in the jejunum. Also, the expression of the tissue-type plasminogen activators uPA and tPA, as well as plasminogen activator inhibitor-1 (PAI-1), known to play a role in MMP activation, increased significantly in the jejunum starting from 6 h following treatment. There was no significant change in tPA or uPA and a decrease in PAI-1 was noticed in the colon.[143] Given the expression profiles of MMPs correlate with the histopathological damage, these results suggest varying roles for MMPs at different stages of mucositis development within the AT.

Irinotecan-induced mucositis manifests typically as early diarrhoea observed in the rats from 2 to 6 h, and a late onset diarrhoea apparent at 72 h after treatment. The latter corresponds with changes in the luminal environment. As was reported for 5FU,[90] goblet cell numbers, composition, and distribution were also altered upon irinotecan treatment.

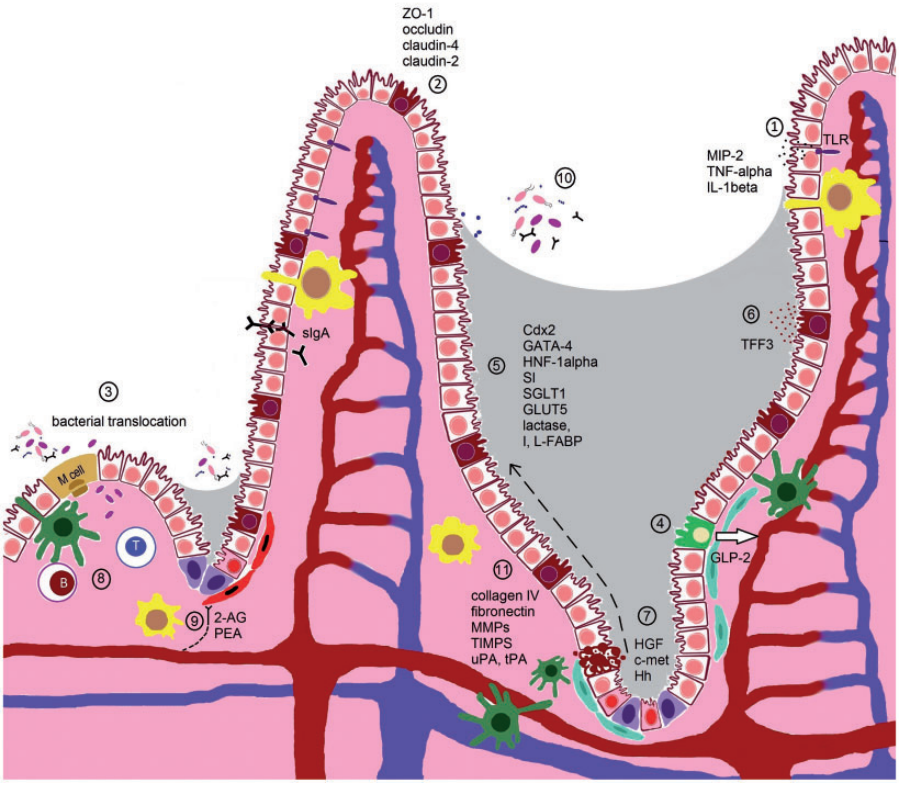

Figure 1 Cellular and molecular targets of MTX, 5FU and irinotecan in the small intestine of the AT. In the small intestine, the barrier provided by the small IECs (white) ensures a segregation between luminal microbiota and the mucosal immune system. In the crypts, the intestinal epithelial stem cells (pink) share their niche with stromal (blue), dentritic cells (green) and macrophages (yellow), and ensure the continuous renewal of the epithelial cell layer. After differentiation, IECs migrate up the crypt–villus axis (dashed) arrow. Goblet (brown) and Paneth (purple) cells secrete mucus and antimicrobial proteins to exclude microbiota. The release of sIgA further contributes to this barrier function. M cells and goblet cells mediate transport of luminal antigens and live microbiota across the epithelial barrier to dendritic cells (green), and macrophages sample the lumen through transepithelial dendrites. Pathogen recognition receptors such as TLRs recognize conserved microbial-associated molecular motifs and pathogen-specific virulence properties. TLRs induce cytokines on ligation to signal molecules via NF-kappaB activation and the MAPK pathway. Cytotoxics MTX, 5FU, and irinotecan impair different normal epithelial intestinal functions via different mechanisms:
(1) MTX enhances the production of ROS and RNS, probably formed by phagocytes in the inflammatory regions of the small intestine, which trigger the activation of TLR4, and downstream NF-kappaB and MAPK pathways leading to elevated levels of MIP-2, TNF-alpha, and IL-1beta.
(2) MTX impairs cell–cell adhesion by destabilizing the interaction between ZO-1 and claudin-4 and altering the normal expression levels of claudin-2 (increased), claudin-4 (decreased) and occludin. Irinotecan has been shown to decrease claudin-1 and occludin expression in both the small and large intestine. This might be due to the increased expression of TNF-alpha, IL-1beta, MIP-2, and TLR4. MTX also causes the formation of RNS that probably act together with ROS in damaging small intestinal cells, either in the MLNs or the spleen.
(3) MTX increases the risk in *Escherichia coli* translocation and both MTX and 5FU have been shown to promote adherence of *Candida albicans.* Microbiota is also detected in irinotecan-treated rats.
(4) MTX stimulates the levels of GLP-2, a peptide secreted from L-type entero-endocrine cells (green) of the distal small intestine and colon, in blood and might therefore be a potential marker for MTX-induced intestinal mucositis. The disruption of basal GLP-2 R signaling seems to play a role in irinotecan-induced intestinal injury as exogenous activation of the GLP-2 receptor ameliorates gut injury.
(5) MTX downregulates the transcription factors Cdx2, GATA-4, and HNF-1alpha, which are important for intestinal development, differentiation, and gene expression. This is correlated with a decreased expression of the SI. Also enterocyte markers lactase, I-FABP, L-FABP, SGLT1, and GLUT5 are downregulated by MTX. In contrast, 5FU stimulates the activity of the Na+-dependent SGLT1 while the SGLT1 protein expression is decreased, resulting in a higher absorption of D-glucose.
(6) MTX alters the expression of TFF3, which are predominantly synthesized by the goblet cells. TFFs play an important role in preserving the integrity of the epithelial barrier.
(7) MTX, 5FU, and irinotecan induce p53-dependent apoptosis and cell cycle arrest in the epithelial stems cells of the deep crypts of the small intestine in the initial stage of mucositis. The degree of apoptosis seems to depend on the expression levels of members of the Bcl-2 family. Irinotecan, MTX, and 5FU also reduce the number of goblet cell numbers. In the mucosal regeneration phase, an upregulation of HGF and c-met can be noticed following MTX treatment while the Hh signaling seems to be important during the repair phase of the gut mucosa damage following 5FU.
(8) GALT in the intestine has been considered a centre of mucosal immunity. 5FU has been shown to reduce the number of lymphocytes at the GALT effector sites and the levels of intestinal and respiratory tract IgA levels.
(9) Endocannabinoids receptors are localized throughout the gut and predominantly located in the enteric nervous system expressed in cholinergic neurons. Receptor activation leads to relaxation in muscle cells (red) of the gut. MTX results in an overactive endocannabinoid system in the gut which might function to reduce intestinal inflammation.
(10) 5FU and irinotecan alter the microbial composition of the gut. A decrease in *Clostridium* spp., *Lactobacillus* spp., and *Streptococcus* spp., and an increase in *Escherichia* spp. could be observed in the jejunum of 5FU-treated rats. In irinotecan-treated rats, a decrease in *Bacteroides* spp., *Lactobacillus* spp., and *Bifidobacterium* spp can be noticed, whereas *Staphylococcus* spp., *Clostridium* spp., and *E. coli* are increased.
(11) Irinotecan and 5FU can cause dramatic changes in the ECM. Irinotecan results in the increase of total collagen deposits around crypts, but a decrease in collagen IV and fibronectin expression in the crypt region. Irinotecan and 5FU further alter the levels of MMP-2, -3, -9, and TIMP-1 and -2 in the jejunum. Also, the expression of the serine proteases uPA and tPA as well as PAI-1, known to play a role in MMP activation, is increased following irinotecan.

Total goblet cell number in the jejunum decreased significantly in the crypts after 48–72 h and in the villus after 24 h. In the colon, the most noticeable decrease was noted after 48–72 h. Mucin staining in the jejunum decreased in both the crypts and villi from 90 min onwards and became more sulphated in treated rats. In the colon, no major changes could be noticed, although a shift towards the sulphated form was seen. Muc2 and Muc4 expression is significantly lowered in the villi and crypts of the jejunum at 12 h and 24–48 h, respectively, and in the colon between 24 and 72 h.[146]

With regards to the alimentary microbiome, changes could be observed in the stomach, jejunum, colon, and faeces of rats. The majority of the changes were seen at 6, 12, and 24 h after treatment and persisted up to 72 h. *Bacteroides* spp., *Lactobacillus* spp., and *Bifidobacterium* spp. were shown to decrease after treatment, whereas *Staphylococcus* spp., *Clostridium* spp., and *E. coli* increased. These data suggest the involvement of the alimentary microbiome in the severity of irinotecan-induced mucositis.[147]

Other groups have used similar animal models (although often in absence of tumors) to identify important mediators and signaling pathways that are involved during irinotecan-induced toxicity, and can be summarized as follows (Table 3).

- It was reported that irinotecan causes BT in mice.[149] Nakao et al. investigated the relationship between disruption of TJs and BT after irinotecan-treatment of rats.[148] Microbiota were detected in 80% of irinotecan-treated rats either in the MLNs or the spleen, while no BT occurred in the control rats. Irinotecan-treated rats also showed decreased claudin-1 and occludin expression in both the small and large intestine, therefore indicating that TJ might be disturbed after irinotecan treatment permitting microbiota and toxins present in the intestinal lumen getting into the deep tissues.[148]
- The disruption of basal GLP-2 R signaling seems to play a role in irinotecan-induced intestinal injury and bacteremia. It was shown that exogenous activation of the GLP-2 R ameliorates experimental small and large bowel gut injury.[150] Lee et al. studied the importance of the GLP-2 R for gut growth and the response to mucosal injury, and host-microbiota interactions in GLP-2 R +/+ and GLP-2 R −/− mice. GLP-2 R −/− mice exhibited significantly enhanced mucosal injury in response to irinotecan and microbial colonization of the small bowel was significantly increased.[151] The authors speculate that the latter may be due to the reduced expression of antimicrobial products derived from Paneth cells in GLP-2 R −/− mice. These data reveal a role for GLP-2 in the control of gut microbial communities.

Conclusion

The data obtained from the three rat models for chemotherapy-induced mucositis generally suggest that there are differences in the mucositis pathobiology depending on the type of chemotherapeutic drug used. Although this seems logical as MTX, 5FU, and irinotecan are known to act via various molecular pathways (Figure 1), this is in contrast to what has been assumed so far. However, this may have implications for the management of mucositis, particularly with respect to the development of treatment regimens for mucositis. Further investigations are required to determine the exact pathways that lead to damage caused by the different drugs. Recent data have shown that the Dark Agouti rat model is suitable to assess the safety and efficacy of chemoprotective agents. Any agents that show response in this model without promoting tumor growth can then be considered for human clinical trials, either singly or in combination.

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