Methotrexate depletes serotonin (5-HT) production and 5-HT_{2B} receptor expression in the small intestine of rats

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Abstract

The indiscriminate, cytotoxic nature of anti-cancer treatments causes gastrointestinal mucositis (GIM), an inflammatory gut state that underpins a constellation of significant clinical and economic consequences, including diarrhoea and increased costs of care. The current lack of effective treatment options has been attributed to a poor understanding of targetable GIM mechanisms. Therefore, this study aimed to quantify how suspected pathobiological contributor, serotonin (5-HT), production and receptor expression changed following chemotherapy, using a well-established, preclinical model of GIM. Tumour bearing Dark Agouti rats received either two doses of methotrexate or saline, and were assessed for validated markers of GIM, diarrhoea and weight loss. Rats were killed 72 hours after initial methotrexate treatment. 5-HT production was assessed in the jejunum and colon by immunofluorescence, in which 5-HT+ cells were counted and normalised to tissue area, and by qPCR, to quantify 5-HT production enzyme, *Tph1*, expression. Further qPCR was conducted for receptors, 5-HT_{2A} and 5-HT_{2B}. Methotrexate-treated rats displayed lower levels of jejunal 5-HT+ cells/μm^2 (control: 0.0051±0.0010, MTX: 0.00045±0.00020, **P=0.0013), as well as relative *Tph1* (control: 470±270, MTX: 41±16, **P=0.0087) and 5-HT_{2B} (control: 9500±3300, MTX: 730±390, **P=0.0079) mRNA expression. These findings suggest a potential anti-inflammatory role of 5-HT in GIM, which may be mediated through the 5-HT_{2B} receptor, and support further investigation of the translational potential of maintaining 5-HT production to minimise local GIM and related systemic toxicities.

Introduction

Although recent and significant advances in medicine have improved cancer survival rates and time living after diagnosis,[1] they have also exacerbated the ever-prominent, yet widely under-recognised physical and psychological consequences of cancer treatment.[2] One such consequence is gastrointestinal mucositis (GIM), an acute side effect of cytotoxic cancer therapy characterised by inflammation of the gastrointestinal mucosa,[3] which occurs when highly regenerative cell populations become unintended targets of non-selective chemo- and radiotherapy.[4] For the 70% of chemotherapy recipients that experience mucositis,[4] they must not only navigate the burden of cancer, but must also endure troublesome and debilitating clinical manifestations, such as diarrhoea, abdominal bloating and significant pain.[3] Furthermore, these symptoms create a multi-faceted burden, encompassing clinical, psychosocial and economic factors, which impacts the affected individual, their family and friends, and the healthcare system.[5, 6]

An extensive GIM knowledge base has been established, particularly in terms of initiating factors and key tissue changes. Fundamental GIM pathogenesis has been classified into five distinct yet overlapping stages, and key hallmark characteristics have been identified as villous atrophy, shallow crypts, ulceration and inflammation.[7, 8] This knowledge assisted in the development of new clinical practice guidelines, with several strategies for *oral* mucositis now exisiting.[9] Unfortunately, this level of progress is unmatched for GIM, with current interventions only mediating symptoms, rather than addressing key underlying mechanisms.[9]

Controlling GIM is particularly challenging as it is initiated by the same cytotoxic mechanisms that contribute to the efficacy of anticancer therapies.[10] To avoid impairing treatment efficacy, novel interventions have attempted to address secondary mechanisms that amplify tissue injury,[11] with particular focus on host-microbe interactions, given their ability to modulate various aspects of mucositis pathobiology.[8] Whilst highly modifiable, this approach is again undermined by the

risk of impairing chemotherapy efficacy, with translocation of luminal microbes known to activate inflammatory pathways responsible for tumour cell death.[12] Thus, more viable and promising molecular therapeutic targets must be identified.

Gut-derived serotonin (5-hydroxytryptamine or 5-HT) is of particular interest in the context of gastrointestinal disease. Of total bodily 5-HT, 90% is synthesised, stored and released from enterochromaffin (EC) cells, an enteroendocrine cell that is ubiquitously expressed in the gastrointestinal tract mucosal lining.[13] The rate-limiting enzyme in EC cell 5-HT production is tryptophan hydroxylase 1 (*Tph1*; Figure 1).[14] Following basolateral excretion, seven groups of serotonergic receptors, some with additional subtypes, facilitate an array of physiological functions of mucosal 5-HT (5-HT released from EC cells).[14] This includes local modulation of gut motility, secretion and fat absorption, as well as distal effects in organs such as the pancreas and liver.[15] In a GIM context, the 5-HT2 receptor family is of particular interest, due to the expression of subtypes, 5-HT_{2A} and 5-HT_{2B}, throughout the gastrointestinal tract, on circular and longitudinal smooth muscle cells, immune cells, enterocytes and neurons.[16, 17] Furthermore, these receptors have a determined role in stimulating gastrointestinal smooth muscle contraction and epithelial cell secretion,[16] two regulatory mechanisms behind diarrhoea, which can be induced by chemotherapy via disruption of the intestinal mucosa.[3]

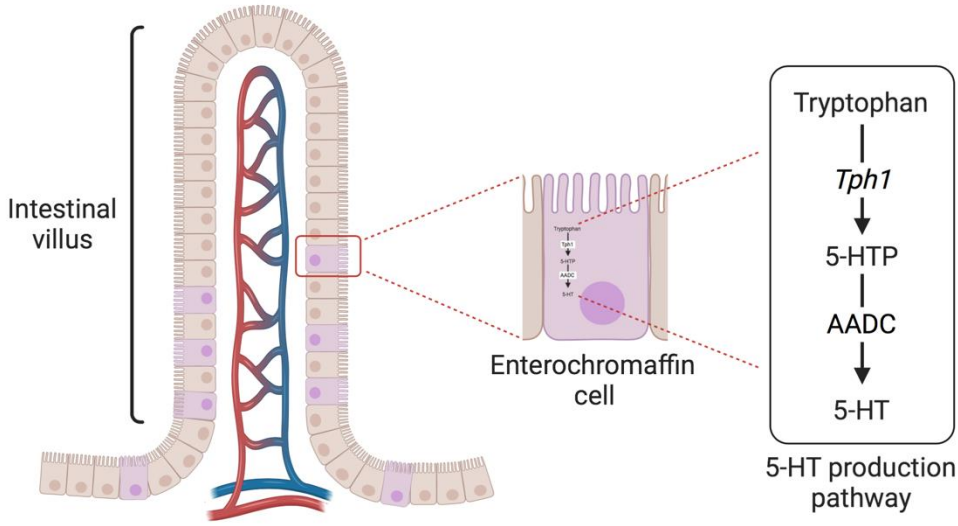

Figure 1. Mechanism of serotonin biosynthesis in enterochromaffin cells. Enterochromaffin cells use rate-limiting enzyme, tryptophan hydroxylase 1 (*Tph1*) to convert dietary obtained amino acid, tryptophan, to the 5-HT precursor, 5-hydroxytryptophan (5-HTP). This product is then decarboxylased by amino acid decarboxylase (AADC) to finally form 5-HT. Image author owned and created using BioRender.com.

Whilst motility and secretion are certainly well described mediators of diarrhoea, their involvement in chemotherapy-induced diarrhoea (due to GIM) is less understood. Perhaps of greater relevance to GIM is the interaction between 5-HT and inflammation; the hallmark feature of GIM.[7] 5-HT is becoming increasingly documented to regulate acute intestinal inflammation through the recruitment and activation of immune cells, including eosinophils and immature dendritic cells.[18] This is supported by investigations in various experimental models of intestinal inflammation, that have identified 5-HT as a key pro-inflammatory mediator.[19-21] Deletion of serotonin reuptake transporter (SERT), an approach used to increase 5-HT availability, intensified inflammation in a model of TNBS-induced colitis, in mice both with and lacking IL-10.[19, 20] Alternatively, reduced 5-HT availability by both *Tph1* inhibition and genetic knockout, attenuated the extent of intestinal inflammation in DSS- and DNBS-induced colitis mouse models.[21] Additionally, the administration of a 5-HT precursor in *Tph1*-deficient mice, bypassing the need for *Tph1*, was shown to increase the severity of colitis, further endorsing the pro-inflammatory

nature of 5-HT in intestinal inflammation.[21] Observational preclinical studies are consistent with these findings, as 5-HT and EC cell levels were increased in TNBS-colitis in guinea pigs,[22] and in infection-induced enteritis in mice.[23]

5-HT has also been implicated clinically, in benign, inflammatory diseases of the gut; albeit the data are heterogeneous in their conclusions. In Crohn's disease and ulcerative colitis, both increases *and* decreases in mucosal 5-HT and EC cells have been observed.[24-26] Lymphocytic colitis patients have also displayed higher densities of 5-HT-immunoreactive cells.[27] While directional contradictions exist, these studies irrefutably imply that 5-HT production is altered in states of intestinal inflammation, suggesting a possible role in GIM pathobiology. Despite this accumulating body of evidence implicating 5-HT in GIM, 5-HT has so far been overlooked and underexplored as a potential, critical candidate in GIM pathobiology.

Study hypotheses

Given the body of evidence implicating 5-HT in the regulation of gut motility and secretion, and most importantly, inflammation, this study hypothesised that:

1. Mucosal 5-HT production increases during GIM due to up-regulation of *Tph1*.
2. The expression of 5-HT gut receptors, 5-HT_{2A} and 5-HT_{2B}, are up-regulated during GIM.
3. 5-HT production and receptor expression are positively correlated to GIM severity, as defined by weight loss, diarrhoea and small intestinal wet weight.

Research aims

Using archival tissue samples from a previously conducted study that used a preclinical rat model of GIM induced by the chemotherapeutic agent, methotrexate (MTX), this study aimed to:

1. Determine if mucosal 5-HT and its production enzyme, *Tph1*, are altered by MTX.
2. Determine if gut receptors, 5-HT_{2A} and 5-HT_{2B}, are altered by MTX.
3. Determine the correlation between the changes in mucosal 5-HT and key outcome measures of mucositis caused by MTX.

Material and Methods

Ethics

The tissue used in this project was obtained from a 2016 study by the University of Adelaide's Cancer Treatment Toxicities Group. Titled DAMX, the study was approved by the Animals Ethics Committee of the University of Adelaide (M-2014-144) and complied with the National Health and Research Council (Australia) Code of Practice for Animal Care in Research and Training (2014).

DAMX Model Description

The DAMX study used the Dark Agouti rat mammary adenocarcinoma (DAMA) model of MTX-induced mucositis, a preclinical model of GIM validated to mirror clinically defined mechanisms and symptomology.[28] Unlike other models of GIM, DAMA is tumour-bearing, ensuring the immunomodulatory properties of a tumour are reflected in the disease mechanisms of GIM. Furthermore, mammary adenocarcinoma cells used to inoculate the rats were isolated from a spontaneously arising tumour, and propagated since their initial collection. This supports their use in an isogenic tumour model, thus avoiding the need to immunosuppress the recipient rat.

Induction of GIM

Twelve female Dark Agouti rats weighing between 130-160 g were injected subcutaneously with mammary adenocarcinoma cells at a concentration of $2x10^7$ cells/mL, on the right flank. Approximately 10 days later, upon tumours reaching 1% of total body weight (where tumour volume = $\frac{(\text{tumour width}^2 \text{ x tumour length})}{2}$ and tumour burden = $\frac{\text{tumour volume}}{\text{body weight}}$ x 100), rats (n=6) were treated with the mucotoxic chemotherapeutic agent, MTX, which was administered as two intramuscular doses (2 mg/kg, 6.25 mg/mL). Control rats (n=6) received a volume equivalent dose of saline. All rats were killed by exsanguination and cervical dislocation 72 hours post initial treatment, to coincide with peak mucosal injury and clinical symptomology.

GIM Outcome Measures (DAMX)

Body weight and diarrhoea were assessed daily, with diarrhoea defined according to a well-established grading system, where Grade 0 = diarrhoea not present; Grade 1 = mild perianal staining; Grade 2 = staining on top of the legs and lower abdomen; Grade 3 = staining over the legs, higher abdomen and continual anal leakage.[29] These criteria were used to assess GIM severity, as they are confirmed and reliable predictors of GIM (Table S1, supplementary material). Small intestinal wet weight was also defined as a primary outcome measure for the DAMX study, based on historical (unpublished) data from our laboratory that indicate its accuracy in objectively detecting GIM. As such, in addition to body weight and diarrhoea, 5-HT-related read outs were also correlated with small intestinal wet weight in this study.

Tissue preparation

Gastrointestinal tract tissue, from pyloric sphincter to rectum, was flushed with cold, isotonic saline following dissection. For project-relevant tissue, jejunum and proximal colon, 1 cm long samples were collected, snap frozen in liquid nitrogen, and stored in RNAlater (Ambion) at -80ºC until time of molecular analysis. In preparation for histological analysis, 1 cm segments were also fixed in 10% neutral buffered formalin and 70% ethanol the next day, processed and embedded in paraffin wax blocks, and stored at room temperature until time of analysis.

Immunofluorescence

5μm sections of archived jejunum and proximal colon samples were cut on a rotary microtome (Leica RM2235), and mounted onto FLEX IHC microscope slides (Flex Plus Detection System, Dako; #K8020). Immunofluorescent analysis was performed for mucosal 5-HT on an automated immunostainer (AutostainerPlus Dako; #AS480), using and following standard Dako reagents and protocols. First, slides were deparaffinised by manual heating (60ºC, 15 mins) and immersion in histolene (3 x 5 mins), before rehydration through graded ethanols (100%, 90%, 70%) and MilliQ water. Next, heat-mediated antigen retrieval was carried out using a buffer (pH 9.0), comprising

of 0.555 g of ethylenediaminetetraacetic acid (Sigma-Aldrich), 1.815 g of Trizma® base (Sigma-Aldrich) and 0.5mL of tween, in 1.5 L of MilliQ water. Using Dako's PT LINK (pretreatment module; Dako; #PT101), the buffer was preheated to 65ºC, at which point the slides were immersed and the temperature raised to 97ºC for 20 minutes, before removal of the slides upon cooling to 65ºC. Next, non-specific staining was blocked using 10% normal horse serum (NHS) in 1x phosphate-buffered saline (PBS). The slides were then incubated in a primary 5-HT goat antibody (kindly provided by Damien Keating, Flinders University) which was diluted in 5% NHS (v/v with PBS) at a concentration of 1:1000 (1 μg/ml). Next, a fluorescently labelled secondary antibody (donkey anti-goat, AlexaFluro488), diluted in 1x PBS, 1% bovine serum albumin and 2% foetal bovine serum to a concentration of 1:250 (0.8 μg/ml), was applied for a further hour. Slides were washed with 1x PBS and counterstained with 1 μg/mL 4',6-diamidino-2-phenylidole (DAPI; Life Sciences; #D1306), before treatment with an aqueous mounting medium (Fluoroshield™, Sigma-Aldrich, #F6182) and coverslipping. 5-HT immunofluorescence was quantified by blinded, manual counts of 5-HT+ cells, which were normalised to tissue area (determined using ImageJ).

Real-Time Polymerase Chain Reaction (qPCR)

RNA Extraction and Assessment of Quantity and Quality

Ribonucleic acid (RNA) was extracted from archived tissue samples being stored in RNAlater at -80ºC, using the Macherey-Nagel NucleoSpin® RNA kit, following manufacturer's guidelines. Upon completion of all appropriate steps, resulting RNA was eluted in 60 μL of RNase-free water, and stored at -20ºC. Total RNA yield (ng/μL) and purity was quantified and determined using a spectrophotometer (ThermoFisher Scientific NanoDrop 1000). Absorbance at 260 nm and 280 nm was measured to determine an A_{260}:A_{280} ratio, which, when 2.1, indicates the absence of organic contaminants and proteins.[30] RNA integrity was assessed by Adelaide Microarray Facility

(SAHMRI), with RNA integrity scores of <6.5, 6.5-8.3 and >8.3, demonstrating poor, moderate and good integrity, respectively.

cDNA Conversion

Following assessment, RNA was converted by reverse transcription into 15μL of complementary deoxyribonucleic acid (cDNA) using a cDNA Synthesis Kit (BioRad iScript^{TM}), following manufacturer's instructions. Each reaction consisted of 4 μL of 5x iScript Reaction Mix, 1 μL of iScript Reverse Transcriptase, and 1 μg of RNA template (made up in nuclease-free water to a volume of 15 μL). Subsequent, thermal cycling involved priming (25ºC, 5 mins), reverse transcription (46ºC, 20 mins) and reverse transcription inactivation (95ºC, 1 min). Sample yields and purities were again assessed using the spectrophotometer, with an A_{260}:A_{280} ratio of 1.8 indicating a pure sample of cDNA.[30]

qPCR

To determine the mRNA expression of the genes encoding for *Tph1*, 5-HT_{2A} and 5-HT_{2B}, qPCR was performed on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Amplification mixtures contained 1 μL of cDNA (100 ng/μL), 5 μL of fluorescent SYBR green dye, 3 μL of nuclease-free water and 0.5 μL of both forward and reverse primers (50 pmol/μL, Table 1). All samples were run in triplicates. Thermal cycling conditions were dependent on primer sets (Table 2). Suitable primers were mostly identified from the literature, including the housekeeping gene ubiquitin C (UBC), which has been validated for stability in GIM models.[31] The 5-HT_{2B} primers were designed using Primer3 (v4.0) and were based on the mRNA sequence for the *Rattus norvegicus* 5-HT_{2B} gene (NCBI). All primers were assessed for quality using NetPrimer (PREMIER Biosoft).

Table 1. Primer sequences and information

Gene	Primer sequence (5'-3')	Tm[3] (ºC)	Product (bp)	mRNA accession No.
Tph1[32]	F: CAAGGAGAACAAAGACCATTC	60.2	185	NM_001 136084.2
	R: CGCAGTCCACAAAAATCTCA	63.8		
5-HT_{2A} receptor[33]	F: AACGGTCCATCCACAGAG	61.2	220	NM_017 254.1
	R: AACAGGAAGAACACGATGC	60.3		
5-HT_{2B} receptor*	F: GGAGAAAAGGCTGCAGTACG	63.8	234	NM_017 250.1
	R: ATAACCAGGCAGGACACAGG	63.9		
UBC[31]	F: TCGTACCTTTCTCACCACAGTATCTAG	58.0	82	NM_017 314.1
	R: AAAACTAAGACACCTCCCCATCA	56.0		

* Designed using Primer 3. All primers synthesised by Sigma-Aldrich.

Table 2. qPCR cycling conditions

Primer set	*Tph1*	5-HT_{2A}	5-HT_{2B}
Hold 1	95ºC, 15 mins	95ºC, 10 mins	95ºC, 10 mins
Extension	94ºC, 15 secs	95ºC, 10 secs	95ºC, 10 secs
Denaturation	55ºC, 20 secs	60ºC, 30 secs	61ºC, 20 secs
Annealing	72ºC, 25 secs	72ºC, 45 secs	72ºC, 30 secs
Repeat	45 cycles	40 cycles	40 cycles
Hold 2	72ºC, 4 mins	N/A	N/A
Hold 3	60ºC, 15 secs	N/A	N/A
Melt	60-99°C, hold 90 secs on step 1, hold 5 secs on next steps	72-95°C, hold 90 secs on step 1, hold 5 secs on next steps	72-95°C, hold 90 secs on step 1, hold 5 secs on next steps

Primer Efficiencies and Quantification of mRNA Expression

Primer efficiencies were assessed using a 3-point standard curve of cDNA template (1, 10, 100 ng/μL). Despite significant optimisation, equal primer efficiencies could not be achieved, meaning the ΔΔCt method of quantification could not be used. Instead, mRNA expression was presented as $2^{-\Delta Ct}$, where $\Delta Ct = \Delta Ct_{(UBC)} - \Delta Ct_{(target\ gene)}$.

Statistical Analysis

GraphPad Prism (v8.0) was used for all statistical analyses. All data were first assessed for normality using the Kolmogorov-Smirnov test. If normally distributed, an unpaired t-test or one-way analysis of variance (ANOVA) was used to determine statistical significance. In cases of non-parametric distribution, a Mann-Whitney or Kruskal Wallis test was used with appropriate post-hoc testing. Diarrhoea scores were assessed between groups using a Chi-squared test. For correlative analyses, a simple XY (Pearson's) correlation was performed, with R squared (r^2)- and P-values generated through linear regression models. In all cases, $P<0.05$ was considered statistically significant.

Results

Methotrexate causes weight loss, diarrhoea and damage to the small intestine of rats

MTX caused significant weight loss which was most severe at 72 hours (control: +5.5±0.75%, MTX: -9.7±0.63%, P<0.0001, Figure 2A) which coincided with peak diarrhoea severity (P<0.0001, Figure 2B). MTX induced significant atrophy in the small intestine, indicated by a decrease in wet weight (control: 4.9±0.15 mg, MTX: 3.6±0.12 mg, P<0.0001, Figure 2C). In contrast, a significant increase in colonic wet weight was observed at 72 hours in MTX-treated rats (control: 1.0±0.045 mg, MTX: 1.3±0.11 mg, P=0.045, Figure 2D).

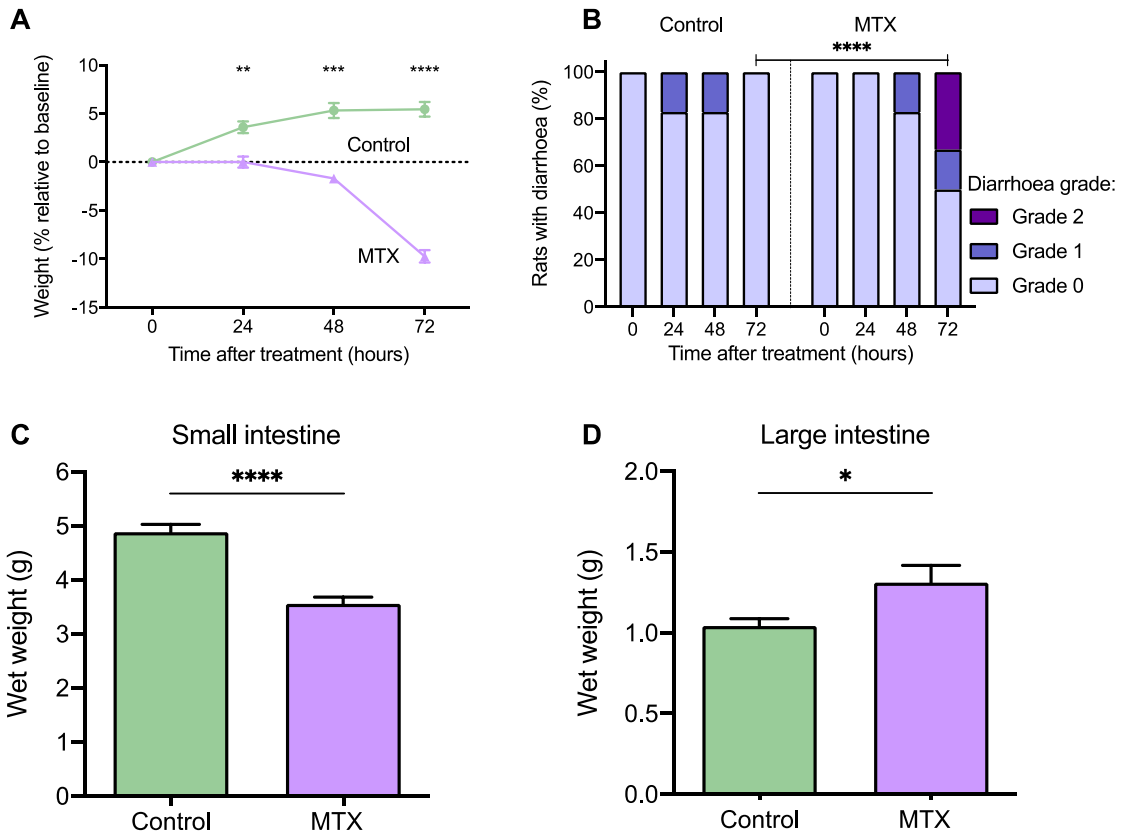

Figure 2. Model validation and clinical phenotype. MTX induces significant **(A)** weight loss (ΔBW%, mean ± SEM); **(B)** diarrhoea (grade scores); **(C)** decrease in small intestinal wet weight (mean ± SEM) and **(D)** increase in large intestinal wet weight (mean ± SEM). Data courtesy of Jenne Tran. 0.01 < P* < 0.05; 0.001 < P** <0.01; 0.0001 < P*** < 0.001; P**** < 0.0001.

Methotrexate impairs the production of mucosal serotonin in the jejunum of rats

MTX decreased the number of 5-HT+ cells/μm^2 in the jejunum of rats (control: 0.0051±0.0010, MTX: 0.00045±0.00020, **P=0.0013, Figure 3A). Similarly, jejunal *Tph1* mRNA expression was also significantly impaired by MTX (control: 470±270, MTX: 41±16, **P=0.0087, Figure 3B). This was in contrast to the colon where no significant changes were observed for both 5-HT+ cells (Figure 3C) and *Tph1* expression (Figure 3D). Representative images for immunofluorescence are shown in Figure 4.

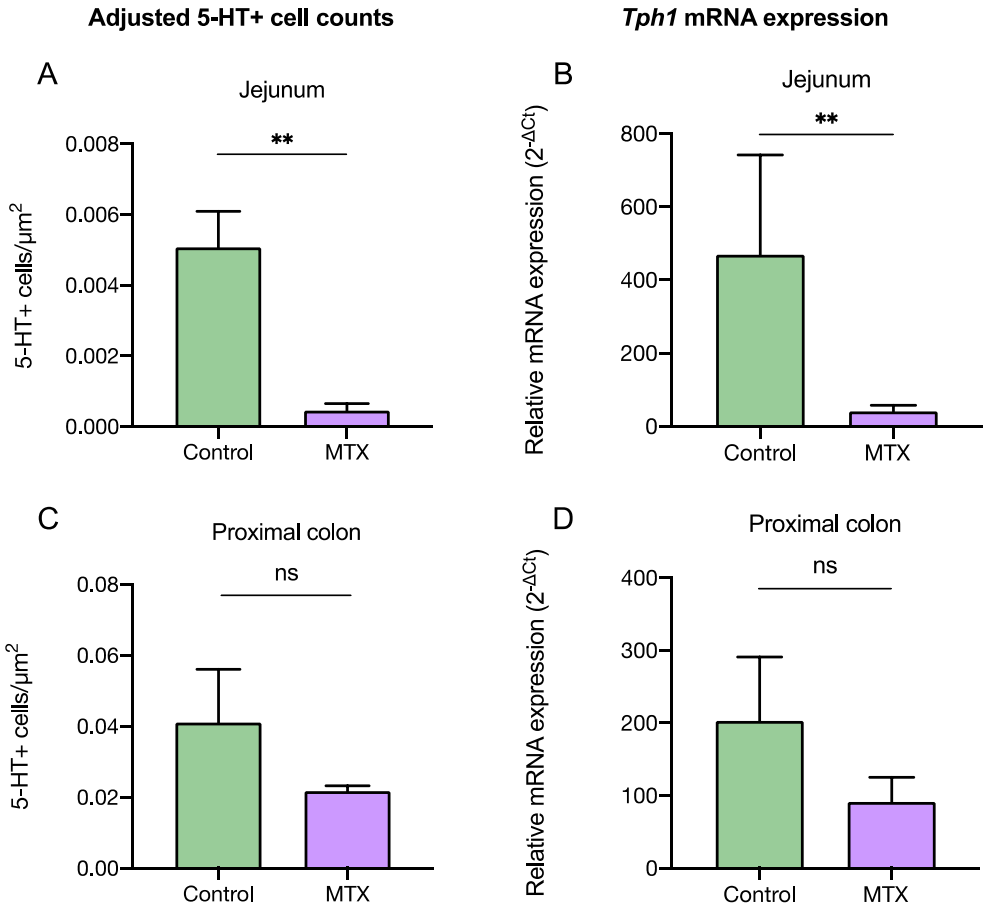

Figure 3. MTX impairs enzymatic production of mucosal 5-HT in the jejunum of rats. Jejunal **(A)** number of 5-HT+ cells/μm^2 and **(B)** relative *Tph1* mRNA expression of control (n=6) and MTX (n=5-6) animals. Proximal colonic **(C)** number of 5-HT+ cells/μm^2 and **(D)** relative *Tph1* mRNA expression of control (n=6) and MTX (n=4-6) animals. Absolute cell counts were adjusted with respect to tissue area (μm^2). Data presented as mean ± SEM. 0.001 < P** <0.01**.**

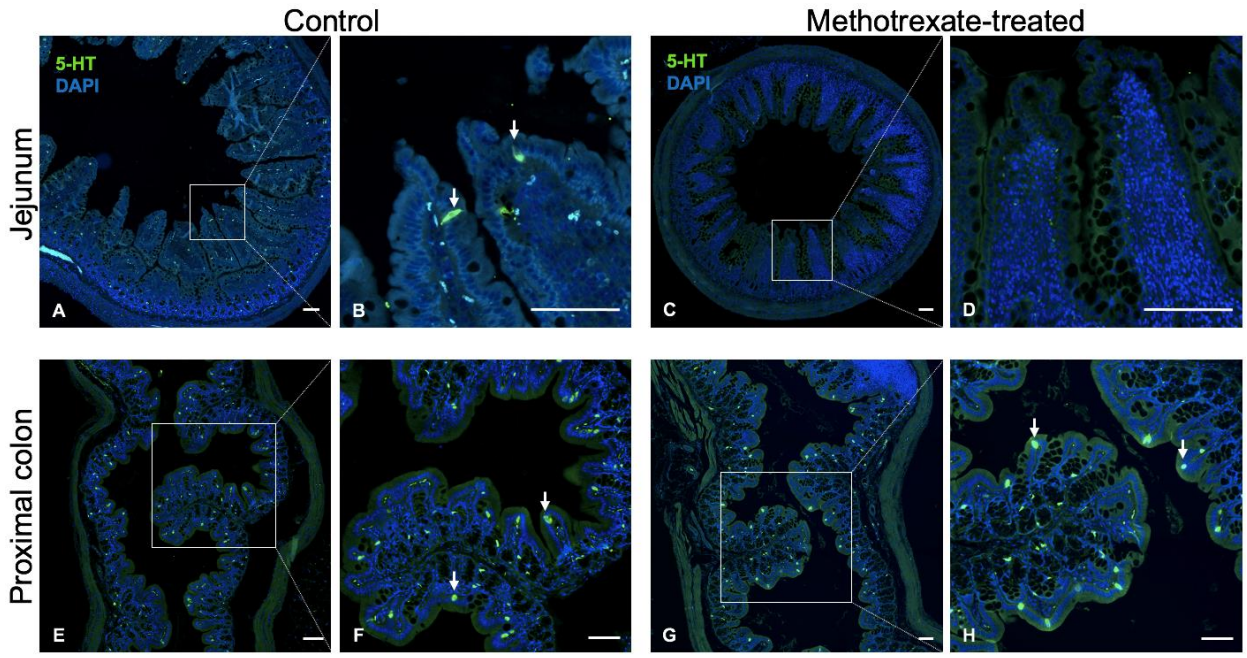

Figure 4. Representative images of 5-HT immunofluorescence in the jejunum and proximal colon of control and MTX-treated rats. Samples were stained with a primary antibody for 5-HT, visualised using an AlexaFluor anti-goat (488 nm, green). Blue counterstaining (DAPI, 405 nm) shows cell nuclei. 5-HT+ cells were considered positively stained cells in the luminal, mucosal lining (examples indicated by arrowheads in **(B)**, **(F)**, **(H)**). Scale bars represent 100 μm. Original magnification, 40x.

Methotrexate down-regulates 5-HT$_{2B}$ receptor expression in the jejunum of rats

There were no statistically significant differences in the mRNA expression of the 5HT$_{2A}$ receptor in the jejunum (Figure 5A) and colon (Figure 5B). In contrast, a significant decrease in the *jejunal* mRNA expression of the 5HT$_{2B}$ receptor was seen following MTX administration (control: 9500±3300, MTX: 730±390, **P=0.0079, Figure 5C). No significant change in 5-HT$_{2B}$ expression was observed in the proximal colon (Figure 5D).

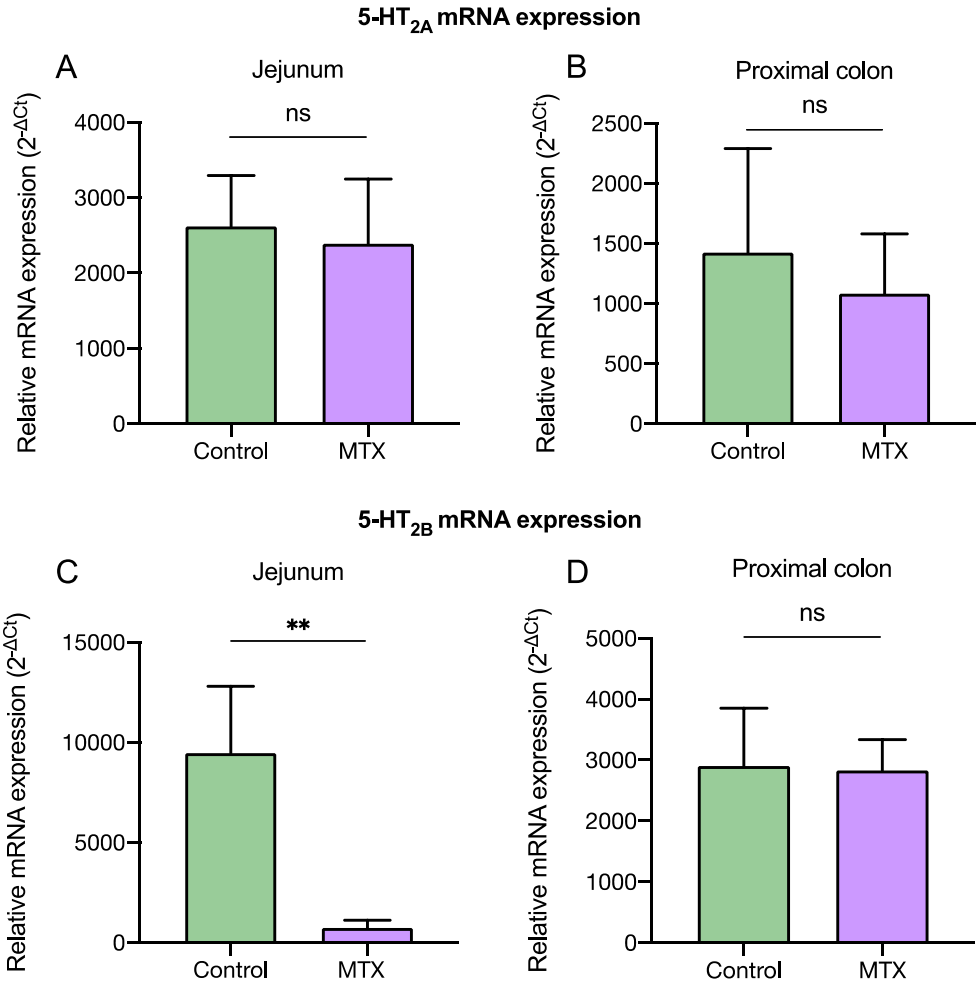

Figure 5. MTX has no effect on the expression of the 5-HT$_{2A}$ receptor in rats, but reduces 5-HT$_{2B}$ receptor expression in the jejunum. Relative mRNA expression of 5-HT$_{2A}$ in the **(A)** jejunum and **(B)** proximal colon of control (n=5-6) and MTX (n=5) rats. Relative mRNA expression of 5-HT$_{2B}$ in the **(C)** jejunum and **(D)** proximal colon of control (n=5-6) and MTX (n=3-5) rats. Data presented as mean ± SEM.

Serotonin expression correlates with methotrexate-induced mucositis

To understand the relationship between 5-HT synthesis and GIM severity, Pearson's correlations were performed between significant 5-HT readouts in the jejunum of rats (5-HT+ cells/μm^2, and *Tph1* and 5-HT_{2B} mRNA expression) and key assessment criteria confirmed to accurately reflect GIM (supplementary material). Weight change relative to baseline (ΔBW%) was significantly correlated with 5-HT+ cells (r^2=0.72, P=0.00050) and *Tph1* mRNA expression (r^2=0.41, P=0.025, Figure 6A). Diarrhoea correlated significantly with *Tph1* (r^2=0.43, P=0.028) and 5-HT_{2B} (r^2=0.58, P=0.010, Figure 6B) mRNA expression. Small intestinal weight was significantly correlated with all three markers (Figure 6C), with the strongest association to 5-HT+ cells (r^2=0.71, P=0.00060), followed by 5-HT_{2B} (r^2=0.65, P=0.0048) and *Tph1* (r^2=0.47, P=0.019) mRNA expression.

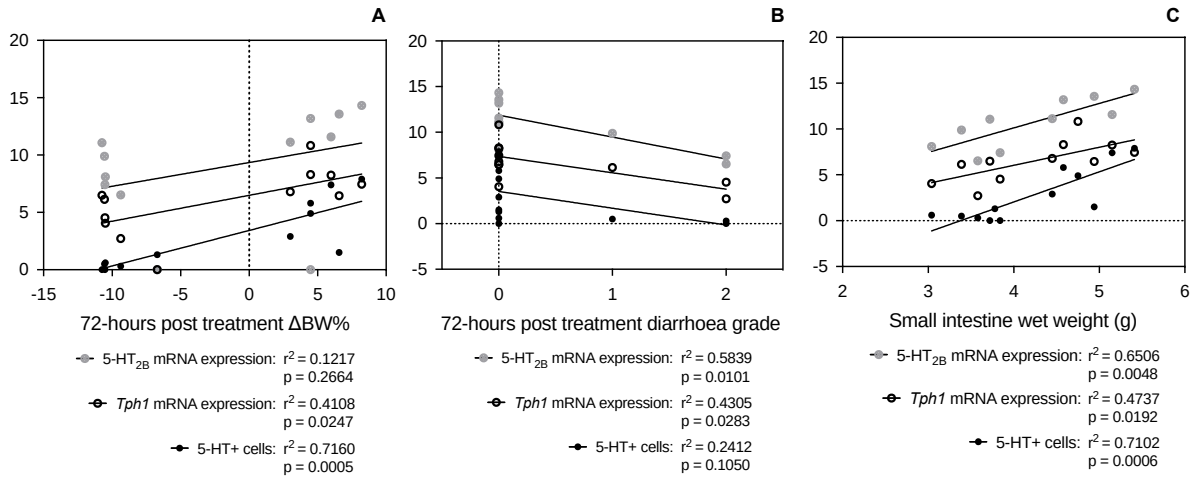

Figure 6. Validated markers of GIM correlate with 5-HT production in the jejunum of rats. Correlations between **(A)** weight change; **(B)** diarrhoea; and **(C)** small intestinal wet weight, and adjusted 5-HT+ cell counts, relative *Tph1* mRNA expression and relative 5-HT_{2B} mRNA expression in the jejunum of rats.

Discussion

Despite significant advances in the understanding of gastrointestinal mucositis (GIM) and supportive care approaches, GIM remains a major dose-limiting toxicity of most anti-cancer therapies, and without effective intervention.[1, 9] A growing body of knowledge implicates serotonin (5-HT) in GIM, and although a lot is known about its multiplicity of physiological roles in the gut, how 5-HT is altered during GIM remains poorly understood. Here, both 5-HT production and 5-HT_{2B} expression were reported to be down-regulated in the jejunum of rats treated with methotrexate (MTX), suggesting a possible anti-inflammatory role of 5-HT in GIM pathobiology, which may be mediated through the 5-HT_{2B} receptor (Figure 7).

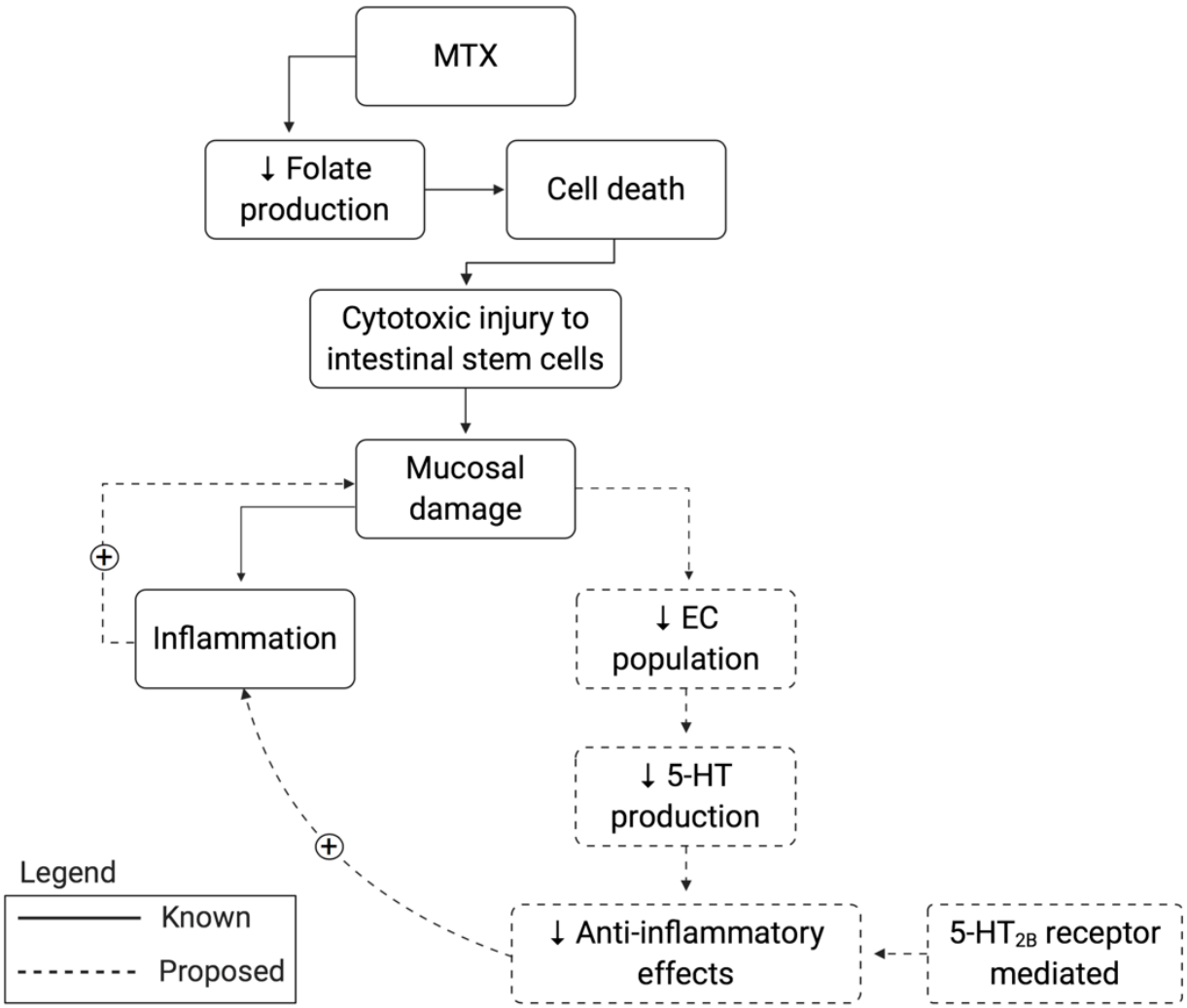

Figure 7. Proposed role of 5-HT in GIM pathobiology, explaining the observed results of this study.

Multiple reports from experimentally-induced colitis implicate 5-HT in inflammatory bowel diseases (IBD), with most supporting a ***pro-inflammatory*** role. It is important to note, that while comparable, IBD and GIM have distinct pathobiologies (chronic, colon-specific versus acute, pan-intestinal), so common mechanisms should be considered and interpreted carefully. Nonetheless, increasing 5-HT availability during colitis via SERT knockout, heightened intestinal inflammation intensity,[19, 20] while decreasing 5-HT availability with either *Tph1* inhibition or knockout, reduced the severity of colitis inflammation.[21] Conversely, and refuting the hypotheses of this study, my results clearly indicate deficient 5-HT production during gut inflammation, with both 5-HT+ cells/μm^2 and the *Tph1* enzyme down-regulated in the jejunum of MTX-treated rats. If considered independently, this observed reduction in 5-HT+ cells might be explainable by considering possible timelines of 5-HT release from enterochromaffin (EC) cells with respect to the analysed time-point (72-hours post MTX treatment). That is, perhaps EC cells responded to MTX by rapid, hypersecretion of 5-HT within 72 hours after treatment. Alternatively, it is possible that MTX causes such severe mucosal damage and significant tissue atrophy, directly decreasing the number of 5-HT+ cells. However, given that cell counts were adjusted to tissue area, and *Tph1* expression itself was also reduced, it is more likely that the fundamental, enzymatic production of 5-HT is altered by MTX-induced GIM.

The concept that 5-HT is pro-inflammatory has largely been based on its positive correlation with IBD. However, an increasing body of research is beginning to challenge this exclusivity, with emerging data suggesting possible ***anti-inflammatory roles*** for 5-HT. For example, decreased 5-HT levels in the inflamed mucosa of IBD patients are proposed to be driven by damage to the cellular constituents responsible for 5-HT production.[26] Similarly, in addition to the loss of 5-HT producing EC cells, ulcerative colitis patients also exhibited depleted mucosal 5-HT and *Tph1* mRNA expression, suggesting an inverse relationship with inflammation and supporting the results of this study.[25]

Although informative, these studies are limited, only detailing *associations* between 5-HT and diseases characterised by mucosal inflammation. More recently, a true anti-inflammatory mechanism was identified for 5-HT. Systemic and selective 5-HT_{2A} receptor activation with agonist (R)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, or (R)-DOI, proved to induce a systemic blockade of TNFα effects,[34] a pro-inflammatory cytokine that is elevated in GIM[7]. This resulted in particularly profound anti-inflammatory effects in the small intestine, as well as prevention of a TNFα-induced increase in circulating IL-6,[34] another cytokine implicated in GIM-inflammation.[7] These findings are particularly relevant to my study, as these anti-inflammatory effects of 5-HT were reported to be dependent on 5-HT_{2A} activation, highlighting the potential for agonism of this receptor to alleviate inflammatory gut disorders.[34] While I identified a change in 5-HT_{2B} expression, together these results indicate that the anti-inflammatory actions of 5-HT may be mediated by the 5HT2 receptor, suggesting 5-HT supplementation and/or 5-HT_2 agonism are potential approaches to alleviate GIM-inflammation.

Excitingly, 5-HT_{2B} agonism to alleviate inflammation has recently shown promise in experiments on a subset of human monocyte-derived dendritic cells (moDCs), $CD1a^+$, which are crucial to both innate and adaptive inflammatory responses.[35] $CD1a^+$ moDCs were treated with a synthetic toll-like receptor 3 ligand, polyinosinic:polycytidylic acid, or polyI:C, to elicit a strong inflammatory response. Following this, receptor activation with a highly selective 5-HT_{2B} agonist proved to decrease both mRNA expression and secreted levels of pro-inflammatory cytokines, TNFα and IL-6, as well as IL-8 and IL-10.[35] In addition, ligation of 5-HT_{2B} blocked the adaptive inflammatory response of T helper (Th) cells, Th1 and Th17. Furthermore, 5-HT_{2B} blocking experiments with an anti-5-HT_{2B} polyclonal antibody, confirmed the proposed, immunomodulatory effects of 5-HT_{2B}. Altogether, these findings, along with the results of my study, promote the candidacy of 5-HT_{2B} as a pharmacological target for both acute and chronic inflammatory disorders.[35]

Before overstating the potential of 5-HT supplementation (through 5-HT_{2B} agonism) as an approach to attenuate GIM-inflammation, it is important to decipher what my results mean in terms of how 5-HT is implicated in GIM pathobiology. That is, is it simply an innocent bystander that is affected by mucosal injury but has no direct part in exacerbating the disease, or, is it an active culprit that contributes to tissue injury and symptomology? To elucidate this, correlative strength between 5-HT readouts and key outcome measures of GIM was evaluated (Figure 6). While this approach cannot dissect its causative contribution, it is evident that 5-HT is intimately involved with mucosal injury and symptomology. This warrants further investigation into its active contribution, despite the inherit challenge in deducing cause and consequence in GIM. One potential approach would be utilising a 5-HT knockout organism in a validated model of GIM. Various methods of manipulating 5-HT availability, such as SERT[19, 20] and *Tph1*[21] knockouts, have already been validated in IBD studies as reliable approaches to dissect causative mechanisms in a complex disease process, where significant collateral damage is evident.

It is also important to acknowledge the existing understanding of 5-HT in cancer therapy, in particular, its well-established role in chemotherapy-induced nausea and vomiting (CINV). Chemotherapy, including MTX, is well-documented to disrupt EC cells, causing a massive, local release of 5-HT, which eventuates in acute- and delayed-onset nausea through the activation of 5-HT_3 and NK_1 receptors in the chemoreceptor trigger zone.[36, 37] Thus, 5-HT's established role in CINV could undermine the viability of therapeutic 5-HT supplementation for the mediation of GIM. Therefore, robust investigation should be done to determine if 5-HT supplementation is able to mitigate GIM-inflammation, and whether such supplementation can be achieved in a gut-localised manner, to avoid CINV side effects. One possible intervention that could facilitate this, is nanotechnology, in which drugs are directed to the site of action. This approach has already shown promise in minimising gut-inflammation in an IBD setting, with a glucagon-like peptide 1 nanomedicine demonstrating preclinical efficacy in a mouse model of colitis.[38]

Whilst there is the potential to exacerbate CINV, it is also important to highlight that 5-HT supplementation may have wider reaching benefits for people with cancer, owing to its pro-neuropsychological mechanisms that affect mood, cognition and sleep.[35, 39] This is particularly important in a supportive oncology setting where mood and sleep disturbances are commonly reported in cancer patients, yet remain without effective intervention.[39] This presents a novel opportunity to simultaneously address multiple side effects of cancer treatment, while also reducing the need for polypharmacy, another adversity affecting cancer patients, particularly the elderly.[40]

Conclusion

The complexity of the pathobiology behind chemotherapy-induced GIM is reflected by the current absence of an effective therapeutic intervention. This study demonstrated, for the first time, that MTX-induced GIM impairs 5-HT production and 5-HT_{2B} receptor expression in the small intestine of rats. In light of a strong relationship with key outcome criteria of GIM, this loss in 5-HT is proposed as a probable *contributor* to mucosal injury and symptomology, via anti-inflammatory pathways (Figure 7). These data support follow up studies that challenge the existing idea of the pro-inflammatory nature of 5-HT, and aim to i) confirm the true contribution of 5-HT to GIM through genetic modification and ii) elucidate whether 5-HT supplementation is a viable therapeutic approach for GIM. Finally, while this approach has the multi-dimensional potential to simultaneously minimise gut injury and enhance cancer-induced impairments in mood and sleeping patterns, it must be approached with particular caution, due to the possibility of exacerbating 5-HT dependent nausea and vomiting.

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Supplementary material

Although histopathological analysis is currently deemed the "gold-standard" in characterising mucositis severity, it is not without limitations, which include the subjective grading of various criteria and the necessity of necroscopy. While non-lethal clinical outcome measures, such as diarrhoea severity and weight loss, accurately represent the clinical phenotype of GIM, they are only assumed to be indicative of fundamental histological damage, as currently, no evidence in the literature has confirmed this. Therefore, to determine which non-lethal, clinical outcome measures most accurately predict the histopathological damage of mucositis, correlative, followed by multivariate statistical analysis, was performed on an abundance of archival rodent (mouse and rat) data. Results revealed diarrhoea severity and weight loss to be the most suitable non-lethal predictors of histological damage, and therefore GIM severity, as indicated by a statistically significant ($P<0.05$) correlation of these factors with markers of histopathological damage (Table S1). These determined, non-lethal predictors, were then used to correlate changes in 5-HT production and receptor expression with mucositis.

Table S1. Multivariable binary logistic regressions of GIM outcome measures relative to histologically confirmed GIM.

Outcome	Non-lethal, clinical outcome measure	Odds ratio (95% CI)	P-value
Combined mucositis (jejunum and colon)	Weight (per 1% increase from baseline)	0.81 (0.69, 0.96)	0.0129
Colon mucositis	Diarrhoea grade: per 1 unit increase	3.82 (1.07, 13.64)	0.0393