Experimental Disruption of Intestinal Mucosal Homeostasis

Exploring the Protective Potential of Melatonin and Misoprostol

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Abstract


The intestinal mucosa serves as a protective layer that separates the intestinal contents from the underlying tissues. It restricts harmful substances, pathogens, and undigested particles from entering the bloodstream. This mucosa also facilitates selective absorption of nutrients, electrolytes, and fluids, allowing essential substances to pass while maintaining a defense against potential threats. The integrity of the mucosa can be disrupted, such as in diseases or by off-target toxicities of chemotherapeutic drugs. A dysfunctional intestinal mucosa can result in inflammation, altered epithelial secretory and absorptive functions, as well as an increased mucosal permeability that may enable bacterial translocation. Chemotherapy-induced intestinal side effects may lead to dose reduction or even discontinuation of the treatment, but also decreasing the patient’s quality of life.

The aim of this thesis was to explore the protective potential of melatonin and misoprostol on experimental disruption of small intestinal mucosal permeability and chemotherapy-induced mucositis.

In Papers I and II an increase in intestinal mucosal permeability was induced by perfusing the jejunal segment with the surfactant sodium dodecyl sulfate (SDS) in rats. Melatonin and misoprostol were found to mitigate the induced increase in permeability. In Paper II it was shown that the melatonin receptor antagonist luzindole completely abolished the protective effect of melatonin on SDS-induced increase in mucosal permeability, showing that the effect of melatonin is receptor-mediated. In Papers III and IV off-target intestinal toxicity of the chemotherapeutic agents doxorubicin (DOX) and 5-fluorouracil (5-FU) were evaluated. In Paper III the progression of intestinal mucositis during seven consecutive days after a single injection of DOX was monitored. It was found that villus atrophy was most distinct after three days. In addition, within the first 24 hours after administration of DOX the most pronounced effect on a decrease in cell proliferation and an increase in crypt cell apoptosis was observed. In Paper IV it was found that daily administration of melatonin fully prevented villus atrophy and reduced the number of apoptotic crypts cells induced by a single injection of 5-FU. Administration of misoprostol increased colonic water contents but had no effect on 5-FU-induced villus atrophy or apoptosis. Furthermore, melatonin reduced 5-FU-induced cytotoxicity in murine intestinal organoids.

In conclusion, the results suggest that melatonin might be a potential candidate for supportive therapy in diseases affecting the small intestinal mucosal barrier and in chemotherapy-induced mucositis.

Keywords: Intestinal barrier dysfunction, intestinal permeability, chemotherapy-induced mucositis, doxorubicin, 5-fluorouracil, apoptosis, proliferation, melatonin, misoprostol
Mit dem Wissen wächst der Zweifel.

Johann Wolfgang von Goethe
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*Shared first authorship, contributed equally to the execution of the study.

All published articles are published in open access journals.
List of Papers not included in this Thesis


Abbreviations

$^{51}$Cr-EDTA  $^{51}$Cr-ethylenediamine tetraacetic acid
5-FU  5-fluorouracil
CIM  chemotherapy-induced mucositis
CL  blood-to-lumen clearance
COX-2  Prostaglandin-endoperoxide synthase 2
cpm  counts per minute
DAB  3, 3’-diaminobenzidine
DOX  doxorubicin
DOXol  doxorubicinol
GI  gastrointestinal
IL  interleukin
IP  intraperitoneal
IV  intravenous
MT$_{1/2}$  melatonin receptor 1/2
NF-κB  nuclear factor kappa-light-chain-enhancer of activated B cells
NO  nitric oxide
ROS  reactive oxygen species
SD  standard deviation
SDS  sodium dodecyl sulfate
SEM  standard error of the mean
SPIP  single-pass intestinal perfusion
TUNEL  terminal deoxynucleotidyl transferase dUTP nick end labelling
TNF-α  tumour necrosis factor α
Wnt  wingless-related integration site
Introduction

The small intestinal mucosa

One of the main physiological functions of the small intestinal mucosa is the digestion and transport of nutrients, water, and ions, as well as the simultaneous restriction of the passage of potentially harmful substances such as allergens, microbiota and toxins [1]. The wall of the intestine is divided in distinct layers (Figure 1). Its base is the serosa, followed by the muscularis externa, the submucosa, and finally the mucosa that faces the luminal content. The intestinal mucosa is a selective and dynamic barrier separating the luminal contents from the systemic circulation. It includes the epithelium, the lamina propria and the muscularis mucosae. The epithelium is a continuous sheet of columnar cells. The lamina propria, underneath the epithelial layer, is a connective tissue for the villi and contains nerve fibres, blood and lymph vessels, as well as immune cells.

The intestinal mucosa includes a single layer of intestinal epithelial cells, over 90% of which are enterocytes [2], which uphold physiological functions in digestion and absorption of nutrients, electrolytes and water. Other prominent types of cells in the intestinal mucosa include the mucin-producing goblet cells, enteroendocrine cells that secrete hormones, and immunomodulating
paneth cells [3]. In the small intestine, the mucosa is folded into finger-like protrusions called villi, in order to increase the surface area by a factor of 5-6 to further facilitate absorption. In addition, each enterocyte has microvilli at the apical membrane that increase the surface area even further [4,5]. At the base of the villi are the crypts of Lieberkühn, which are made up of the intestinal stem cells responsible for the continuous renewal of the villus epithelium. While the villi can be seen as the sites of electrolyte, fluid, and nutrient absorption, the crypt regions appear to be responsible mostly for electrolyte and water secretion [6]. Passage across the epithelium can occur via transcellular (through the cells) or paracellular (between the cells) pathways (see Figure 2).

Figure 2. Schematic illustration of passage over the intestinal epithelium, indicating trans- and paracellular permeability as well as important proteins. Adapted from Brunner et al. 2021 [7]. Created with BioRender.com.

Transcellular passage occurs through epithelial cell membrane proteins at both the apical and basolateral sides that allow for selective active and passive transport of substances. It is dependent on membrane carriers and channels that often require direct or indirect use of energy. Drug molecules however, can also cross the transcellular pathway passively [8]. Molecular paracellular diffusion across the epithelium is a passive process driven by electrochemical, hydrostatic and osmotic gradients. It is strictly regulated by the junctional complex at the apical portion of the intestinal epithelial cells to uphold systemic homeostasis [9,10]. The junctional complex consists of tight junctions, adherens junctions, desmosomes, and gap junctions, with the tight junctions being the most important component for restricting passage of small solutes across the transcellular pathway [11]. Paracellular permeability is dynamic and can change due to a number of physiological and pathophysiological stimuli [12].
Dysfunction of the intestinal mucosa

Dysfunction of the intestinal mucosa is associated with a range of gastrointestinal (GI) and systemic diseases and disorders, such as inflammatory bowel disease, irritable bowel syndrome, gastric and duodenal ulcers, type 1 diabetes, and fatty liver disease [13]. The increased intestinal mucosal permeability observed in these conditions can enable bacterial translocation and even trigger the development and proliferation of serious conditions including sepsis and multi-organ failure [14,15]. Reversing pathological increases in intestinal mucosal permeability may aid in combatting underlying diseases as well as alleviate symptoms such as diarrhoea, improving the quality of life for patients [16].

Intestinal permeability can be evaluated in multiple ways. While in vitro methods such as the Caco-2 assay [17] or ex vivo methods such as the Ussing chamber [18] can be useful regarding simple measurements as they provide a high simplicity and reproducibility, they lack the complexity of functioning neuro-endocrine and blood supply systems. Additionally, intestinal permeability can be evaluated by measuring changes in transepithelial electrical resistance. While this is a good measure of how small ions permeate the epithelium, electroneutral movements are not detectable [19]. Another method is the evaluation of the clearance of various permeability markers, such as lactulose, mannitol, or $^{51}$Chromium-ethylenediaminetetraacetic acid ($^{51}$Cr-EDTA) [1]. These probes can be administered orally, luminally, or intravenously (IV) and their clearance can be determined by measuring their concentration in the urine, blood, or luminal perfusate. In this thesis we have chosen to use $^{51}$Cr-EDTA or $^3$H-mannitol IV and determine their clearance into the luminal perfusate (blood-to-lumen clearance).

Experimentally induced insults to the intestinal mucosa

While generally associated with diseases and disorders, an increase in intestinal mucosal permeability may also be experimentally induced. Surfactants, such as sodium dodecyl sulfate (SDS), have been investigated for their use as intestinal permeability enhancers to improve intestinal absorption of low permeability drugs [20]. The amphiphilic, surface-active properties of surfactants allow some of them to be incorporated into the cell membrane lipid bilayer, thus causing an increase in its fluidity [21]. In the case of SDS, increased luminal exposure may result in lysis of the cell membrane [22]. Combined, the membrane effects lead to a loss of membrane integrity and an increase in intestinal mucosal permeability of hydrophilic molecules and some polar drugs [23]. The effect of SDS on intestinal solute permeability is both concentration- and time-dependent and shows the biochemical and histological characteristics of intestinal mucosal injury [24,25]. Altogether, this makes SDS a viable
agent for studying dysfunction in the intestinal barrier as well as potential treatment strategies thereof.

Chemotherapy-induced intestinal mucositis

Intestinal epithelial stem cells proliferate rapidly and migrate along the crypt-villus axis. At the villus tip they are shed and the epithelium is replaced completely without any loss of function within three to five days [26]. As anti-cancer drugs mainly affect proliferating cells, the GI tract is especially vulnerable to off-target toxicities. Consequently, chemotherapy-induced mucositis (CIM) is one of the most common and serious adverse effects of anti-cancer drugs. It is a complex GI complication that affects more than 40 % of all cancer patients [27–30]. The incidence and severity of CIM depend on both the type of chemotherapeutic used, as well as the dosing schedule, where some high-dose myeloablative chemotherapeutics such as busulfan, etoposide and doxorubicin (DOX) lead to an incidence rate of over 90 % [27,31]. CIM may lead to a variety of pathologies such as diarrhoea, mucosal ulceration, nausea, pain, and bacterial translocation sepsis through an increased intestinal permeability. If its symptoms are severe, it can result in dose reduction and thus, a lower anti-cancer treatment effect [32].

Figure 3. The progression of chemotherapy-induced intestinal mucositis in rats. In addition to ROS generation, DNA damage to proliferative cells causes apoptosis and induces the release of inflammation mediators. This can lead to inflammation, ulceration, crypt hypoplasia, villus atrophy, as well as bacterial infiltration. About one week after a single dose of a chemotherapeutic drugs (two weeks in humans), the normal histology and function of the small intestine are restored. This figure was adapted from [32] and created with BioRender.com.
In 2009 a five stage model for oral CIM was proposed, which can also be applied to intestinal CIM. The five stages are: initiation, primary damage response, amplification, ulceration and tissue inflammation, and finally healing [33]. The first stage, initiation, consists of the generation of reactive oxygen species (ROS) as well as direct damage to DNA that causes strand breaks. During the second stage, primary damage response, chemotherapy, radiation, and the generated ROS initiate the activation of numerous transcription factors (e.g. NF-κB, Wnt and p53) and their associated pathways within seconds. This leads to the generation of a number of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, resulting in cell injury and death. The third stage, amplification, consists of a large number of positive and negative feedback loops. For example, TNF-α can induce NF-κB to amplify its response, increasing the extent of tissue injury occurring. In the fourth stage, ulceration is caused by the damages and apoptotic changes to the epithelium resulting from previous stages. During this stage, morphological changes to the villi of the intestine can be observed, as an increase in already occurring apoptosis and shedding of cells at the villus tip is no longer compensated by cellular renewal along the villus axis, thus leading to villus atrophy. In addition, the ulcers enable bacteria to penetrate into the submucosa where they stimulate further secretion of pro-inflammatory cytokines and recruitment of immune cells to the intestinal region. Further complications can be caused by reduced uptake of nutrients, fluids, electrolytes, and medications as well as other issues associated with an increased intestinal mucosal permeability. The final, fifth stage describes the healing of the intestinal mucosa, in which the inflammatory pathways from earlier stages are reduced and an increase in epithelial proliferation, differentiation and maturation can be observed [33,34].

Two chemotherapeutics shown to cause CIM in patients are DOX and 5-fluouracil (5-FU) [35]. DOX is an anthracycline commonly used in various anti-cancer therapies. Its main mechanisms of action are the activation of different types of programmed cell death using intercalation into nucleus DNA, thus inhibiting the biosynthesis of macromolecules as well as inhibiting topoisomerase II’s interactions with DNA, and the intracellular generation of ROS. Its active metabolite doxorubicinol (DOXol) acts in the same manner [36]. 5-FU is an anti-metabolite drug widely used in a variety of anti-cancer treatments. Its effect stems from its incorporation into macromolecules such as DNA and RNA, and inhibiting their normal function, as well as inhibiting essential biosynthetic processes [37]. As there currently is no effective way to avoid or treat for CIM, there is a strong need for preventive and/or supportive treatment strategies [32].
Potential treatment strategies for increased intestinal permeability and CIM

An increase in intestinal mucosal permeability is often caused by injury or dysregulation of the epithelial barrier [13,14,38]. The recovery of epithelial integrity is vital not only in improving the quality of life in patients, but may also aid in combatting underlying diseases. As such, substances promoting epithelial homeostasis are interesting candidates for the prevention or treatment of a pathologically increased intestinal permeability.

While part of the damage to the intestinal tissue in the development of CIM is directly caused by induction of cell death by the chemotherapeutic agent administered, a large portion of this off-target toxicity can likely be attributed to the effects of ROS and the inflammatory response resulting from the induction of cell death [33]. Common inflammatory signalling cascades in this process involve substances such as NF-κB, IL-1, IL-6, and TNF-α [34,39]. Therefore, substances intervening with ROS or the aforementioned inflammatory cascades, as well as those inhibiting apoptosis in healthy cells are interesting candidates for the prevention or treatment of CIM.

Melatonin

Melatonin is well known for its role in regulating the circadian rhythm of the body. Interestingly, the GI tract is the greatest source of extra-pineal melatonin, where it is synthesized by the enterochromaffin cells [40]. There it is involved in for instance duodenal mucosal bicarbonate secretion, acid secretion, and the regulation of duodenal and jejunal mucosal permeability [41,42]. As a pharmaceutical, melatonin has been shown to have positive effects as a treatment for inflammatory bowel disease in both mice [43] and humans [44], as well as for irritable bowel syndrome [45]. In addition, it mitigates increases in intestinal mucosal permeability and motility induced by luminal exposure to ethanol, an effect that is abolished by the administration of the melatonin receptor antagonist luzindole, suggesting that melatonin acts through activation of G-protein coupled melatonin receptors (MT₁ and MT₂) [42]. Furthermore, melatonin has been shown to be a potent scavenger of free radicals, through which it exerts protective effects on radiation-induced increases in intestinal permeability [46–49]. This process occurs both through direct detoxification of reactive oxygen and nitrogen species, as well as by stimulating antioxidant enzymes and suppressing prooxidant enzymes [50]. Additionally, melatonin attenuates IL-1β [51] and lipopolysaccharide [52] induced inflammation in Caco-2 cells, characterized by increased levels of IL-6, IL-8, COX-2 and nitric oxide (NO). It was suggested that the protective effect shown in the IL-1β-induction study was associated with a reduction of NF-κB activation and that the reduced release of IL-6 was not melatonin-receptor-mediated as it could
not be modified by the addition of luzindole. However, while proposing an antioxidative component to its effect, Mannino et al. 2019 allude to the need to further investigate the mechanisms [51]. The combination of its two mechanisms of action makes melatonin an interesting candidate for treating pathologically increased intestinal permeability in general as well as CIM.

Misoprostol

Misoprostol is a drug that is most commonly used for the prevention and treatment of ulcers and mucosal perturbation caused by nonsteroidal anti-inflammatory drugs [53,54]. It is a synthetic E-type prostaglandin analogue that unfolds its effects through the G-protein-coupled prostaglandin E receptors 1-4. Activation of these receptors mediates protective mechanisms against mucosal damage and they are involved in epithelial homeostasis [55]. The cytoprotective effects of misoprostol are achieved through the inhibition of the parietal cell causing reduced gastric acid secretion. In addition, misoprostol stimulates mucus and bicarbonate secretion, and downregulates production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF. It also acts through the activation of adaptive cell survival pathways [53,56]. Common adverse effects of misoprostol include diarrhoea, abdominal pain, hyperthermia, nausea, and vomiting [57]. In 2020, Dahlgren et al. showed the capability of misoprostol to reduce the increases in jejunal mucosal permeability induced by 15-min luminal exposure to SDS, where it also showed a synergistic effect with melatonin [58]. These effects and mechanisms of action make it a promising candidate for intervening in pathologically increased intestinal permeability as well as some aspects of CIM.
Aims

The overall aim of this thesis was to elucidate and increase the mechanistic understanding of how two substances that have shown to exert protective effects in the gastrointestinal (GI) tract, melatonin and misoprostol, may be used to prevent or treat pathological increases in intestinal mucosal permeability as well as chemotherapy-induced mucositis (CIM). For the individual studies the aims were as follows:

In Paper I, the specific aim was to investigate the effects of melatonin and misoprostol on sodium dodecyl sulfate (SDS)-induced increases in jejunal mucosal permeability. To test the hypothesis that surfactant-induced increases in mucosal permeability can be inhibited by melatonin and misoprostol, we used a single-pass intestinal perfusion model in rats *in vivo*.

In Paper II, the specific aim was to elucidate the impact of the melatonin receptor antagonist luzindole and the nicotinic acetylcholine receptor antagonist mecamylamine on melatonin-mediated protective effects on SDS-induced increases in jejunal mucosal permeability.

In Paper III, the specific aim was to investigate the mechanisms of doxorubicin in the initiation and progression of chemotherapy-induced mucositis.

In Paper IV, the specific aim was to examine the effects of melatonin and misoprostol on 5-fluorouracil-induced changes of jejunal epithelial cell proliferation, apoptosis, villus length and colonic water content to test the hypothesis that melatonin and misoprostol can reduce chemotherapy-induced mucositis.
Experimental methods

The rat as an experimental model

In general, the use of \textit{in vivo} models allows for the monitoring of numerous integrative physiological parameters regulated by dynamic feedback mechanisms involving the immune and neuroendocrine system. A complexity that is limited in \textit{in vitro} models but that is vital for investigating multifaceted pathologies such as increased intestinal permeability or chemotherapy-induced mucositis (CIM), as well as the evaluation of potential treatments [16,34]. Rats have been used as an experimental model organism for over a century [59]. The anatomy and physiology of the rat are well characterised. It is a mammal that shows similarities to humans in several aspects, such as the characteristics of its gastrointestinal (GI) tract, that make it invaluable as a model organism in preclinical research [60]. Furthermore, the rat GI tract has shown to provide a good translational value when predicting intestinal drug absorption in humans [61,62].

As such, the rat was chosen as a model organism for elucidating regulation of intestinal permeability and CIM in this thesis. Healthy male Wistar HAN rats (strain code 273) at least 6 weeks old were included. After transport to Uppsala, all animals were allowed to acclimatise for at least one week in the animal laboratory facility, Uppsala University, prior to the start of the experiments and were allowed water and food \textit{ad libitum}. Housing conditions were 21-22 °C at a 12 h – 12 h light-dark cycle. All studies were approved by the local ethics committee for animal research (registration numbers C64/16 for \textbf{Paper I-II} and 5.8.18-06777/2020 for \textbf{Paper III-IV}).

Induction and treatment of CIM

In \textbf{Paper III-IV}, a rat model for chemotherapy-induced intestinal toxicity was established and evaluated. In these studies, the animals were administered chemotherapeutic drugs for the induction of CIM, as described in Table 1. Additionally, in \textbf{Paper IV}, rats were administered daily intraperitoneal (IP) injections of 10 mg/kg melatonin and/or 1 mg/kg misoprostol for four days, starting one day before the single IP injection of 200 mg/kg 5-fluorouracil (5-FU).
Table 1. Dose, administration route and corresponding and commonly used clinical dose for the two chemotherapeutic drugs, doxorubicin (DOX) and 5-fluorouracil (5-FU) used to investigate chemotherapy-induced mucositis (CIM) in Paper III-IV. Drugs were administered either intraperitoneally (IP) or intravenously (IV).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Corresponding clinical dose (mg/m²)</th>
<th>Commonly used clinical dose (mg/m²)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline</td>
<td>IV/IP</td>
<td>-</td>
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<tr>
<td>DOX</td>
<td>10</td>
<td>IV/IP</td>
<td>60</td>
<td>40-75</td>
<td>[64]</td>
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<tr>
<td>5-FU</td>
<td>200</td>
<td>IP</td>
<td>1200</td>
<td>1300</td>
<td>[65]</td>
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Single-pass intestinal perfusion

To determine the small intestinal permeability in rats, a single-pass intestinal perfusion (SPIP) model was used. For this, rats were anesthetised with an IP injection of 5-ethyl-5-(1´-methyl-propyl)-2-thiobarbiturate (Inactin®, 180 mg/kg). Body temperature was kept at 37-38 °C by a heating pad controlled by a rectal thermistor probe. Catheters were placed in the femoral artery and vein. The arterial catheter was connected to a pressure transducer to continuously monitor systemic arterial blood pressure, as well as enabling blood sampling during the experiments. The venous catheter allowed for infusion of saline, drugs, and the administration of mucosal permeability probes. After an abdominal incision was made, a jejunal segment (10-12 cm) was cannulated, placed outside of the abdomen [66], and covered with polyethylene wrap in order to conserve fluid and temperature. A schematic illustration of the experimental setup is shown in Figure 4. During the first 45 min after surgery the intestinal, respiratory, and cardiovascular functions were allowed to stabilise before initiating the experiments. During this recovery period the jejunal segment was perfused with phosphate-buffered perfusate solution (pH 6.5, 8mM, 37 °C). The segments were then perfused with different solutions for different periods of time, depending on the experimental setup in the different papers. The luminal perfusion rate was at all times 0.2 mL/min, controlled via a peristaltic pump. After the end of experiments, the length and tissue weight of the perfused segments were determined.
In **Paper I-II**, the intestinal mucosal paracellular permeability probe $^{51}$Cr-EDTA was administered intravenously (IV) as a bolus of 75 µCi (0.4 mL) followed by a continuous IV infusion at a rate of 50 µCi per hour (1 mL/h) throughout the experiments. To reverse surgery induced ileus of the intestine, parecoxib (10 mg/kg) was given IV after the surgery [67]. Subsequently, after a 45 min baseline period, the intestinal segments were perfused with different combinations of perfusates.

In **Paper III**, the paracellular permeability probe $^3$H-mannitol was administered IV as a bolus of 0.25 µCi (0.1 mL), followed by a continuous IV infusion at a rate of 0.5 µCi/h (0.1 mL/h) throughout the experiments. Following the stabilisation period, the intestinal segments were perfused with a phosphate-buffered perfusate solution for 75 min.

In all SPIP experiments, the perfusate leaving the intestinal segment was collected at 15-min intervals and weighed throughout the experiment. Blood samples (< 350 µL) were collected from the femoral artery at the start and end of the experiments. The collected blood samples were centrifuged and plasma was taken for further analysis. Luminal perfusate and blood plasma samples were analysed for $^{51}$Cr-EDTA (**Paper I-II**) or $^3$H-mannitol (**Paper III**) activity (cpm). $^{51}$Cr-EDTA activity was measured in a gamma counter (1282 Compugamma), whereas samples containing $^3$H-mannitol were mixed with an appropriate scintillation cocktail and analysed in the liquid scintillation analyser Tri-Carb 2910 TR. A linear regression analysis of the plasma activity values
was made to calculate a corresponding plasma value for each time point a perfusate sample was taken. The blood-to-lumen clearance (CL) of the two permeability probes was calculated using Equation (1) [68]:

\[
CL = \frac{c_{\text{perfusate}} \times Q_{\text{in}}}{c_{\text{plasma}} \times \text{wet tissue weight}} \times 100
\]  

Where \( c_{\text{perfusate}} \) and \( c_{\text{plasma}} \) are the activities (cpm/mL) in the perfusate and plasma, respectively, and \( Q_{\text{in}} \) is the flow rate (mL/min) into the segment. Clearance is expressed as mL/min/100 g wet tissue weight. This blood-to-lumen clearance represents the small intestinal mucosal barrier function (paracellular permeability) [69,70]. In Paper I-II the area under the permeability curve that represents the total blood-to-lumen clearance was determined and compared between groups. In Paper III, the average blood-to-lumen clearance was determined and compared between groups.

Morphological examination of the small intestine

To examine the morphology of the intestine in Paper III-IV, the villus height and the crypt depth were measured on paraffin-fixed jejunal tissue slides stained with hematoxylin-eosin. For each animal, ten villi and their corresponding crypts were measured. The average villus height and crypt depth of each animal were subsequently determined.

Figure 5. Illustration of measurement of the height of ten villi and depth of corresponding crypts to determine the mean for each rat. Scale bar represents 100 µm.
Immunohistochemical examination of the small intestine

Different immunohistochemical staining protocols were used to determine the levels of cell proliferation and apoptosis (Paper III-IV), as well as the presence of the MT2 melatonin receptor (Paper IV). As all of these protocols can have a certain level of variability between separate incidences, all samples within each study were analysed simultaneously for one marker at a time.

The level of cell proliferation in small intestinal samples from rats was determined through an immunohistochemical staining for Ki67, a tightly regulated protein that plays a vital role during proliferation and as such, is commonly used as a marker thereof [71]. The staining was performed using Ki67 antibody, a horseradish peroxidase-3, 3’-diaminobenzidine (DAB) Detection IHC kit according to the manufacturer’s instructions, as well as hematoxylin (Paper III) or methyl green (Paper IV) as counter staining. Microscopy images were processed with an ImageJ macro to automatically quantify the amount of DAB staining (Ki67 positive). Within this macro, the colours of the image were first deconvoluted and split into DAB and counter staining. A threshold was used to determine the percentage of the image that was stained by DAB. In a separate step, the image was converted to greyscale and a higher threshold was applied to determine the percentage of the image that contained actual sample, in order to correct the determined DAB stained area for different samples. A ratio between these two percentages was then calculated, to determine the percentage of stained area within a sample. A graphical representation of the process is displayed in Figure 6.

Figure 6. Representation of the 3, 3’-diaminobenzidine (DAB) staining quantification in a small intestinal sample. The unprocessed microscope image (a) is subjected to colour deconvolution to split the image into the counterstain portion (b) and the DAB portion (c). A threshold is then set to determine the DAB stained sample area (d). To determine the percentage of the image containing sample, a higher threshold is applied (e). Image from [72].
To show the presence of the MT$_2$ melatonin receptor in Paper IV, an immunohistochemical staining was performed using an MT$_2$ antibody, a horseradish peroxidase-DAB Detection IHC kit according to the manufacturer’s instructions, as well as methyl green as counter staining.

To detect apoptosis, samples were stained with a TUNEL kit according to the manufacturer’s instructions. The number of stained cells in the crypt regions were then manually counted and divided by the number of crypts in each image to determine the number of apoptotic cells per crypt. While the used TUNEL assay may detect other forms of programmed cell death than just apoptosis [73,74], cells stained with this detection kit will be referred to as apoptotic in this thesis in accordance with previous studies [75–77].

**Colonic water content**

In order to determine the colonic water content (Paper IV), the colon of each rat was removed. The entire faecal matter was then collected and weighed, before placing it in an oven (50 °C) until all water had evaporated (~3 days). It was subsequently weighed again, and the percentage of water content was then calculated as a measure of diarrhoea. In comparison to other methods such as metabolic cages or different grading systems, this method has the advantages of being purely quantitative as well as not having to take into account coprophagy in rats [78].

**Statistical analysis**

To determine statistical significance of differences between treatment groups, ANOVA analyses with Šidák’s multiple comparison post hoc test were conducted and comparisons with $p < 0.05$ were considered significant. All comparisons were tested for equality of group variance and normality of residuals with a Brown-Forsythe and Shapiro-Wilk test, respectively. If the distribution was non-normal, the non-parametric Kruskal-Wallis test with Dunn’s multiple comparisons post hoc test was used. If the group variance was not equal, a Brown Forsythe ANOVA with a Dunnet T3 post hoc test was used. All data analysis was conducted in GraphPad Prism.
Results and discussion

Experimentally increased small intestinal permeability

An injured or dysregulated intestinal barrier is commonly associated with an increased mucosal permeability, infiltration of pathogenic substances, as well as potential sepsis and organ failure [14,15,38]. In Paper I-II, sodium dodecyl sulfate (SDS) was used in a single-pass intestinal perfusion (SPIP) model in rats to disrupt the intestinal mucosal barrier. SDS is a pharmaceutical surfactant that has been reported to increase the intestinal absorption of low permeability drugs in a concentration- and time-dependent manner [23–25,79], through incorporation into the lipid bilayer of epithelial cells, thus destabilising the cell membrane and tight junction complex [21,80].

![Figure 7. Effect of 5 mg/mL sodium dodecyl sulfate (SDS) in the luminal perfusate for 15 min (a) or 60 min (b) on jejunal paracellular permeability (blood-to-lumen $^{51}$Cr-EDTA clearance (CL$_{Cr-EDTA}$)) adapted from Paper I-II. SDS significantly increased the permeability. Values are means (± SEM).](image-url)

Perfusion of the rat jejunum with control buffer lead to a consistently low jejunal mucosal permeability with a total blood-to-lumen $^{51}$Cr-EDTA clearance (CL$_{Cr-EDTA}$) of 9.2 ± 1.3 (Figure 7a) and 14.0 ± 2.0 mL/100g (Figure 7b) for the 120 and 165 min experiments respectively. The addition of SDS to the perfusate to a final concentration of 5 mg/mL significantly increased the total CL$_{Cr-EDTA}$ about 6 fold to 59.5 ± 7.7 (p < 0.01, Figure 7a) and 15 fold to 204.0 ± 22.8 mL/100g (p < 0.01, Figure 7b) for the 15 and 60 min exposure respectively. As can be seen in Figure 7, the SDS-induced increase in jejunal mucosal permeability is more substantial after longer exposure, supporting the time-dependent effect proposed previously [25]. It was also shown that the induced
increase is transient. While a nearly complete return to baseline permeability could be observed after 15 min exposure to SDS, this decline appears to take longer after 60 min exposure.

Treatment of experimentally increased small intestinal permeability

Melatonin and misoprostol have previously been shown to effectively mitigate increases in jejunal mucosal permeability induced by 15 min luminal exposure to SDS [58]. While the addition of melatonin at 100 µM to the perfusate lead to a 50 % reduction of an SDS-induced increase in total $CL_{Cr-EDTA}$ [58], we observed an even more substantial reduction of about 75 % from $59.5 \pm 7.7$ to $15.9 \pm 1.8$ mL/100g ($p < 0.01$, Figure 8a). The addition of melatonin at concentrations of 10 and 100 µM to the perfusate in animals subjected to 60 min SDS-exposure resulted in a reduction of the total $CL_{Cr-EDTA}$ of about 50 % ($p < 0.05$, from $204.0 \pm 22.8$ to $91.4 \pm 18.2$ and $100.3 \pm 5.7$ mL/100g respectively). This suggests that a plateau has been reached regarding the dose, at which a higher dose no longer results in a stronger effect. To evaluate a dose-response for the protective effect of melatonin, multiple lower doses would have to be investigated. While it has been shown that release of melatonin in the duodenal segment induced by intracerebroventricular injection of phenylephrine increased the luminal concentration from 5 to 60 ng/mL (22 to 261 nM) [81] and melatonin content in the rat jejunal tissue was found to be approximately 0.5 ng/g [82], exact local physiological concentrations are unknown. The use of melatonin in this study was meant to simulate exogenous treatment in order to alleviate an increased intestinal mucosal permeability. The results obtained are in line with previous studies showing that melatonin mitigates ethanol-induced increases in intestinal mucosal permeability [42].

The addition of 10 µM misoprostol to the perfusate in a SPIP study has previously lead to a 38 % reduction in increased total $CL_{Cr-EDTA}$ induced by 15 min luminal exposure to SDS [58]. When adding misoprostol at the same concentration to the perfusate in animals exposed to 60 min luminal SDS, we observed a significant reduction in total $CL_{Cr-EDTA}$ of 75 % from $204.0 \pm 22.8$ to $50.0 \pm 8.6$ mL/100g ($p < 0.01$, Figure 8c). This more substantial reduction compared to previous results may be explained by the larger increase in jejunal mucosal permeability induced by 60 rather than 15 min of exposure to SDS. A lower concentration of 1 µM misoprostol lead to a smaller, 50 % reduction of the SDS-induced increase in total $CL_{Cr-EDTA}$ to $102.0 \pm 10.6$ mL/100g ($p < 0.05$, Figure 8c). This indicates a clear dose response for the protective effect of misoprostol, which has not been shown in this experimental design previously.
Figure 8. The effect of luminal melatonin, misoprostol, or a combination of the two on jejunal paracellular permeability (blood-to-lumen $^{51}$Cr-EDTA clearance (CL$_{Cr-EDTA}$)) increases induced by 15 min (a) or 60 min (b-d) luminal exposure to 5 mg/mL sodium dodecyl sulfate (SDS) adapted from Paper I-II. a) The addition of melatonin (100 µM) completely abolished the increase in permeability induced by 15 min SDS exposure. b) Melatonin at both 10 and 100 µM reduced the increase in permeability induced by 60 min SDS exposure by half. c) Misoprostol reduced the increase in permeability induced by 60 min SDS exposure by 50% and 75% at 1 µM and 10 µM respectively. d) The combination of melatonin (100 µM) and misoprostol (10 µM) reduced the increase in permeability induced by 60 min SDS exposure by 75%. Values are means (± SEM).

As melatonin and misoprostol exert their effects through different mechanisms, the combination of the two substances was investigated. As shown in Figure 8d, the addition of 100 µM melatonin and 10 µM misoprostol resulted in a reduction of total CL$_{Cr-EDTA}$ induced by 60 min of 5 mg/mL SDS of approximately 75 %, from 204.0 ± 22.8 to 56.6 ± 7.3 mL/100g (p < 0.01). The magnitude of the effect observed was similar to that of 10 µM misoprostol alone, thus not corroborating results from a previous study in which a combination of 100 µM melatonin and 10 µM misoprostol lead to a stronger protective effect than either substance alone [58]. This may be explained by the longer duration of the experiments in this study, which could have given the opportunity for misoprostol to exert more of its regulatory effects. The effects of melatonin and misoprostol on experimentally induced increases in intestinal mucosal permeability shown in this study warrant further investigations of the two substances as potential treatments for pathologically increased intestinal mucosal permeability.
Mechanism of the effect of melatonin

Melatonin displays multiple protective and regulatory effects in the gastrointestinal (GI) tract [41–49]. While some of these, such as the reduction of radiotherapy-induced increases in intestinal permeability, are exerted through the role of melatonin as a potent scavenger of reactive oxygen species (ROS), others, such as the reduction of ethanol-induced increases in intestinal permeability, are mediated through G-coupled melatonin receptors. Furthermore, a number of intestinal functions, such as duodenal motility and transmucosal fluid and electrolyte flux are controlled by nicotinic acetylcholine receptors in the enteric nervous system [83]. As such, it has been reported that nicotinic receptor blockage reduces hypotonicity-induced increases in duodenal mucosal permeability [84]. To elucidate the mechanisms behind the protective effect of melatonin in the current study, the effects of the potent melatonin receptor antagonist luzindole [85,86] and mecamylamine, a non-selective, non-competitive antagonist of nicotinic acetylcholine receptors [87], on SDS-induced increases in intestinal mucosal permeability were evaluated.

The addition of 100 µM luzindole and 100 µM melatonin to the perfusate prior to the 15 min exposure to 5 mg/mL SDS resulted in a total CL$_{Cr-EDTA}$ of 60.1 ± 11.0 mL/100 g (Figure 9a). This value is significantly higher than that of animals treated with 100 µM melatonin (15.9 ± 1.8 mL/100g, p < 0.05) and similar to that of animals subjected to SDS without additional treatment (59. 5 ± 7.7 mL/100 g, p > 0.99). Thus, the protective effect of melatonin on the SDS-induced induced increase in jejunal mucosal permeability was completely abolished by the addition of luzindole, suggesting that the effect of melatonin is receptor-mediated rather than antioxidative.
Perfusion with mecamylamine reduced the SDS-induced increase in total CLCr-EDTA by 55% from 59.5 ± 7.7 to 26.8 ± 4.4 mL/100g (p < 0.05, Figure 9b). This is in contrast to ethanol-induced permeability increases, where nicotinic inhibition had no effect [42]. This suggests that SDS- and hypotonicity-induced increases in intestinal mucosal permeability may be mediated by different mechanisms than ethanol-induced increases. Further investigations of the mechanisms are, however, warranted.

Chemotherapy-induced intestinal mucositis

In Paper III and IV, two different chemotherapeutic agents, doxorubicin (DOX) and 5-fluorouracil (5-FU), were used to induce chemotherapy-induced mucositis (CIM) in healthy rats. While the main focus of Paper III was to further elucidate the processes occurring during the progression of CIM after a single injection of DOX, in Paper IV the potential treatment effects of the previously investigated GI protective substances melatonin and misoprostol on 5-FU-induced CIM were investigated.

Morphological examination of the small intestine

Intestinal villus atrophy, caused by a cytostatic-induced disturbance of the rate of cell proliferation and apoptosis, is a hallmark morphological effect of CIM and often used to quantify the degree of off-target small intestinal toxicity in pre-clinical animal models [88,89]. It has therefor been selected as a relevant endpoint to determine the severity of CIM as well as the effect of supportive treatments in these studies.

The small intestinal villus height underwent the strongest reduction at 72 h after an intraperitoneal (IP) injection of 10 mg/kg DOX. Villus height was reduced by 38% from 475 ± 80 (control) to 293 ± 49 µm (p < 0.001, Figure 10a), which returned to a value close to the control after 168 h (448 ± 29 µm). This is in line with previous data where a 10 mg/kg IP injection of DOX lead to a 43% reduction in the villus length in mice [88]. As the morphological changes observed were most pronounced at 72 h after injection with DOX, this time point was used for the evaluation of treatment effects in Paper IV.

A single IP injection with 200 mg/kg 5-FU lead to a significant reduction in villus height of 38% from 252 ± 25 to 157 ± 29 µm (p < 0.05, Figure 10b), similar to a 32% reduction observed 48 h after a single 400 mg/kg injection of 5-FU in a previous rat study [90]. Daily treatment with melatonin alone (291 ± 63 µm, p < 0.001) or in combination with misoprostol (300 ± 81 µm, p < 0.001) completely abolished the villus height reduction induced by 5-FU,
whereas the monotreatment with misoprostol (204 ± 53 µm) had no significant effect on this villus height reduction.

Figure 10. Effect of exposure to chemotherapeutics as well as treatment effects of melatonin (Mel) and misoprostol (Mis) on villus height, adapted from Paper III and IV. In a) the effect of a single 10 mg/kg intravenous injection of doxorubicin (DOX) over time is shown. For b) the control and 5-FU groups were re-used from [91] and compared to rats treated with daily injections of 10 mg/kg melatonin and/or 1 mg/kg misoprostol for four days starting one day before the injection of 200 mg/kg 5-FU. Each symbol represents the mean value from a single animal based on ten separate measurements. The statistical analysis was performed with an ANOVA analysis with Šidák’s multiple comparisons post hoc test. Significant comparisons are indicated by one (p < 0.05) or three (p< 0.001) stars.

Melatonin displayed a clear protective effect, which may be exerted through its anti-inflammatory and anti-oxidant properties. However, under certain conditions melatonin can also display pro-oxidant and anti-proliferative effects. This is shown to mainly occur in severely injured cells, as well as cancer cells [92], and depends, among other mechanisms, on the activation of NF-κB, driving melatonin synthesis and transforming melatonin into a pro-apoptotic molecule [93,94]. This unique combination of protection in healthy cells and potential pro-apoptotic effects in cancer cells makes melatonin a promising candidate for further evaluation as a supportive treatment in cancer patients.

Immunohistochemical examination of the small intestine
As the healthy intestinal epithelium is replaced continuously without any loss of its function, there is a balance between ongoing apoptosis and proliferation of stem cells that then migrate along the villus axis to replace shedding cells [26]. While spontaneous apoptosis can occur in the crypt region in rare cases,
an increase in apoptosis may lead to severe gut pathology [95]. Exposure to chemotherapeutic agents may affect either of these two processes.

The average number of apoptotic cells per crypt after exposure to DOX increased significantly at 24 h from $0.3 \pm 0.2$ (control) to $2.1 \pm 1.1$ ($p < 0.01$, Figure 11a). While a similar average number was observed already after 6 h ($2.2 \pm 2.0$), it was not significantly different from control ($p = 0.8$). The wider spread of data in this group may point to an inter-individual variability in the time of onset of DOX-induced apoptosis. The average number of apoptotic cells per crypt was $0.8 \pm 0.8$ after 72 h and $0.7 \pm 0.3$ after 168 h, suggesting a return to baseline levels at these time points. Although it has previously been reported that DOX-induced apoptosis mainly occurs in a subset of stem cells more prone to cytotoxic damage located at positions 3-6 in the crypts [96], the methods used in our studies did not allow for such detailed evaluation. The timeline of apoptosis observed in this study is in agreement with previous data, where an increase in apoptosis was observed at 6 and 24 h after DOX administration [88]. As the most pronounced increase in apoptosis in small intestinal crypts occurs soon after DOX exposure, these data suggest that supportive treatment aiming to reduce apoptosis should be most effective during the first 24 h after the administration of chemotherapy.

At 72 h after exposure to 5-FU, the number of apoptotic cells per crypt was $2.1 \pm 0.9$, a value not significantly higher than that observed in control animals ($1.0 \pm 0.7$, $p = 0.25$, Figure 11b). In animals administered 5-FU melatonin reduced the number of apoptotic cells per crypt from $2.1 \pm 0.9$ to $0.7 \pm 0.7$, a value that was very close to obtain statistical significance ($p = 0.052$). As was elucidated in Paper III, the strongest increase in apoptotic cells occurs at time points earlier than 72 h, suggesting that the effect of melatonin on chemotherapy-induced apoptosis in intestinal crypts should be evaluated already after 24 h to further investigate a potential anti-apoptotic effect that could be an explanation for the observed protection from villus atrophy.
Figure 11. Effect of exposure to chemotherapeutics as well as treatment effects of melatonin (Mel) and misoprostol (Mis) on the number of apoptotic cells per crypt, adapted from Paper III and IV. In a) the effect of a single 10 mg/kg intravenous injection of doxorubicin (DOX) over time is shown. For b) the control and 5-FU groups were re-used from [91] and compared to rats treated with daily injections of 10 mg/kg melatonin and/or 1 mg/kg misoprostol for four days starting one day before the injection of 200 mg/kg 5-FU. Each symbol represents the mean value from a single animal. The statistical data analysis was performed with a Kruskal-Wallis test with Dunn's multiple comparisons post hoc test. Significant comparisons are indicated by two (p < 0.01) stars.

In addition to an increase in apoptosis, a reduction in cell proliferation could affect the chemotherapy-induced morphological changes observed, as proliferating progenitor stem cells compensate for cells shedding from the villus tips under normal circumstances [97]. As such, proliferation in the intestinal epithelium is another key factor in the progression of CIM and a potential target for supportive treatment.

The administration of DOX resulted in a significant decrease in proliferation after 24 h (1.3 ± 0.9 %) when compared to control animals (5.5 ± 1.7 %, p < 0.01, Figure 12a), whereas no changes could be observed at the other evaluated time points. While these data are in agreement with a previous rat study, in which 200 mg/kg irinotecan caused similar changes in proliferation, Al-Dasooqi et al. (2011) also observed a 50 % increase in proliferation at 96 h past chemotherapy exposure [75].
Figure 12. Effect of exposure to chemotherapeutics as well as treatment effects of melatonin (Mel) and misoprostol (Mis) on the area of sample stained for the proliferation marker Ki67, adapted from Paper III and IV. In a) the effect of a single 10 mg/kg intravenous injection of doxorubicin (DOX) over time is shown. For b) the control and 5-FU groups were re-used from [91] and compared to rats treated with daily injections of 10 mg/kg melatonin and/or 1 mg/kg misoprostol for four days starting one day before the injection of 200 mg/kg 5-FU. Each symbol represents the mean value from a single animal. The statistical data analysis was performed with a Kruskal-Wallis test with Dunn’s multiple comparisons post hoc test. Significant comparisons are indicated by two (p < 0.01) stars.

Treatment with melatonin or misoprostol did not have a significant effect on the observed levels of proliferation at 72 h after 5-FU exposure. As a pronounced decrease in proliferation induced by chemotherapy occurs already after 24 h, followed later by an increase at 96 h during the healing phase, it cannot be clearly determined if melatonin has an effect on proliferation in the current setup. For this, additional time points both earlier and later should be investigated.

As the precise mechanisms through which melatonin may exert its protective effects in CIM are unknown, we tested whether the MT2 melatonin receptor was present in the rat small intestine. As can be seen in Figure 13, the receptor was prominently expressed in the villus section of the rat jejunum in animals administered both 5-FU and melatonin. In rats administered 5-FU alone, the MT2 receptor was instead prominently expressed in the crypt regions. These data suggest that exogenous treatment with melatonin may lead to a change in expression pattern or an attenuation of melatonin receptors, a process observed in the rat liver [98] and superchiasmatic nucleus [99].
Figure 13. Expression of the MT2 melatonin receptor in rat jejunum adapted from Paper IV. Animals were administered 5-FU and melatonin (a-c) or 5-FU alone (d-f). Original microscopy pictures are shown in a and d. Colour deconvolution was performed to split the different colour channels and show only the DAB portion (b, e). Finally, a threshold was set to highlight the areas stained with a higher intensity (in red) from unspecific background staining and (c, f).

Single-pass intestinal perfusion experiments

Studies evaluating CIM usually solely focus on investigating mechanisms in its development [100,101], however there rarely is a link to key intestinal functions. As discussed previously, the main function of a healthy intestinal mucosa is its ability to absorb nutrients and fluids, while providing a protective barrier restricting the passage of harmful bacteria, viruses, xenobiotics and other pathogens [1].
While it has been shown that the induction of CIM with commonly used chemotherapeutic agents such as methotrexate increases intestinal permeability [102], the SPIP experiments conducted in Paper III showed the opposite. Jejunal mucosal permeability to the permeability marker mannitol [103] was significantly reduced at 6, 24, and 168 h after DOX exposure (Figure 14). This could be explained by an immediate sealing of tight-junction complexes as a reaction to the induced injury, a protective mechanism commonly observed in the intestinal mucosa [9] to uphold its barrier function and avoid the development of an uncontrolled “leaky gut” [38]. One proposed mechanism is the redistribution of tight-junction proteins in order to maintain barrier integrity after TNF-induced apoptosis [26].

**Colonic water content**

Diarrhoea is a frequent symptom of CIM, affecting more than 10% of patients depending on cancer type and treatment [104]. Chemotherapy-induced diarrhoea may occur due to a direct toxic effect on the intestinal epithelium, disturbing the delicate balance of ion and water absorption and secretion. One likely cause is villus atrophy, reducing the epithelial absorptive surface, while not impacting secretion, thus leading to an imbalance in net fluid flux. The degree of diarrhoea was determined through the percentage of water in the colonic content of rats.
Figure 15. Colonic faecal water content (%) reflects diarrhoea 72 h after dosing of 5-Fluorouracil (5-FU, 200 mg/kg) and melatonin (Mel, 10 mg/kg daily starting 24 h before 5-FU) and/or misoprostol (Mis, 1 mg/kg daily starting 24 h before 5-FU). Figure adapted from Paper IV. Each point represents the percent colonic water content from a single animal, the horizontal line signifies the group mean. The dotted line at 80 % represents a threshold from which diarrhoea can be assumed. The statistical analysis was performed with a Brown-Forsythe and Welch ANOVA test with Dunnett’s T3 multiple comparisons test. Comparisons with p < 0.05 were considered significant, indicated by one star.

The results from all groups tested in Paper IV are shown in Figure 15. The faecal water contents in untreated control animals were 63 ± 3 %. A single dose of 5-FU (200 mg/kg) did not influence the faecal water content (61 ± 5 %), contrary to effects observed in the clinic [105]. This may be attributed to a dose effect, as two doses of 150 mg/kg 5-FU on consecutive days induced diarrhoea in rats [106], suggesting a re-evaluation of dosing regimen is required when investigating 5-FU-induced diarrhoea in rats. It also indicates, however, that chemotherapy-induced diarrhoea is not only caused by villus atrophy, as the observed atrophy in this study did not cause diarrhoea. Similarly, melatonin administration in animals subjected to 5-FU did not significantly change the faecal water content (69 ± 8 %). Interestingly, all groups of animals subjected to 5-FU and misoprostol displayed a significant increase in faecal water content. When misoprostol and melatonin were co-administered, the water content was 71 ± 4 % and when misoprostol was given alone it was 82 ± 13 %. Both groups were significantly different to the 5-FU subjected group (p < 0.05). This is in concurrence with diarrhoea being a common side effect of misoprostol [57].
Conclusions and future perspectives

This thesis aimed to elucidate and increase the mechanistic understanding of how melatonin and misoprostol exert a protective effect in the gastrointestinal tract of rats, and to evaluate their potential as treatments for pathological increases in intestinal mucosal permeability and chemotherapy-induced mucositis (CIM). Additionally, it aimed to increase the understanding of the progression of CIM and its processes, while establishing a relevant model in our laboratory.

In Paper I, it was shown that both melatonin and misoprostol substantially reduce increases in jejunal mucosal paracellular permeability induced by 60 min exposure to sodium dodecyl sulfate (SDS). Importantly, the combination of the two did not display a larger effect than either monotreatment. While a clear dose-related response could be observed for misoprostol, this was not the case for melatonin, indicating that further studies are required to establish an effective dose regimen for melatonin. Studying lower doses may also unmask an increased effect of combining the two substances. Furthermore, while a full return to baseline permeability is observed within 60 min after 15 min SDS exposure, this was not the case after the 60 min exposure. This suggests that a longer recovery period should be investigated.

In Paper II, we demonstrated that the protective effect of melatonin is receptor-mediated rather than antioxidative, as it could be fully reversed by the addition of the melatonin receptor antagonist luzindole. As luzindole displays a higher affinity for the MT2 than the MT1 melatonin receptor, these results also lead to the choice to stain for this receptor in Paper IV. Furthermore, the non-selective, non-competitive acetylcholine receptor antagonist mecamylamine reduced SDS-induced increases in jejunal mucosal permeability, suggesting an involvement of these receptors in the processes by which SDS increased permeability, similar to hypotonicity-induced permeability increases. However, further research into the mechanisms is warranted, as the used single-pass intestinal perfusion (SPIP) model in rats displays strong limitations regarding the resolution for molecular processes.
In Paper III, we showed the progression of CIM over time. While villus height was substantially reduced by day three after doxorubicin (DOX) dosing, it recovered by day seven. An increase in apoptosis in the crypts and a decrease in cell proliferation occurred within the first day after doxorubicin (DOX) administration, preceding any morphological damage. While molecular mechanisms are difficult to evaluate in this in vivo model, the added complexity of feedback and signalling mechanisms is crucial for understanding the intestinal response to chemotherapeutic exposure. The timeline established in this study resulted in the choice of 72 h after administration of chemotherapy for the evaluation of treatment effect in Paper IV, as it showed the largest extent of morphological damage. Furthermore, the data suggests that potential treatment strategies affecting apoptosis or proliferation should be applied within the first day after chemotherapy.

In Paper IV, we showed that 5-fluorouracil (5-FU)-induced villus atrophy could be treated effectively with daily (pre-) treatment with melatonin in rats. We also demonstrated that melatonin increased cell viability in murine intestinal organoids exposed to 5-FU. While no significant changes in apoptosis and proliferation could be observed in the rat intestine, this could be due to the timeline of the progression of CIM, where both processes are most extensively affected within the first day. This warrants the need for the investigation of additional time points prior to 72 h, as well as dose finding studies and extended mechanistic investigations. However, the data from this study presents melatonin as a strong candidate for supportive therapy for CIM, especially given the potential anti-cancer effects melatonin may exert.
When thinking about the largest body surface exposed to external threats, the skin will immediately come to mind for most people. However, the intestinal tract with its many folds and finger-like protrusions is more than 10 times larger, and comes into contact with everything we eat and drink. Because of this, it is very important for the intestine to provide a barrier protecting the “inside” of the body from the entry of dangerous substances. At the same time, it is of course vital that this barrier allows the body to absorb all the nutrition and fluids we need to survive.

When this barrier is disrupted, harmful substances or microorganisms, such as bacteria, can pass through to our blood and lead to serious disease and, possibly, death. Disruptions like this can happen as a result of diseases and disorders, as well as from medication overuse of for instance NSAIDs including ibuprofen. It may also happen as side effects from cancer treatment with chemotherapy or radiation, which may necessitate treatment discontinuation, of course reducing the chance of the treatment being effective.

In this doctoral thesis, I have studied two different substances, melatonin and misoprostol, to see if they can help protect the intestine from such damages. For this, we did experiments in healthy rats, as it is important for the understanding of complex immunological processes to observe them in living systems. First, we induced a slight damage to the small intestinal mucosa in rats, to then evaluate if melatonin and/or misoprostol could help prevent these insults.

Melatonin is most well-known for its role in sleep. It is a common remedy for overcoming jetlag or helping people to fall asleep. However, it has also been shown to be present in the intestine and to protect different types of cells from damage. Misoprostol is a pharmaceutic drug that is used to treat ulcers resulting from overusing NSAIDs. Because these substances have shown protective effects before, they were of interest to treat other damages as well.

In the first study, the rat small intestine was subjected to a detergent, deliberately compromising its natural mucosal barrier. When we administered melatonin or misoprostol, we saw a remarkable protection against the weakening
effects of the detergent. Melatonin is known to mediate its protective effects mainly via two major ways. Firstly, melatonin can act as an antioxidant by neutralising reactive oxygen species, molecules notorious for causing cellular damage within the body. Secondly, melatonin interacts with receptors that are present on cells, which in turn lead to processes protecting the cells. In the second study, we could show that melatonin protects the intestinal barrier from breaking after being in contact with the detergent, through interacting with receptors. We did this by administering luzindole, a substance that blocks the melatonin receptors, together with melatonin. Through blocking the receptors, we eliminated one way for melatonin to exert its protective effect and saw that it was completely nullified.

In the third study, we looked closer at what happens to the intestine after chemotherapy treatment. We could show that the finger-like protrusions in the small intestine were shorter three days after giving chemotherapy. We could also see that before that, more cells in the intestine died and fewer of them multiplied than is the case normally.

In the last study, we wanted to see if we could prevent the damage to the intestine caused by chemotherapy by giving the chemotherapy-exposed animals daily doses of melatonin and/or misoprostol. The animals that were treated with melatonin were fully protected from the shrinking of the finger-like protrusions in the intestine caused by chemotherapy. However, misoprostol did not protect the animals from the effects of chemotherapy at all.

These findings not only deepen our understanding of the protective mechanisms orchestrated by melatonin, but also open new avenues for potential therapeutic interventions to fortify intestinal health. Because of that, we think that melatonin should be investigated more closely, to maybe give to cancer patients receiving chemotherapy to improve both their quality of life, and the quality of their treatment.
Acknowledgement

The work in this department was carried out at the department of Neuroscience (April 2019 – December 2021) and the department of pharmacy (April 2019 – September 2020), as well as the Department of Medical Cell Biology (December 2021 – January 2024) and the Department of Pharmaceutical Biosciences (September 2020 – January 2024). The studies were financially supported by Vetenskapsrådet, Cancerfonden, and the Scientific Domain of Medicine and Pharmacy, Uppsala University. I would also like to thank the Animal Facility at Uppsala University for taking good care of our animals.

Now for a part that I dreaded to write ever since I found out it was a thing in fear that I will forget someone – reflecting on all the help, input, contributions and support I received and the wonderful people that provided it.

The supervisors
My main supervisor Markus, thank you for all the time and effort you put into bringing me through this. I could always rely on you making the time for me, supporting me and helping me out with any issue I might bring to your door. I really appreciate all that you have done for me!
Hans, thank you for the discussions, input, and (sometimes) random anecdotes. It seems like no matter what topic, you always have the right paper to reference and the right people to ask at hand!
David, thank you for bringing me in on the pharmaceutical side of things and giving me a home in your old office. Your hands-on help and the ability to bounce things off you really helped me a lot!

Others I shared my time at BMC with
Fredrik, thank you for the great cooperation during the second half of my PhD! We might not have always had the same approach to tackle issues, but I believe it improved both of our work to get input and help from the other!
Oliver, you were not only the right guy to help Fredrik and me with our project, but always the right guy to ask about pretty much everything. I am still impressed by how you manage to do so many things and do them so well!
Ilse, thank you for both discussions and input on all projects, presentations and other problems that have occurred during my time as a PhD student. Your questions and feedback have always helped me to improve!
Luna, always one step ahead! Thank you for allowing me to “leech” off your experiences with all the complicated processes of getting this book printed. Although you were mostly on the other side of the country, the times you were in Uppsala were always a blast!

Janis, the other half of the German office in the corridor. I did not expect to get along with a Schalke fan that well. Thanks for allowing me to vent, share milk and the occasional German curse word with you.

Femke, thank you for the fun meetings, the live podcast, the scientific advice, and collaborations, as well as all the support both in teaching and writing matters.

Maria, thank you for all your help. You have provided so much time in hands-on work, writing, coffee breaks, lunch breaks that turn into a Greek birthday, and so much more. I could not have gotten this done without your support!

All the others in the D3:3 and 4 corridors, thanks for fun times during the rare times I joined for lunch, the times at the department days, the spexes and defence parties and of course while organizing ULLA! You made me as a half-outsider feel very at home at the department!

The PQP group, thanks for all the productive seminars and the few shared Christmas parties. I’m glad we ended up in the same group!

The students, Clara, Anton, and Nathalie – thank you for all your contributions as well as the fun times at after works we got to share!

The Smålands nation crowd. If I would list all you of, I would bankrupt the department for printing this, but you know who you are. Thank you for making me quickly feel at home in Sweden after moving here. For being open enough to welcome my family to gasques, beer pong and kubb matches. For making free time fun even during Covid and other difficulties. For making me want to stay after my master’s degree. For all the nonsense and stupidity that made me laugh. For everything, thank you!

Mama & Papa, ihr habt mir über die Jahre sowohl die Voraussetzungen, als auch die Unterstützung gegeben, die ich gebraucht habe um dieses Buch fertigzubringen. Ich verdanke euch so viel, dass man es kaum in Worte fassen kann. Danke für alles!

Michael & Shan, ich kann gar nicht sagen wie froh ich bin wieder engeren Kontakt mit euch zu haben. Ich freu mich jetzt schon auf alle zukünftigen Zusammenkommen, ob im hohen Norden oder im tiefsten Süden.
Christian, du bist sicherlich nicht ganz unschuldig an meiner ursprünglichen Studienwahl. Also habe ich dir den ganzen Stress zu verdanken… Ich bin froh über jede wissenschaftliche oder hart unwissenschaftliche Unterhaltung die wir führen, und, dich zu haben.

Hans & Trudi, meine ursprüngliche Fluchtmöglichkeit vor den zuvor genannten. Danke für alle Urläubchen, Gespräche und Unterstützung… und Nutella!

Katrin & Julian, danke für eure Offenheit, eure Freundschaft, eure Unterstützung. Und danke für eure wundervollen Kinder!

Eda, thank you for your undying support, your patience, your genius, your ability to help me, nudge me and ground me. Thank you for letting me vent and agree with me when I’m reasonable, but to tell me off when I’m not. You’re the reason I’m able to write this right now.

Should you feel forgotten after reading this, I refer you to the second paragraph. Let’s blame it on a very poor memory and a preoccupation with other things – like writing the rest of this book.

Bis die Tage
Karsten Peters, November 2023
References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)