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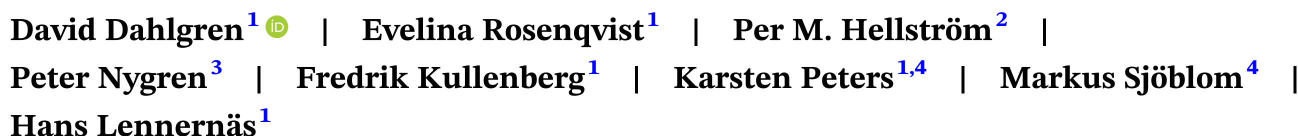
Evaluation and validation of chemotherapy-specific diarrhoea and histopathology in rats



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Abstract

Chemotherapy-induced mucositis is characterized by diarrhoea and villous atrophy. However, it is not well-understood why diarrhoea arises, why it only occurs with some chemotherapeutics and how it is related to villus atrophy. The objectives in this study were to determine (i) the relationship between chemotherapy-induced diarrhoea and villus atrophy and to (ii) establish and validate a rat diarrhoea model with clinically relevant endpoints. Male Wistar Han IGS rats were treated with saline, doxorubicin, idarubicin, methotrexate, 5-fluorouracil, irinotecan or 5-fluorouracil+irinotecan. After 72 h, jejunal tissue was taken for morphological, apoptotic and proliferative analyses, and faecal water content and change in body weight were determined. All treatments except methotrexate caused a similar reduction (≈42%) in villus height, but none of them altered mucosal crypt cell proliferation or apoptosis. Doxorubicin, idarubicin, irinotecan and 5-fluorouracil+irinotecan caused body weight reduction, but only irinotecan and idarubicin caused diarrhoea. No direct correlation between diarrhoea and villus height or body weight loss was observed. Therefore, studies of the mechanisms for chemotherapy-induced diarrhoea should focus on functional factors. Finally, the irinotecan and idarubicin diarrhoea models established in this study will be useful in developing supportive treatments of this common and serious adverse effect in patients undergoing chemotherapy.

KEYWORDS

apoptosis, chemotherapy-induced mucositis, cytostatics, diarrhoea, proliferation, toxicity, villus atrophy, weight loss

1 | INTRODUCTION

Cancer is the second largest cause of global premature death before the age of 70.[1] The last decades have seen substantial improvements in drug treatment of cancer with the introduction of immunotherapy and selective or targeted therapies such as hormones and protein kinase inhibitors. Nonetheless, cytotoxic chemotherapeutics are

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still the most common pharmacological treatment, due to their broad application for many cancers and cost efficiency.[2]

Chemotherapeutics induce desired toxic effects in proliferating cancer cells by targeting *general* cellular functions, such as DNA replication, mitosis and pivotal signal transduction pathways. This means that normal cell growth and development throughout the body are also affected by nontargeted mediated toxic effects. The entire intestinal epithelium is continuously renewed every 4–5 days from rapidly dividing stem cells in the crypts of the intestinal epithelium.[3] The consequence is off-target gastrointestinal (GI) toxicity or chemotherapeutics-induced mucositis. It affects up to 80% of all cancer patients undergoing chemotherapy (over 1 million patients annually in the United States and Europe[4,5]), as well as patients treated with some of the targeted antineoplastic drugs.[6,7] Clinically, it is primarily associated with a crippling diarrhoea,[8] but anorexia, pain, nausea and sepsis are also common. The diarrhoea severely reduces the quality-of-life in patients, often necessitates a dose reduction that delimit the therapeutic effectiveness, can be fatal and its management is associated with substantial health care costs.[9] For some especially GI toxic drugs, such as 5-fluorouracil (5-FU) and irinotecan (IRI), about one third are severe cases, that is, diarrhoea grades 3 and 4[10] which means more than seven bowel movements above normal per day and even hemodynamic disturbances.

Key symptoms of diarrhoea include loose stool consistency, increased frequency and/or urgency of bowel movements and faecal incontinence, with or without pain. Diarrhoea arises because of the following: (i) an increased fluid and electrolyte secretion, (ii) a reduced fluid and electrolyte absorption, (iii) an increased intestinal motility and/or (iv) exudative diarrhoea, in which disruption of the epithelium causes leakage of water, electrolytes, mucus, proteins and cells.[11,12] Causes of gut dysregulation of fluid flux and motility include infection, inflammation, allergies, bacterial imbalance, malabsorption and, as mentioned, chemotherapy. In the latter, diarrhoea typically occurs within days from onset of treatment and tapers off after a few days or weeks. Chemotherapy directly induces diarrhoea by altering gut integrity, motility and normal secretory functions and possibly indirectly, by affecting the gut microbiota.[13]

Chemotherapy-induced diarrhoea (CID) is currently treated with drugs that decrease intestinal motility, intravenous fluids to substitute for hypovolemia, to set the intestines at rest and by treatment postponement and dose reduction.[14] Frequently, these strategies need to be combined. However, CID is notoriously difficult to prevent and treat. It occurs only with some chemotherapeutics, for reasons that are largely unknown[13,15] A better understanding of the underlying mechanisms is therefore necessary for improved supportive care.

We have previously shown that three mechanistically different chemotherapeutics, doxorubicin (DOX), 5-FU and IRI, cause a similar 30% reduction in villus height but differ considerably with respect to their effects on the mucosal barrier.[16] The epithelial permeability of mannitol is increased 2.5-fold for 5-FU, 1.3-fold for IRI and reduced 0.5-fold for DOX. As mentioned, 5-FU and IRI are also the two chemotherapeutics that cause the most outspoken diarrhoea clinically, especially in combination with each other.[8] Consequently, we were interested if our observations regarding the role of the mucosal barrier with different chemotherapeutics in rats agreed with CID in patients. We were also interested in determining the relationship between chemotherapy-induced villus atrophy and epithelial stem cell death and proliferation. Taken together, this is expected to improve the in-depth knowledge of the pathology of mucositis in general and for diarrhoea in particular. It would also enable us to establish a rat model for CID for development and evaluation of new supportive treatment strategies for this common and serious side effect.

The primary objectives of this rat in vivo study were to evaluate the effect of a single dose of five chemotherapeutics and one combination—DOX, idarubicin (IDA), methotrexate (MTX), 5-FU, IRI and 5-FU + IRI—on body weight loss, jejunal villus atrophy (villus height, crypt depth, programmed cell death and proliferation) and diarrhoea, as well as the relationship between villus height reduction and diarrhoea and body weight loss. A secondary objective was to validate and further establish the in vivo rat diarrhoea model for future evaluation of intervention strategies using clinically relevant experimental endpoints. Rats were dosed with saline (control) or chemotherapeutics. At 72 h, when villus atrophy in rodents is most extensive,[17,18] jejunal segments were excised for morphological and immunohistochemical analyses, and the total colonic luminal faeces was desiccated for analysis of water content. The rat was selected as model species as it is considered to be translationally relevant, and it is well-established in preclinical studies of CID.[19]

2 | MATERIALS AND METHODS

2.1 | Chemicals

Atropine sulphate, Accustain formalin solution (10%, neutral buffered), dimethyl sulfoxide, ethanol, phosphate

buffered saline (PBS, pH 7.4) tablets and Inactin (thiobutabarbital sodium) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride was purchased from Merck KGaA (Darmstadt, Germany). 5-FU Teva (solution for injection, 50 mg/ml), IRI Actavis (solution for infusion, 20 mg/ml) and MTX Ebewe (solution for injection, 100 mg/ml) were purchased from Apoteket AB (Stockholm, Sweden). DOX HCl and IDA HCl were purchased from Toronto Research Chemicals (Toronto, Canada). Transferrin Ki67 antibody (ab16667), horseradish peroxidase - DAB detection IHC kit (ab64261) and TUNEL Assay Kit - HRP-DAB (ab206386) were purchased from Abcam, Cambridge, UK.

2.2 | Study drugs

Five chemotherapeutics were used in this study. 5-FU (solution, 50 mg/ml), IRI (solution 20 mg/ml) and MTX (solution, 100 mg/ml) were obtained in ready-to-use form. Stock solutions (100 mM) of DOX hydrochloride and IDA hydrochloride in dimethyl sulfoxide were prepared and diluted to 5 mg/ml in saline on the day of drug administration (final dimethyl sulfoxide concentration <5%). Inactin (50 mg/ml) and atropine (0.1 mg/ml) were dissolved in saline and used within the recommended stability time.

2.3 | Animals

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[20] The study was approved by the local ethics committee for animal research (5.8.18-17 754/2019) in Uppsala, Sweden. Conventional male Wistar Han IGS rats (strain code 273) weighing between 240 and 440 g (6–10 weeks) were procured from Charles River Co. (France). They were delivered to the animal laboratory facility at Uppsala University, Sweden, at least 1 week before the experiment. Before and during the experiments, the rats were kept in enriched cages with free access to food and water at a 12:12 h light–dark cycle, 21–22°C, 60% relative humidity.

2.4 | Study design

This study included seven experimental study groups (each $n = 6$). Five groups received a single dose of one of five chemotherapeutics, one group received a fixed-dose combination of 5-FU and IDA and the control group received saline. Table 1 shows the drugs, doses, administered volumes, administration routes and corresponding human doses. Doses were selected based on clinical relevance, our previous experience and published data.[21] Prior to dosing with IRI alone, or with a combination of 5-FU + IRI, each rat received a subcutaneous injection of atropine (0.02 mg) to avoid some of the immediate but transient cholinergic side effects of IRI.[22,23]

The rats were weighed on day 0 and at 72 h, to monitor the change in body weight induced by the different drug treatments. At 72 ± 2 h, the study was terminated by an intraperitoneal injection of a 5% w/v inactin solution (180 mg/kg). Immediately thereafter, the proximal jejunum was excised for morphological, apoptotic and proliferative analyses. The entire colon and its contents were removed and evaluated for faecal matter water contents, as described below. A study protocol was established before this study. It included information on rat ID and body weight, dose(s), dosing time and

TABLE 1 Dose, volume, administration route and corresponding and commonly used clinical dose for a control and six experimental study groups ($n = 6$ in each)

Group	Abbreviation	Dose (mg/kg)	Volume (ml)	Route	Corresponding clinical dose (mg/m^2)[50]	Commonly used clinical dose (mg/m^2)	Reference
Control	-	Saline	1	i.v.	-	-	-
Doxorubicin	DOX	10	0.4–0.7	i.v.	60	40–75	[51]
Idarubicin	IDA	2	0.1–0.2	i.v.	12	10–12	[52]
Methotrexate	MTX	40	0.1–0.2	i.v.	240	600–3000	[53]
5-Fluorouracil	5-FU	200	1–2	i.v.	1120	1300	[54]
Irinotecan	IRI	200	2–3	i.p.	1120	125–350	[55]
5-Fluorouracil + Irinotecan	5-FU + IRI	133 + 133[a]	1 + 2[a]	i.p.	800 + 800	1000 + 125	[56]

Note: The two irinotecan formulations were administered intraperitoneally (i.p.) because the dosing volume was too large for intravenous (i.v.) administration.
[a]The two doses and volumes are for 5-fluorouracil and irinotecan, respectively.

volumes, sampling times, type of body samples and sample handling procedure. A humane endpoint was defined, and animal well-being was monitored twice daily to avoid unnecessary suffering. Confounders were not controlled for in the study.

2.5 | Diarrhoea

The colon was removed in its entirety, and the luminal contents emptied into 50 ml Falcon tubes. The tubes were thereafter placed in an oven at 50°C, for at least 24 h until all water evaporated. Diarrhoea was expressed as percent colonic faecal water content (wet weight - dry weight) /wet weight × 100. Water content of 55%–70% is normal, and faecal pellets are solid. About 70%–80% corresponds to an increased water content but with still solid pellets, and at 80% and above, pellets are loose and watery.

2.6 | Morphological and histochemical analysis of the jejunum

Tissue samples were rinsed with room temperature saline and fixed in 10% formaldehyde for 24 h, then transferred to 70% ethanol. They were then embedded in paraffin and microtome-sliced (Microm Cool-Cut HM 355 S) at 5 μm and dried overnight. Sections were deparaffinized and serially rehydrated (submersion for 3 min × 2 in solutions of xylene, 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol followed by distilled water) prior to the stainings described below.

Haematoxylin–eosin staining of the tissue sections was carried out according to standard practice.[24] The histological jejunal samples were analysed for villus height and crypt depth, to quantify villus atrophy and crypt proliferation, respectively. Ten villi and crypts were measured (Figure 1) for each rat and the mean value selected.

To monitor cell proliferation, immunohistochemistry with the Ki67 antibody with a horseradish peroxidase/DAB detection kit was performed according to the manufacturer's guidelines. In short, slides were washed in PBS with Tween-20, and peroxidase activity was blocked using H_2O_2 block. A DIVA-decloaking chamber was used to retrieve crosslinked antigens. Nonspecific background staining was blocked using Protein Block solution. Primary Ki67 antibody was added in a 1:1000 dilution of PBS-Tween, incubated for 1 h at 37°C and thereafter exposed to with biotinylated goat antirabbit antibodies and streptavidin peroxidase at room temperature for 10 min. DAB was added to the slides for 2 min and rinsed. PBS was used for in between washing steps.

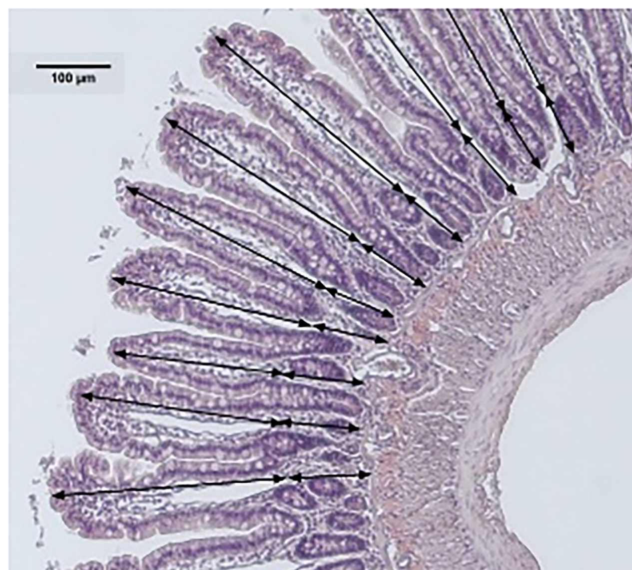

FIGURE 1 Ten measurements of villus height (finger-like protrusions) and crypt depth (area between villus bottom and submucosa) were used to determine the mean for each rat. The arrows show the height of each villus and crypt. Scale bar = 100 μm.

Finally, slides were counterstained with haematoxylin, dehydrated and mounted.

To detect programed cell death (e.g. apoptosis), the samples were stained with a TUNEL Assay Kit according to the manufacturer's instructions.

All images were acquired using a Zeiss Axio Vert microscope equipped with a Zeiss Axiocam 208 colour camera and Zeiss A-Plan 10x/0,25 Ph1 objective. Villus height and number of stained cells per crypt (TUNEL) determinations were performed in Fiji ImageJ, and DAB was automatically quantified with a macro (supporting information S1).

2.7 | Statistics

Data are presented for each individual animal and as group mean ± SD. Data collected included villus height, crypt depth, apoptotic cells per crypt, proliferation (% stained sample area), colonic water content (%) and body weight reduction (%). The values were compared against the saline control using a one-way, unpaired ANOVA, with a post hoc Dunnett's comparison test. All comparisons were tested for normality of residuals and equality of group variance with the tests Shapiro–Wilk and Brown–Forsythe, respectively. If group variance was not equal, regular ANOVA analysis was replaced by a Brown–Forsythe ANOVA test with a Dunnett T3 post hoc test. If a nonnormal distribution appeared, the

nonparametric Kruskal–Wallis test with Dunn's multiple comparisons post hoc test was applied. A p-value <0.05 was regarded significant in all analyses. Statistic tests and graphs were made in GraphPad Prism 9.1.2. The sample size ($n = 6$) was selected based on previous experience of the effect and variability (body weight loss, villus length and permeability) following chemotherapy treatment to rats.[16] The total number of rats used in the study was 42.

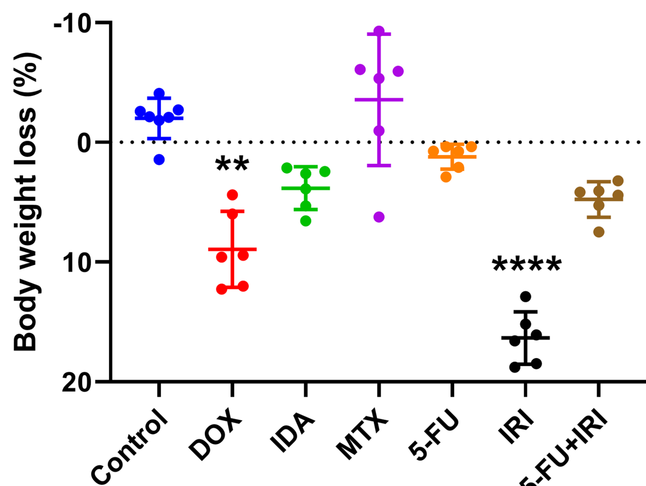

FIGURE 2 The mean (±SD) and individual body weight loss 72 h after dosing with saline (control) or with doxorubicin (DOX, 10 mg/kg), idarubicin (IDA, 2 mg/kg), methotrexate (MTX, 40 mg/kg), 5-fluorouracil (5-FU, 200 mg/kg), irinotecan (IRI, 200 mg/kg), or 5-FU + IRI (133 + 133 mg/kg). The dotted line indicates no change in body weight (0%) after 72 h. For details of the treatments, see Table 1. *Significance, p-value < 0.05–0.0001, was evaluated against control, Kruskal–Wallis test, with a post hoc Dunn's comparison test.

3 | RESULTS

3.1 | Body weight

The percent change in body weight from dosing at time 0 until 72 h afterwards is shown in Figure 2. Control rats gained on average 2.0% body weight over the 3 days, while there was significant body weight reduction in groups dosed with DOX (8.9 ± 3.2%) and IRI (16.3 ± 2.2%) alone.

3.2 | Villus height and crypt depth

Figure 3A–G displays representative pictures of the jejunal mucosa from the seven different treatment groups (Table 1). Figure 4A,B shows the mean (±SD) jejunal villus height and crypt depth at 72 h following dosing with saline (control), DOX, IDA, MTX, 5-FU, IRI or 5-FU + IRI (Table 2). The villus height decreased significantly for all chemotherapeutics compared with the control (442 ± 40 μm). The mean reduction was similar (36%–49%) for all treatments except MTX (20%). For crypt depth, there was a significant 32% increase for IRI compared with the control (148 ± 22 μm).

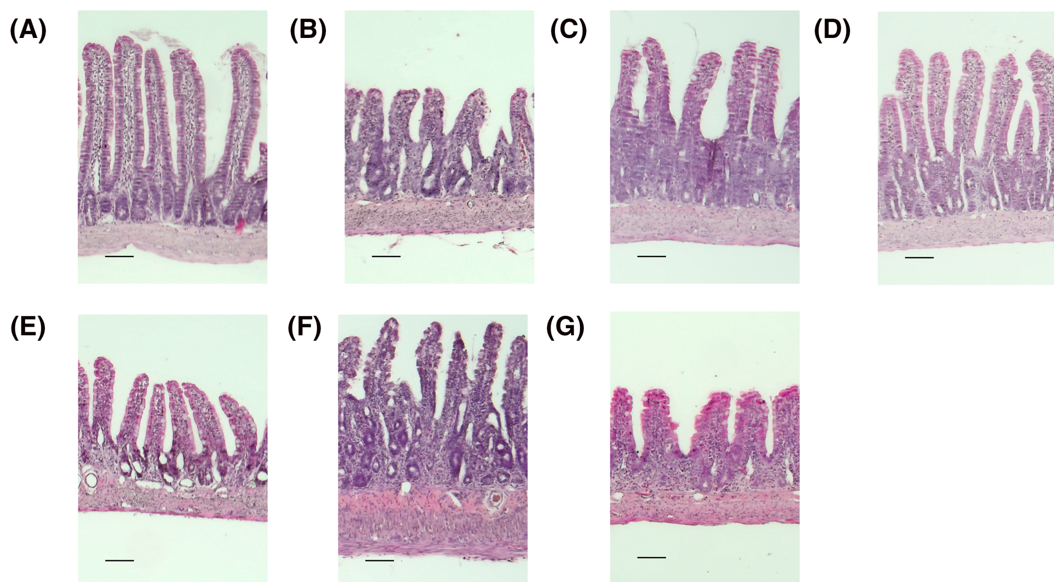

FIGURE 3 Haematoxylin–eosin staining of the rat jejunal mucosa 72 h after dosing with (A) saline, (B) doxorubicin - DOX, (C) idarubicin - IDA, (D) methotrexate - MTX, (E) 5-fluorouracil - 5-FU, (F) irinotecan - IRI, and (G) 5-FU + IRI. For details of the treatments, see Table 1.

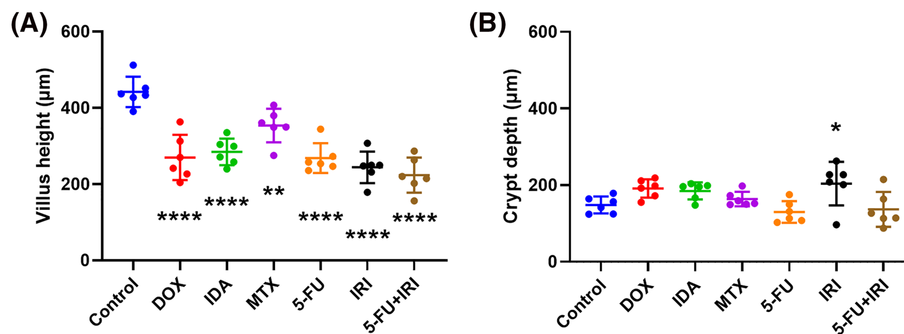

FIGURE 4 The mean (±SD) and individual (A) villus height and (B) crypt depth, measured 72 h after dosing with saline (control) or with doxorubicin (DOX, 10 mg/kg), idarubicin (IDA, 2 mg/kg), methotrexate (MTX, 40 mg/kg), 5-fluorouracil (5-FU, 200 mg/kg), irinotecan (IRI, 200 mg/kg) or 5-FU + IRI (133 + 133 mg/kg). For details of the treatments, see Table 1. *Significance, *p*-value < 0.05–0.0001, was evaluated against controls, one-way, unpaired ANOVA, with a post hoc Dunnett's comparison test.

TABLE 2 The mean (±SD) body weight loss, diarrhoea, villus length and crypt depth, proliferation and cell death, 72 h after dosing with saline (control) or with doxorubicin (DOX, 10 mg/kg), idarubicin (IDA, 2 mg/kg), methotrexate (MTX, 40 mg/kg), 5-fluorouracil (5-FU, 200 mg/kg), irinotecan (IRI, 200 mg/kg) or 5-FU + IRI (133 + 133 mg/kg)

Group	Weight loss (%)	Diarrhoea (% water content)	Villus length (µm)	Crypt depth (µm)	Proliferation (% tissue staining)	Cell death (cells/crypt)
Control	−2.0 ± 1.7	63.5 ± 3.4	442 ± 40	148 ± 22	2.6 ± 2.9	0.25 ± 0.18
DOX	**8.9 ± 3.2****	75.0 ± 8.3	**270 ± 59******	191 ± 24	3.4 ± 1.8	0.87 ± 0.78
IDA	3.8 ± 1.8	**78.6 ± 11.0****	**285 ± 35******	185 ± 22	6.1 ± 5.1	0.085 ± 0.085
MTX	−3.6 ± 5.5	72.5 ± 8.9	**354 ± 44****	164 ± 19	2.1 ± 2.0	0.066 ± 0.074
5-FU	1.2 ± 1.0	60.6 ± 5.3	**268 ± 39******	130 ± 28	3.0 ± 2.0	0.19 ± 0.21
IRI	**16.4 ± 2.2******	**85.2 ± 12.0*****	**244 ± 41******	**204 ± 57***	1.8 ± 1.5	0.44 ± 0.25
5-FU + IRI	4.8 ± 1.5	69.1 ± 3.3	**224 ± 46******	137 ± 46	2.0 ± 1.5	0.34 ± 0.11

Note: Significance (in bold), *p*-value was evaluated against controls, one-way, unpaired ANOVA with a post hoc Dunnett's comparison test or Kruskal–Wallis test, with a post hoc Dunn's comparison test.
*$p < 0.051$. **$p < 0.01$. ***$p < 0.005$. ****$p < 0.001$.

3.3 | Diarrhoea

Figure 5 shows mean (±SD) percent of colonic faecal water at 72 h posttreatment with saline (control), DOX, IDA, MTX, 5-FU, IRI or 5-FU + IRI (Table 2). There were significant increases in water content compared with the control (63.5 ± 3.4%) following dosing with IDA (78.6 ± 10.7%) and IRI (85.2 ± 12.3%). Diarrhoea was consistently observed with IRI, whereas three animals in the IDA group had none. 5-FU (60.6 ± 5.3%) and MTX (72.5 ± 8.9%) alone did not cause diarrhoea and neither did the combination of 5-FU + IRI (69.1 ± 3.3%) administered at a lower dose (133 + 133 mg/kg).

3.4 | Proliferation and programmed cell death

Figure 6A,B shows represenative pictures of the stainings for proliferation (Ki67-DAB) and programmed cell death (TUNEL), respectivly. Figure 6C,D shows the mean (±SD) jejunal crypt proliferation and programmed cell death for the control rats and 72 h after dosing with DOX, IDA, MTX, 5-FU, IRI and 5-FU + IRI (Table 2). There was no difference compared with control animals with regard to either proliferation (2.6 ± 2.9% of sample stained) or programmed cell death (0.25 ± 0.18 cells per crypt).

4 | DISCUSSION

Antineoplastic drug therapy is notorious for its side effects, in particular chemotherapy-induced intestinal mucositis. It is characterized by morphological changes, such as villus atrophy, together with severe diarrhoea. Villus atrophy arises as a result of stem cell death in the crypts of the mucosal epithelium of the small intestine. Under normal conditions, proliferating progenitor stem

cells replace the continuous shedding of intestinal epithelial cells from the tip of the villi about every 4–5 days.[3] When this renewal process is compromised, the height of the villi is reduced. Following chemotherapy to rodents, maximum histological injury occurs after about 3 days.[17,18] In our study, DOX, IDA, 5-FU, IRI and 5-FU + IRI decreased villus height by 36%–49% after 72 h, in agreement with similar studies.[25]

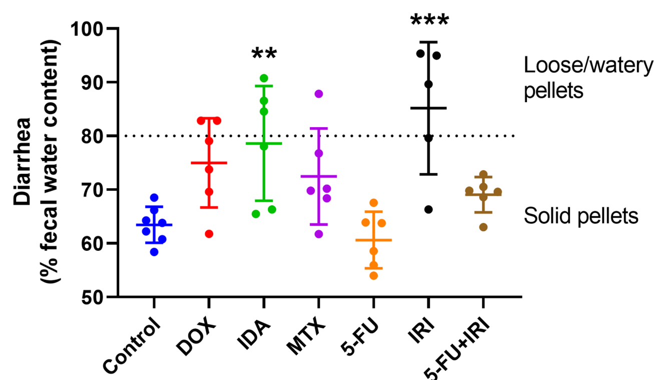

FIGURE 5 The mean (±SD) and individual colonic faecal water contents (%) that reflects diarrhoea, determined 72 h after dosing with saline (control) or with doxorubicin (DOX, 10 mg/kg), idarubicin (IDA, 2 mg/kg), methotrexate (MTX, 40 mg/kg), 5-fluorouracil (5-FU, 200 mg/kg), irinotecan (IRI, 200 mg/kg) or 5-FU + IRI (133 + 133 mg/kg). For details of the treatments, see Table 1. *Significance, p-value < 0.05–0.0001, was evaluated against controls, one-way, unpaired ANOVA, with a post hoc Dunnett's comparison test.

However, clinically, these drugs have different GI toxicity profiles, especially with regard to incidence and severity of diarrhoea.[26] As such, this would indicate that CID is not primarily a result of villus atrophy. The current study corroborates this as we clearly show that the occurrence, and severity of diarrhoea was not related to the villus atrophy induced by the different drugs. Diarrhoea was most pronounced for IRI and to some extent for IDA, while it did not occur for DOX, MTX, 5-FU and 5-FU + IRI, despite similar effects for all study drugs on villus height reduction.

We must therefore look for other mechanisms that can contribute to CID. One hypothesis is that the incidence of CID may be related to unspecific leakage from a compromised mucosal barrier, that is, exudative diarrhoea.[12,16] However, reported rat data show that in vivo leakage of mannitol across the jejunal epithelium has the following rank order: 5-FU > IRI > DOX, when administered at the same doses and time point as in this study.[16] As this study showed no diarrhoea for DOX and 5-FU, but severe diarrhoea for IRI, it seems that jejunal leakage of mannitol and diarrhoea are not correlated. Rather, an inverse association is implied, as 5-FU actually reduced diarrhoea in the rats in our study. It is possible that permeability data from the colon would provide different results or that a correlation would be observed if a barrier marker other than mannitol were used.[16] We suggest that future studies should focus on other permeability markers, include both the small and large intestine and look for other possible causes of CID as a function of the drug mechanism of action.[27] Other

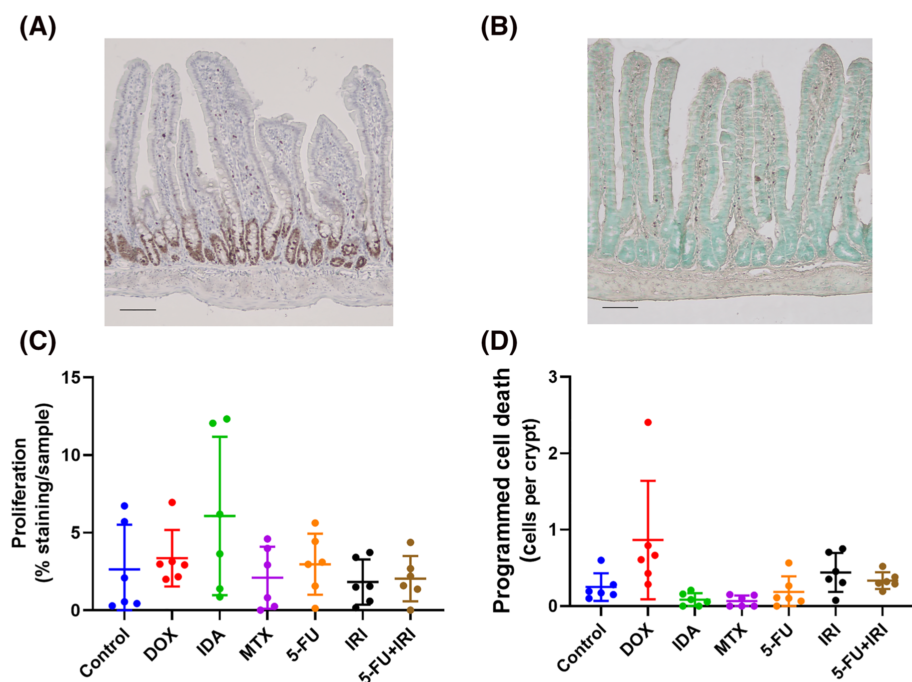

FIGURE 6 Representative pictures of the stainings for (A) proliferation (Ki67-DAB) and (B) programmed cell death (TUNEL). *Scale bar = 100 μm.* The mean (±SD) and individual jejunal crypt (C) proliferation and (D) programmed cell death (e.g. apoptosis) determined 72 h after dosing with saline (control) or with doxorubicin (DOX, 10 mg/kg), idarubicin (IDA, 2 mg/kg), methotrexate (MTX, 40 mg/kg), 5-fluorouracil (5-FU, 200 mg/kg), irinotecan (IRI, 200 mg/kg) or 5-FU + IRI (133 + 133 mg/kg). For details of the treatments, see Table 1.

possible causes of CID are discussed below, for example, differences in disposition routes of the cancer drug, microbiota, immunological response, ubiquitylation that affects key protein function(s) and dysregulation of GI physiology including water secretion and gut motility.

Biliary secretion can lead to high intestinal luminal concentration of chemotherapeutics close to the epithelial stem cells, which causes local toxicity and thereby triggers diarrhoea. For instance, both DOX and IRI (and their metabolites) are biliary secreted to a large extent in the rat.[28,29] On the other hand, oral administration of the inactive metabolite of IRI, SN-38G—which is transformed by luminal bacteria in the GI lumen to the active form, SN-38—does not induce any intestinal damage.[30] Furthermore, DOX does not give rise to an exceptionally high incidence or severity of mucositis, despite its high biliary secretion. Finally, oral dosing of 5-FU results in lower incidence of CID than intravenous dosing.[26] Thus, it seems that biliary secretion is not a primary determinant of GI toxicity with chemotherapy. Still, chemotherapeutics could indirectly contribute to CID by altering the number and diversity of luminal bacteria. For instance, IRI- and 5-FU-induced CID is associated with changes in the gut microbiome in the rat,[31,32] and germ-free or antibiotic-treated mice sometimes experience less outspoken CID.[33,34] Future studies with this model will focus on these processes in colon.

Chemotherapy also induces an immune response associated with release and activation of a number of pro-inflammatory cytokines that contribute to mucositis.[26] The selectivity of inhibition, the upregulation of important signalling pathways, protein modifications by ubiquitylation and alterations in metabolism are evident. For instance, DOX affects gene expression and biological pathways in human organoids from the small and large intestine.[35] Notably, the genes are involved in the cell cycle regulation, the p53 signalling pathway and oxidative stress, and there are significant differences between small and large intestinal organoids. This type of advanced in vitro-based mechanistic studies of the inflammatory response may improve understanding of the incidence and severity of diarrhoea induced by different chemotherapeutics.[35] However, advanced neuroendocrine feedback loops—which need to be considered for any complete understanding—are not present in this in vitro system.

Chemotherapeutics could modify intestinal transit and fluid secretion/absorption by interfering with the enteric nervous system[36] and/or by disturbing hormonal regulation of intestinal functions.[37,38] Regarding motility, there was no correlation clinically between degree of diarrhoea and small intestinal transit time following fluoropyrimidine dosing.[39] However, colonic motility and transit time may still be involved, but the impact of different chemotherapeutics on mucosal secretory functions is currently not well-established.[10] Future experiments will investigate changes in ion and water fluxes across the small and large intestinal mucosa following different chemotherapies and dosing schedules of them.

In our study, body weight loss was the parameter most relevant as a clinical reflection of patient status. Many different factors may contribute to body weight loss, such as diarrhoea, endocrine processes, malabsorption, cachexia and effects contributing to less intake of water and nutrients, like nausea, pain or malaise. This study did not aim to establish a causative correlation between body weight loss and any of these parameters. Generally, we found no correlation between diarrhoea and bodweight loss. Nonetheless, IRI induced the largest effect on both diarrhoea and body weight loss in this study. The connection between the two is further supported by the finding that oral administration of probiotics reduces both IRI-induced body weight loss and severe diarrhoea incidence in rats.[40]

As discussed above, there is an incomplete mechanistic understanding of the pathology and development of diarrhoea following treatment with different chemotherapeutics and their dosing regimens.[10] Identification of the pathophysiological changes induced by chemotherapy is fundamental for the development of supportive interventions for this GI condition, which currently lacks any effective prevention and treatment.[41] To fill these knowledge gaps and enable a high degree of clinical translation, an in vivo model is needed that captures the major physiological and pathophysiological factors and their neuroendocrine feedback signalling. Collectively, these determine the severity of diarrhoea and progress of local inflammation. The combination of all these factors is particularly important when evaluating mechanisms and supportive interventions targeted toward the complex physiological, biochemical and microbiological environment of the GI tract.[19] For this purpose, the rat is a useful in vivo model. Accordingly, our study showed that a single dose of 200 mg/kg of IRI to Wistar rats gave a consistent and severe diarrhoea after 72 h, which is in accordance with reported data from Dark Agouti rats.[23] The same IRI dose also caused a substantial body weight reduction that was most likely related to the diarrhoea. This experimental, albeit clinically relevant rat diarrhoea, model may be useful for evaluation of new strategies for prevention and treatment of IRI-induced diarrhoea in particular and potentially for CID in general.

However, it should be mentioned that 5-FU did not give rise to diarrhoea in our rat model, despite its high clinical incidence.[26] This is possibly related to dose and a higher metabolic clearance in rat. All single doses were

selected to optimize GI symptoms on the basis of experience, published data and clinical relevance, while avoiding unnecessary animal suffering. Different mechanisms of drug-induced diarrhoea, as discussed above, may not be perfectly reflected in rat or in the specific rat strain used for this study. To evaluate the latter, we will replicate this rat diarrhoea study in Sprague Dawley rats.

Another important aspect of in vivo diarrhoea models is time from dosing. One way to account for this is to monitor diarrhoea on a daily basis. Indeed, most reported rat studies rely on this method of qualitative quantification, in which severity and incidence is based on scoring of faecal texture and perianal staining of the coat.[32,42,43] An obvious advantage with this monitoring approach is that this enables repeated observations from each animal. However, qualitative scoring lacks a quantitative determinations of diarrhoea and is highly variable, as opposed to determination of faecal water contents in this study. It should also be highlighted that we have been unsuccessful in correlating colonic faecal water content to the scoring proposed in the qualitative determination of diarrhoea: It is only in the most extreme cases of diarrhoea that we observed any clear signs of it by external monitoring of the rat faeces. We prefer and advocate the method of diarrhoea quantification in our study, especially in cases where subtle differences and response to treatments are investigated or in cases where chemotherapy leads to an increased colonic faecal water content without any qualitative signs of diarrhoea. The quantification method is also expected to provide more conclusive dose–response data for any drug development project, and a lower variability enables the use of less animals. Still, the two methods are nonexclusive and could be combined.

Villus atrophy is related to an imbalance in crypt cell death and proliferation. Peak apoptosis after anthracycline dosing occurs within the first 24 h,[44] and mitosis is reduced between 6 and 96 h.[45] Other groups have seen an increase in proliferation and reduced apoptosis between 72 and 96 h after 5-FU dosing using the BrdU and caspase3 activity assays, respectively.[46–48] However, in our study, using the Ki67 and TUNEL assays, we were unable to detect any significant changes in proliferation and programmed cell death at 72 h, most likely as the renewal process is robust and rather fast.[49] These methods and time points are consequently not suitable for investigating CID treatments.

This study and diarrhoea model have an obvious limitation, as partly discussed above. The clinical pattern of CID for the various drugs was not fully reproducible. This may be due to drug dosing, timing of diarrhoea, body weight loss and villous status assessments as well as intestinal sampling from only the proximal jejunum. Furthermore, the healthy and young Wistar rat per se may only partly reflect the GI sensitivity to chemotherapeutics seen in elderly humans. Thus, the experimental in vivo model needs to be refined, expanded with respect to diarrhoea-inducing cancer drugs and further corroborated prior to use in investigations of strategies for prevention and supportive treatment of CID. It is also encouraged to validate all preclinical rat data also in female rats to enable optimal translation value of the model. Nonetheless, the irinotecan-induced diarrhoea from rat in this study corresponds very well to what is observed in humans as well as in other preclinical models such as mouse,[19] emphasizing the usefulness of rodents for studying CID in humans.

5 | CONCLUSION

This in vivo study established an experimental, albeit clinically relevant diarrhoea model in Wistar rats by using a single dose of 200 mg/kg of irinotecan and a time point of 72 h. It also showed that villus atrophy was pronounced and similar in degree following dosing of five different chemotherapeutics at clinically relevant doses and combinations. However, diarrhoea was only observed with two of them, irinotecan and idarubicin. Consequently, small bowel villus atrophy itself was not predictive of diarrhoea. Future studies should investigate other mechanisms in both the small and large intestine for chemotherapy-induced diarrhoea. Improved understanding of this relationship would contribute to development of supportive treatments for this common and serious adverse drug effect.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
Data are available on request.

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