May cannabinoids prevent the development of chemotherapy-induced diarrhea and intestinal mucositis? Experimental study in the rat

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Running title: 5-FU, cannabinoids & gut

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ABSTRACT

Background: The antineoplastic drug 5-fluoruracil (5-FU) is a pirimidine analog, which frequently induces potentially fatal diarrhea and mucositis. Cannabinoids reduce gastrointestinal motility and secretion and might prevent 5-FU-induced gut adverse effects. Here we asked whether cannabinoids may prevent diarrhea and mucositis induced by 5-FU in the rat. **Methods**: Male Wistar rats received vehicle or the non-selective cannabinoid agonist WIN 55,212-2 (WIN; 0.5 mg kg^{-1} $injection^{-1}$, 1 injection day^{-1}, 4 consecutive days) by intraperitoneal (ip) route; on the first 2 days, animals received also saline or 5-FU (150 mg kg^{-1} $injection^{-1}$, cumulative dose of 300 mg kg^{-1}). Gastrointestinal motor function was radiographically studied after barium contrast intragastric administration on experimental days 1 and 4. Structural alterations of the stomach, small intestine and colon were histologically studied on day 4. PAS staining and immunohistochemistry for Ki67, chromogranin A and CD163 were used to detect secretory, proliferating and endocrine cells, and activated macrophages, respectively. **Key results:** As shown radiographically, 5-FU induced significant gastric emptying delay (on days 1 and 4) as well as diarrhea (on day 4). WIN did not significantly alter the motility curves obtained for either control or 5-FU-treated animals but tended to reduce the severity of 5-FU-induced diarrhea and increased permanence of barium from day 1 to the beginning of day 4 in 5-FU-treated animals. 5-FU-induced mucositis was severe and not counteracted by WIN. **Conclusions and Inferences**: 5-FU-induced diarrhea, but not mucositis, was partly prevented by WIN at a low dose. Cannabinoids might be useful to prevent chemotherapy-induced diarrhea.

KEYWORDS: 5-fluorouracil, gastrointestinal motility, chemotherapy-induced adverse effects, cannabinoids, diarrhea.

KEY POINTS:

- Mucositis and diarrhea are debilitating side effects associated to cancer chemotherapy, but still lacking optimal clinical management. New therapeutic approaches are required.
- In the presence of histologically demonstrated mucositis, the antineoplastic drug 5-fluorouracil delayed gastric emptying and induced diarrhea. The cannabinoid agonist WIN 55,212-2 at a low, non-psychoactive dose partially reduced diarrhea, but not mucositis.
- This is the first experimental study showing that cannabinoids may have a role to counteract chemotherapy-induced diarrhea.

Due to aging and lifestyle changes, global cancer incidence is predicted to significantly increase in the next years, and also the toxic manifestations arising from treatment. Gastrointestinal mucositis affects a large population of the oncology patients (40%-100% depending on the particular treatment schedule) (1,2,3). It has a huge clinical and economic impact because it increases the prevalence of pain, infection and hemorrhage leading to impaired quality of life and higher time and cost of hospitalization (4). Moreover, patients may require reductions in dosing or may no longer be able to continue cancer therapy in severe cases (5). 5-fluorouracil (5-FU), a pyrimidine analog frequently used to treat breast or colorectal cancer (CRC), induces mucositis in 50-80% of patients, resulting in abdominal bloating as well as vomiting and diarrhea (4,6).

Mucositis is probably the main factor involved in chemotherapy-induced diarrhea (CID), characterized by an imbalance between absorption and secretion in the gut (6,7). CID is potentially fatal due to dehydration (which may compromise cardiovascular and renal function and trigger electrolyte disorders), and rupture of the intestinal barrier (which may cause infection and sepsis) (7,8). CID affects 25% of CRC patients receiving 5-FU as single agent (6-13% with severe diarrhea, grades 3/4) and can be severe in up to 40% receiving combination chemotherapy (9,10,11).

Mucositis and its associated diarrhea management are still limited to analgesics, antibiotics, and antidiarrheal and mucosal protective agents. However, these are only palliative and frequently non-effective (10,12,13,14). Thus, mucositis and CID remain an unmet medical problem, requiring evaluation of new treatment options.

Cannabinoids exert potent effects on the gastrointestinal tract (15,16). Cannabinoid agonists are empirically used in the clinic to prevent chemotherapy-induced nausea

and vomiting (CINV) and these effects have been confirmed and characterized in different animal models (15,16,17). Interestingly, however, heavy cannabis smokers develop hyperemesis (18), which might be due to gastric dysmotility. In fact, high (centrally-acting) doses of cannabinoids intensely delayed gastric emptying after acute, daily and intermittent administration in the rat without tolerance development (19,20,21). Cannabinoids also reduce diarrhea associated to a number of conditions (22,23). In experimental animals, non-selective, CB_1 and CB_2 selective agonists prevented diarrhea induced by different stimulants (24,25,26). Activation of both CB_1 and CB_2 cannabinoid receptors might be useful against CID, due to their respective anti-motility/anti-secretory, and anti-inflammatory effects (15,27). To our knowledge, cannabinoid agonists (exogenously administered) have never been tested in animal models of 5-FU-induced diarrhea/mucositis.

Therefore, our aims were: to characterize the effects of 5-FU on gastrointestinal motility in the rat using radiographic techniques (which may non-invasively provide interesting morphological and dynamic data of each gastrointestinal region functioning under pathological conditions and in response to treatment, 28); to determine whether a low non-psychoactive dose of the cannabinoid agonist WIN 55,212-2 (WIN) is able to prevent 5-FU-induced diarrhea; to characterize the effects of WIN on 5-FU-induced mucositis and other histologic alterations of the gut wall.

MATERIALS AND METHODS

The experiments were designed and performed in accordance with the European and Spanish legislation on care and use of experimental animals (2010/63/UE for

animal experiments; Real Decreto 53/2013), and were approved by the Ethic Committee at Universidad Rey Juan Carlos (URJC).

Animals and treatment

Male Wistar rats (250–300 g at the beginning of the experiment) were obtained from the Veterinary Unit of URJC, and housed (4/cage) in standard transparent cages (60 cm x 40 cm x 20 cm), under environmentally controlled conditions (temperature = 20 ºC; humidity = 60%), with a 12 h light/12 h dark cycle. Animals had free access to standard laboratory rat chow (Harlan Laboratories Inc.) and tap water.

Rats received one intraperitoneal (ip) injection of WIN (0.5 mg kg^{-1}) or its vehicle (0.5 mL) each day for 4 consecutive days (experimental days 1-4). In addition, rats received saline (2.5 mL) or 5-FU (150 mg kg^{-1}, ip) for 2 days starting on day 1 (cumulative dose of 300 mg kg^{-1}), 30 min after WIN or its vehicle. Doses were chosen based on pilot studies and the literature (see below). The protocol followed is summarized in Fig. 1 – Supplementary Material.

Throughout experiment (days 1-4) body weight, food intake and water intake, as well as signs of general toxicity, were recorded. Gut motility studies were performed on days 1 and 4 in one group of animals (n = 32). In a parallel group of animals that received the same treatments and whose body weight, and food and water intake were similarly evaluated (n = 26), samples were obtained from the small intestine to perform histological studies. Details of gut motility and histological studies are described below.

Schedule of 5-FU and cannabinoid administration

In pilot experiments, we used a single dose of 150 mg kg^{-1} by the ip route (29,30). However, 4 days after administration, we could not see any radiographic sign of diarrhea, upon which to test the possible antidiarrheal effect of cannabinoids. In fact, this is probably a very low dose compared to that used in humans (5-FU in the standard FOLFIRI regimen for CRC is dosed at 2400 mg m^{-2}, and it has been calculated that the dose of 400 mg kg^{-1} in rats would correspond to 2222 mg m^{-2} in patients: 31,32). Therefore, we decided to administer a second dose on the following day (cumulative dose of 300 mg kg^{-1}, similar to others: 33, iv route; 34, oral route). This schedule was effective to induce diarrhea radiographically observable and was then adopted for our study, although we assume that the 5-FU dosage is probably still lower than that used in clinical chemotherapy.

Regarding WIN, the dose chosen (0.5 mg kg^{-1}) did not induce significant central effects, except for slight analgesia, did not significantly alter gastric motility either in acute or repeated administration for 14 days, but slightly delayed small intestinal transit (19,20), which could be beneficial for preventing 5-FU-diarrhea. Taking into account these previous data from our own laboratory, we performed an invasive test using the charcoal method (Vera et al, 2011: 35), which confirmed that this dose is effective to slightly but significantly decrease gastrointestinal motor function in naïve animals (see Fig. 2 – Supplementary Material for methodological details and results of this pilot test). This dose was used thereafter for our study.

Gut motility experiments

Radiographic techniques were applied in order to non-invasively analyze alterations in gastrointestinal motility induced by 5-FU and the cannabinoid (28). For this, 20 min after the first 5-FU/saline dose (day 1) and 20 min after the fourth WIN/vehicle administration (day 4), 2.5 mL of a suspension of barium sulfate (Barigraph ® AD, Juste SAQF, Madrid, Spain; 2 g mL^{-1}, temperature = 22 ºC) was administered per *os*. Plain facial radiographs of the gastrointestinal tract were obtained using a CS2100 (Carestream Dental, Spain) digital X-ray apparatus (60 kV, 7 mA), and X-rays were recorded on Carestream Dental T-MAT G/RA film (15 x 30 cm) housed in a cassette provided with regular intensifying screen. Exposure time for X-ray shots was adjusted to 0.02 s and focus distance was manually fixed to 50 ± 1 cm. Immobilization of the rats in prone position was achieved by placing them inside adjustable hand-made transparent plastic tubes, so that they could not move. Habituation to the recording chamber prior to commencement of the study did not significantly alter gastrointestinal motility (28). To further reduce stress, rats were released immediately after each shot (immobilization lasted for less than 2 min). X-rays were recorded at different times (immediately and 1, 2, 4, 6, and 8 h: T0-T8) after administration of the contrast medium. While X-ray shooting, the qualified investigator remained, behind a lead screen, at least 2 m away from the X-ray source.

Analysis of the radiographs was performed by a trained investigator blind to the drug administered. Alterations in gut motility were semi-quantitatively determined from the images by assigning a compounded value to each gastrointestinal region considering the following parameters: percentage of the region filled with contrast (0-4); intensity of contrast (0-4); homogeneity of contrast (0-2); and sharpness of the gut region profile (0-2). Each of these parameters was scored and a sum (0-12 points) was

made. The X-ray images were also morphometrically analyzed with the aid of an image analysis system (ImageJ 1.38 for Windows, National Institute of Health, USA, free software: http://rsb.info.nih.gov/ij/) and the alterations in size of stomach and caecum were studied.

Finally, severity of diarrhea was specifically assessed applying the following score to the appearance of the colorectum on the X-rays: 0 – no diarrhea; 1 – mild diarrhea (both liquid and fecal pellets); 2 – severe diarrhea (only liquid). In addition, since some barium contrast from day 1 radiographic analysis could still be seen in the gut on day 4 at T0, we analyzed the presence of these "shadows" for the different experimental groups. For this, a further score was applied to each intestinal region on each T0 (day 4) X-ray: 0 – no barium content remaining from day 1; 1 – barium content remaining from day 1. Afterwards, the values obtained for the different intestinal regions were summed to give the final value (0-3 points) of the intestinal "shadows" on the X-ray.

Histopathological analysis of gastrointestinal regions

On day 4, samples were obtained from the stomach (fundus and body), terminal ileum (at least 10 cm oral to the ileocaecal junction) and colon of 4-7 animals per experimental group, fixed in buffered 10% formalin and embedded in paraffin. Sections of 5 μm were stained with conventional hematoxylin-eosin (HE), Van Gieson´s stain, PAS or prepared for immunohistochemistry. They were studied under a Zeiss Axioskop 2 microscope equipped with the image analysis software package AxioVision 4.6 to calculate the morphometric parameters. The analysis was made by triplicate in 5-8 random fields measured in 20-40x objective microphotographs per

section and specimen. The experimenter was blind to the treatment received by the rat from which the sample under analysis was obtained.

Histological damage of the ileum was evaluated in sections stained with HE using criteria adapted from Galeazzi et al. (36). A numerical score of 0–9 was assigned to each section considering general loss of mucosal architecture (graded 0–3, absent to severe), extent of inflammatory cell infiltrate (graded 0–3, absent to transmural), crypt abscess formation (0–1, absent or present), goblet cell depletion (0–1, absent or present) and muscular layer thickness (0–1, normal to reduced). The number of damaged villi, inflammatory infiltrates per linear centimeter of intestine and thickness of both muscle layers were also measured. The number of goblet cells per villi was counted after PAS staining. Submucosa thickness was measured after staining with Van Gieson to detect collagen fibers. The colon was evaluated according to Saccani et al. (37). The numerical score in this case was 0-13 considering epithelial damage (graded 0–3, normal to severe), inflammatory cells infiltration (from 0 to 4, absence to severe involving submucosa), separation of muscle layer and muscularis mucosae (from 0 to 2, normal to severe) and goblet cell depletion (0–4, absent to present).

For immunohistochemistry, samples were washed with phosphate buffered saline (PBS) with 0.05% Tween 20 (Calbiochem, Darmstadt, GER). Thereafter sections were incubated for 10 min in 3% (vol vol^{-1}) in hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with 1% PBS-BSA or calf serum for 30 min to minimize nonspecific binding of the primary antibody. Pilot experiments performed to determine the optimal antibody dilution showed that some samples needed to be pretreated by boiling in 10 mM citrate buffer for 30 min. Sections were then incubated overnight at 4 °C with the following antibodies: monoclonal mouse anti-human chromogranin A (1:800; Thermo Scientific), to assess the number of

enteroendocrine cells in epithelium per 30 villi, monoclonal mouse anti-rat CD163 (1:100; AbD Serotec, Oxford, UK), as a marker of activated macrophages, and monoclonal mouse anti-human Ki67 (1:600; Novocastra, Newcastle, UK), as a proliferation marker (38). After incubation, samples were washed with PBS-Tween. The peroxidase-based kit Masvision (Master Diagnostica, Granada, Spain) was used as secondary antibody. Samples were counterstained with hematoxylin and coverslips mounted with Eukitt mounting media (O. Kindler GmbH & Co, Freiburg, Germany). To determine the level of non-specific staining, the preparations were incubated without the primary antibody.

Compounds and drugs

Barium sulfate (Barigraf® AD, Juste SAQF, Madrid, Spain) was suspended in tap water and continuously hand-stirred until administration. Charcoal, gum Arabic, 5-FU and WIN 55,212-2 were purchased from Sigma-Aldrich (Spain). 5-FU and WIN 55,212-2 were suspended in saline (sonicated for about 1.5 h) and Tocrisolve, respectively (Tocris, Cookson, Bristol, UK).

Statistical analysis

Data are presented as the mean values ± SEM. Differences were analyzed using Student´s t-test with Welch's correction where appropriate, or one- or two-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test. Severity of diarrhea was evaluated using χ^2. Values of $p<0.05$ were considered significantly different.

RESULTS

As shown in Fig. 1, 5-FU significantly reduced body weight gain and food intake, but it did not significantly modify water intake. WIN alone did not significantly modify any of those parameters, and did not further significantly alter the values obtained in 5-FU treated rats.

Gastrointestinal motility study

Compared to control animals, 5-FU (1st dose, 150 mg kg^{-1}) delayed gastric emptying on day 1, the difference being significant 6 and 8 h after contrast. No significant alterations of the motility curves were observed for small intestine, caecum or colorectum (Fig. 2A). These results were confirmed also in the morphometric analysis for the stomach and caecum (Fig. 2B). WIN (at 0.5 mg/kg, which in the invasive study was effective to reduce upper gastrointestinal transit, see Fig. 2 – Supplementary Material) did not significantly alter any of these parameters in control or 5-FU-treated animals. The only exception was that in WIN+5-FU-treated animals the stomach size remained practically unaltered from 0 to 8 h after contrast, whereas in vehicle+5-FU-treated rats the stomach size decreased a bit, the difference between these groups being statistically significant at T4 (Fig. 2A and 2B). Representative images of the different treatments, taken 8 h after barium, can be seen in Fig. 2C.

On experimental day 4 (2 days after the 2nd dose of 5-FU, cumulative dose 300 mg kg^{-1}), not only gastric motility, but also small intestinal and colorectal motility were altered by the antineoplastic drug. Thus, in 5-FU-treated rats, gastric emptying was significantly delayed, emptying of small intestine was significantly slower and motility

in colorectum was also significantly delayed. WIN did not significantly modify these curves obtained with our semiquantitative score system either in saline- or 5-FU-treated rats (Fig. 3A).

Interestingly, on day 4, the size of the stomach immediately after contrast was significantly lower than on day 1 in the groups of animals that received 5-FU (Fig. 3 – Supplementary Material). Throughout the experiment on this day, the change in size of stomach and caecum was similar to that in control animals (Fig. 3B). However, the caecum of the animals treated with 5-FU did not fill homogeneously with contrast: instead of spreading throughout the whole organ, barium accumulated in some area of it, and it was to some extent difficult to define the organ edges, compared to those in saline-treated rats, with or without WIN (Fig. 3C). The quantitative analysis of the proportion of the organ intensely filled with barium showed that there was a significant decrease in this parameter in 5-FU-treated rats (Fig. 3B'). Once again, WIN did not alter the results in this analysis either in saline- or 5-FU-treated rats.

Afterwards, X-rays were evaluated to more specifically analyze diarrhea. Thus, we categorized diarrhea as mild or severe and determined the % of animals in each group (vehicle+5-FU and WIN+5-FU) that had diarrhea (mild + severe) or severe diarrhea (Fig. 4A). As shown in Fig. 4A', WIN tended to reduce the % of animals with diarrhea, particularly severe diarrhea, although the difference did not reach statistical significance. Animals treated with saline instead of 5-FU showed no signs of diarrhea.

Finally, a further analysis was performed after realizing that on day 4 some barium contrast given on day 1 was still present in the small and large intestine of 5-FU-treated animals in X-rays taken immediately after contrast administration (T0) (Fig.

4B). This barium looked as “shadows” within each intestinal region and therefore we valued its presence and compared the results for the rats treated with vehicle+5-FU or with WIN+5-FU. As shown in Fig. 4B’, the presence of barium within the intestines of animals treated with WIN+5-FU was significantly higher than that remaining in animals treated with vehicle+5-FU. “Shadows” were not found in any animal receiving vehicle+saline or vehicle+WIN (see a representative image of a saline-treated rat at T0 in Fib. 4B).

Histopathological analysis

The histological pattern in HE stained sections of the stomach is shown in Fig. 4 – Supplementary Material (A-D). Compared to control animals (fig. 4SA), damage was observed in the fundus area after 5-FU treatment (Fig. 4SB), with the typical keratinized epithelium being disorganized, vacuolated and infiltrated with lymphocytes up to the muscular layer. Treatment with WIN did not modify these results, with the animals treated with WIN alone being similar to the saline group and the ones treated with 5-FU and WIN similar to 5-FU alone (not shown). In the same way, 5-FU produced apical damage of the gastric glands at the body area (Fig. 4SC for control and 4SD for 5-FU-treated rats). Again, WIN administration did not induce any further effect when used alone or together with 5-FU (not shown).

Regarding the small intestine, there was a clear damage caused by 5-FU, alone or with WIN, with hypertrophy of crypts and lymph vessels within the villi (Fig. 5A-D), and lymph nodules occupying all the gut wall thickness (not shown). In fact, 5-FU evoked statistically significant structural changes in the intestinal wall (Fig. 5E). More specifically, villi height and the number of enterocytes per villus significantly

decreased in 5-FU-treated animals (Fig. 6A-B). In contrast, the populations of goblet (Fig. 6C) and enteroendocrine cells (Fig. 6D) did not significantly change (Fig. 5SA-A' and Fig. 5SB-B' show representative images for PAS staining and immunohistochemistry for chromogranin A, in vehicle+saline- and vehicle+5-FU-treated animals). Regarding the non-mucosa components of the gut wall, submucosa thickness significantly increased in 5-FU-treated animals (Fig. 6E). In the same way, muscle layer thickness also increased with 5-FU treatment both in the circular (Fig. 6F) and the longitudinal layers (Fig. 6G). Immunohistochemistry with Ki67 antibody to detect proliferating cells confirmed the damage caused by 5-FU (Fig. 6SA-D). WIN treatment did not exert any significant effect on ileum structure, neither alone nor in combination with 5-FU (Fig. 6).

The histological structure of the colon is shown in Fig. 7 (A-E). Damage was clear after treatment with 5-FU (Fig. 7B) and 5-FU+WIN (Fig. 7D); ulcers and damage in mucosal architecture were evident (Fig. 7B and 7D), and large Peyer´s patches were also clearly seen (not shown). Both elements, namely mucosa damage and lymphatic nodules proliferation, contributed to the detrimental effect caused by 5-FU shown in the quantitative analysis (Fig. 7E).

Finally, an immunohistochemical study of the presence of activated macrophages (using anti-CD163 antibody) was performed in both ileum and colon. As seen in Fig. 8, macrophage infiltration significantly increased after 5-FU treatment in ileum (but not colon), and WIN did not significantly modify the results obtained in saline- or 5-FU-treated rats.

DISCUSSION

Here we asked if cannabinoids might be useful to prevent the development of chemotherapy-induced diarrhea (CID). For this study, in rats, we used the antineoplastic drug 5-FU, and the non-selective cannabinoid agonist WIN, at a non-psychoactive dose. Besides weight gain loss and food intake reduction, 5-FU induced gastrointestinal dysmotility and diarrhea, which could be observed *in vivo* by radiographic means, as well as mucositis and other changes in gut wall structure. WIN did not prevent mucositis, but tended to reduce diarrhea induced by 5-FU, suggesting that cannabinoids might indeed be useful to prevent and/or treat this debilitating condition.

General health parameters

In agreement with other studies in experimental animals, 5-FU reduced body weight and food intake (34,39). This may be explained by the concomitant reduction in food intake, but other factors could also contribute. An increase in energy expenditure does not seem likely to be involved, because chemotherapy usually induces fatigue and reduces locomotor activity (40,41). In addition, tissues involved in metabolic use of the nutrients absorbed, such as the liver, might be affected by chemotherapy (42), although these possibilities were not specifically addressed here. In contrast, we observed diarrhea, which may favor malnutrition and dehydration, contributing to weight gain reduction. Dehydration might have triggered an increase in water intake, but this parameter did not significantly change. The occurrence of mucositis may have contributed to gastrointestinal dysmotility and diarrhea (see below), but also to gastric dysmotility, which, in turn, may have contributed to reduce food intake.

Importantly, WIN, alone or with 5-FU, did not significantly alter any of the general health parameters measured, probably due to the low dose used (19,20,21).

Effects on the stomach

In our radiographic analysis, the first dose of 5-FU delayed gastric emptying. This might be related to nausea and emesis occurring during 5-FU chemotherapy (4,6). Although apoptosis, the first process in mucositis development, occured in the crypts from mouse ileum only 6 hours after 5-FU (43), gastric dysmotility at this time-point was probably not due to established mucositis, which requires more time to occur. Cisplatin-induced nausea and emesis (as well as gastric dysmotility and distension), involve serotonin release from enterochromaffin cells (44), and thus these effects are sensitive to 5-HT_3 antagonists (45,46). Delayed gastric emptying observed here 6-8 hours after 5-FU might as well involve serotonin release, since plasma serotonin was significantly increased 24 h after the administration of a dose of 50 mg kg^{-1} 5-FU in mice (47), although, in contrast with cisplatin, maybe as a response to the production of inflammatory cytokines (48,49).

On experimental day 4 (2 days after the second 5-FU administration), delayed gastric emptying was more apparent, although this did not involve gastric distension. Gastric dysmotility on day 4 might reflect the toxic consequences of 5-FU administration. Soares et al. (30) described, also in rats, delayed gastric emptying and intestinal transit of liquids that outlasted mucosal inflammation resolution. Their *in vitro* assays revealed hypercontractility of the deep muscle of the stomach and duodenum 3 and 15 days after a single dose of 5-FU (150 mg/kg), corresponding to the inflammatory and post-inflammatory phases of mucositis, respectively. In addition, in cultured

smooth muscle cells, 5-FU inhibited cell proliferation, induced apoptosis, and promoted changes in the cellular and nuclear morphology (50). Possibly other components of the gastric wall, which was damaged by 5-FU treatment both in fundus and body (see Fig. 4, Supplementary material), or its extrinsic innervation, may also be altered, as has been shown for isolated gastric preparations from cisplatin-treated patients (51).

WIN had little effect on gastric motor function, either in control or 5-FU-treated animals, on day 1 or day 4. This was expected since low WIN doses, devoid of central effects (namely catalepsy), did not alter gastric emptying or size in previous radiographic studies (19,20,21).

Effects on the small and large intestine

The effects of 5-FU or WIN, alone or combined, on small or large intestinal motor function, were negligible on experimental day 1. Thus, 5-FU does not seem to induce any "acute" effect that may modify intestinal motor function in the few hours after its administration. This was also found after the first dose of cisplatin (28, 52,53). WIN at 0.5 mg kg^{-1} significantly reduced upper gastrointestinal motor function in an invasive study (see Fig. 2S), and tended to delay emptying of small intestine and arrival of barium to caecum and colorectum, as seen in previous studies (19,20,21).

Gastrointestinal mucositis is most prominent in the small intestine, but occurs also elsewhere in the gut (6). On day 4, typical features of mucositis were evident upon histological examination, including reduced villi height, reduced numbers of enterocytes/villus and proliferating cells in the crypts, as well as increased infiltration of activated macrophages in ileum (30,54,55).

Radiographically, emptying of small intestine on day 4 was significantly delayed but arrival of barium to caecum (which may reflect small intestinal transit) was not. Upper gastrointestinal transit, invasively measured (30), was altered 3 days after 5-FU (150 mg/kg), with delayed gastric emptying (see above), but accelerated small intestinal transit and duodenal hypercontracitility (which was more intense after mucositis resolution) in organ bath studies. These effects may explain why arrival of barium to the caecum was not altered. Vacuolization and neutrophil infiltration (30) might have contributed to an increased thickness of the muscle layers in the small intestine (present study). In addition to inflammation-related effects on the muscle (and maybe other motor components), direct actions of 5-FU on the smooth muscle cells (50) could also contribute to accelerated transit and small intestinal hypercontractility (present study, 30).

Interestingly, in 5-FU-treated rats, barium did not distribute homogeneously within the caecum, and required much longer time to reach the colorectum. This uneven distribution of barium within the caecum may be due to fluid accumulation, excessively produced in the small intestine after 5-FU treatment. In fact, the rat caecum functions as a reservoir in conditions of small intestinal hypersecretion, and the cecectomized rat was suggested to be a good model of diarrhea (56). Other factors including dysbiosis, already described in 5-FU-induced mucositis (8,57), and altered contractility, also likely in this intestinal organ, may have contributed to delayed arrival of barium to colorectum in 5-FU-treated rats. The contribution of all these factors will be specifically analyzed in future studies.

Once reached by barium, maximal filling of colorectum was much lower than in control animals. This may be due to the paucity of barium arrival, but also to the presence of diarrhea, which consequently interfered with adequate formation of fecal

pellets and avoided barium to remain for a long time in this organ. Moderate to severe diarrhea was radiographically observable in 5-FU-treated animals, in similar proportions as previously found for similar doses in rats (33). 5-FU-induced diarrhea might reflect higher water content within the intestines, due to increased secretion and/or reduced absorption, associated to mucositis, but altered motor function might have also contributed. In fact, permanence within the intestines at T0 of barium administered on day 1 ("shadows") in 5-FU-treated rats (but not in saline-treated animals), suggests the antineoplastic drug altered intestinal motor function even before day 4, at least in some animals. *In vitro* experiments in mice also suggest that contractility and peristalsis of colorectum are altered after 5-FU treatment (58).

In spite of the low, non-psychoactive dose of WIN used in this study, which did not alter gastrointestinal motor function *per se* and did not prevent most effects induced by 5-FU, including mucositis and macrophage infiltration, the cannabinoid reduced the incidence of diarrhea, particularly severe diarrhea, in 5-FU-treated animals. Cannabinoids have been able to reduce diarrhea associated to many other inflammatory conditions of the colon, through activation of both CB_1 and CB_2 receptors (22,23,24,25,26). Although clinical evidence is still lacking, it has already been suggested that the antidiarrheal cannabinoid effects might be useful during chemotherapy (59). This is the first research addressing this possibility in experimental animals. Future work will ascertain the mechanisms involved.

The increased presence of "shadows" on day 4 from barium given on day 1 in animals treated with WIN+5-FU, compared to those treated with 5-FU only, suggests an anti-motility effect of the cannabinoid, even at this low dose, which might be due to an increased expression of CB_1 receptor, as was found in other gut inflammation models (60), whereas epithelial permeability might not be modified, at least not by a

direct CB_2-mediated mechanism (27). More research is needed to determine the exact mechanisms of the possible antidiarrheal effect of cannabinoids in chemotherapy-treated animals.

Concluding remarks

The effects of 5-FU on gastrointestinal motility have been characterized by radiographic means. Delayed gastric emptying, altered caecum motor function and diarrhea are present during the inflammatory phase of 5-FU toxicity (mucositis).

The cannabinoid agonist WIN, at a low dose, seemed to exert an antidiarrheal effect. New experiments will determine the receptor involved and whether other cannabinoid drugs, higher doses or other patterns of administration, alone or together with other drugs may be more useful to reach complete protection against diarrhea and, hopefully, against mucositis associated to chemotherapy.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

CONTRIBUTIONS

RA and JAU designed the study and wrote the manuscript. RA, IPG, RG and GV performed the functional experiments. IPG and RdA performed the X-ray and histological analyses, respectively. AELP performed the morphometric analysis. MIMF contributed essential intellectual input and financial support. All authors read and accepted the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. Effect of 5-FU on general health parameters in the rat. Rats received WIN (0.5 mg kg^{-1} day^{-1}, 4 consecutive days, ip) or its vehicle (Veh, 0.5 mL), followed by 5-fluorouracil (5-FU, 150 mg kg^{-1} day^{-1}, 2 consecutive days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}) or saline (2.5 mL). Thus, the following 4 groups were used: Veh+Saline (control, *n* = 8); Veh+5-FU (*n* = 12); WIN+Saline (*n* = 4); WIN+5-FU (*n* = 8). Body weight gain (A), food intake (B) and water intake (C) were recorded at the end of the 4 experimental days. Data represent mean ± SEM. **p*<0.05, ****p*<0.001 *vs* control (one-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test).

Figure 2. Effect of 5-FU on GI motor function in the rat – day 1. Gastrointestinal motor function was evaluated by radiological methods (see text). Rats received WIN (0.5 mg kg^{-1} day^{-1}, 4 days, ip) or its vehicle (Veh, 0.5 mL), followed by 5-FU (150 mg kg^{-1} day^{-1}, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}) or saline (2.5 mL). Thus, the following 4 groups were used: Veh+Saline (*n* = 8); Veh+5-FU (*n* = 12); WIN+Saline (*n* = 4); WIN+5-FU (*n* = 8). Twenty min after the first dose of 5-FU or saline, barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered and X-rays obtained 0-8 h after contrast. A: Semiquantitative analysis of motility in the stomach, small intestine, caecum and colorectum. B: Morphometric analysis of the stomach and caecum sizes. Data represent mean ± SEM. **p*<0.05, ****p*<0.001 *vs* control (two-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test). C: representative images of animals of the 4 treatment groups, 8 h after contrast administration. Scale bar: 30 mm.

Figure 3. Effect of 5-FU on GI motor function in the rat – day 4. Gastrointestinal motor function was evaluated by radiological methods (see text). Rats received WIN (0.5 mg kg^{-1} day^{-1}, 4 days, ip) or its vehicle (Veh, 0.5 mL), followed by 5-FU (150 mg kg^{-1} day^{-1}, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}) or saline (2.5 mL). Thus, the following 4 groups were used: Veh+Saline (*n* = 8); Veh+5-FU (*n* = 12); WIN+Saline (*n* = 4); WIN+5-FU (*n* = 8). On day 4 (2 days after the second dose of 5-FU or saline), 20 min after the fourth dose of WIN or vehicle, barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered and X-rays obtained 0-8 h after contrast. A: Semiquantitative analysis of motility in the stomach, small intestine, caecum and colorectum. B: Morphometric analysis of the stomach and caecum sizes. B': Proportion of the caecum intensely stained with barium contrast 8 h after barium administration. Data represent mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001 *vs* control (one- or two-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test; for more clarity, in A and B symbols are only shown for WIN+5-FU but the same statistical significance was found for Veh+5-FU). C: representative images of animals of the 4 treatment groups, 8 h after contrast administration. Scale bar: 30 mm.

Figure 4. Specific radiographic analysis of 5-FU-induced diarrhea and effect of WIN. Rats received WIN (0.5 mg kg^{-1} day^{-1}, 4 days, ip) or its vehicle (Veh, 0.5 ml), followed by 5-FU (150 mg kg^{-1} day^{-1}, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}) or saline (2.5 ml). Thus, the following 4 groups were used: Veh+Saline (*n* = 8); Veh+5-FU (*n* = 12); WIN+Saline (*n* = 4); WIN+5-FU (*n* = 8). On day 1 (after the first dose of 5-FU) and 4 (2 days after the second dose of 5-FU or saline, 20 min after the fourth dose of WIN or vehicle), barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered and X-rays obtained 0-8 h after contrast. A:

Representative images of mild (upper panel) and severe (lower panel) diarrhea; in mild diarrhea the colon seems to contain both liquid and fecal pellets, whereas in severe diarrhea only liquid is seen in the colon. A': % of rats showing diarrhea (mild+severe, upper panel) or only severe diarrhea (lower panel) on the X-rays taken on experimental day 4; only 5-FU-treated animals were considered (none of saline-treated animals showed diarrhea on X-rays); data were statistically evaluated by means of χ^2 (although $p>0.05$ in both cases, a tendency to a reduction of diarrhea, particularly severe diarrhea, was noted) B: Representative X-rays obtained from control (left panel) or 5-FU-treated animals (right panel) on day 4, immediately after intragastric contrast administration. B': Quantitative analysis, immediately after intragastric administration (T0), of the intestinal barium given on day 1 still remaining within the gut on day 4 (radiopaque "shadows"); only 5-FU-treated rats were considered (none of saline-treated animals showed shadows on day 4 at T0), co-treated with either WIN or its vehicle; data represent mean ± SEM, $^{**}p<0.05$ *vs.* Veh+5-FU (Student's t-test).

Figure 5. Effect of 5-FU on the general structure of the rat ileum. Histological samples were obtained on experimental day 4 and embedded in paraffin sections. A: Tissue sample from control animals treated with Vehicle+Saline (0.5 and 2.5 mL, respectively). B: Sample from an animal treated with Vehicle+5-FU (5-FU: 150 mg kg^{-1} day^{-1}, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}). C: Ileum from a rat that received WIN+Saline (WIN: 0.5 mg kg^{-1} day^{-1}, 4 days, ip). D: Sample from an animal injected with WIN+5-FU. Bar: 100 μm. (E) Quantitative analysis. Bars show mean values ± SEM for organ damage: control (white), vehicle+5-FU (red), WIN+Saline (green) and WIN+5-FU-treated animals (black). Each group consisted of

6 rats. *p<0.05, **p<0.01 *vs.* control (one-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test).

Figure 6. Quantitative analyses of the effect of 5-FU on specific structural features of the rat ileum. Bars show mean values ± SEM for distinct parameters. A: Villi height. B: Number of enterocytes/villus. C: % goblet cells. D: Number of enteroendocrine epithelial cells. E: Submucosa thickness. F: Circular muscle thickness. G: Longitudinal muscle thickness. Animals were treated with Vehicle+Saline (0.5 and 2.5 mL, respectively, white), Vehicle+5-FU (5-FU: 150 mg kg^{-1} day^{-1}, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}, red), WIN+Saline (WIN: 0.5 mg kg^{-1} day^{-1}, 4 days, ip, green) or 5-FU+WIN (black). Each group consisted of 6 rats. *p<0.05, **p<0.01, ***p <0.001 *vs.* Vehicle+Saline (one-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test).

Figure 7. Effect of 5-FU on the general structure of the rat colon. Histological samples embedded in paraffin sections. A: Tissue sample from a control animal treated with Vehicle+Saline (0.5 and 2.5 mL, respectively). B: Sample from an animal treated with Vehicle+5-FU (5-FU: 150 mg kg^{-1} day^{-1}, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}). C: Colon from a rat that received WIN+Saline (WIN: 0.5 mg kg^{-1} day^{-1}, 4 days, ip). D: Sample from an animal injected with 5-FU+WIN. Bar: 100 μm. E: Quantitative analysis. Bars show mean values ± SEM for organ damage; control (Vehicle+Saline, white), Vehicle+5-FU (red), WIN+Saline (green) and 5-FU+WIN-treated animals (black). Each group consisted of 6 rats. *p<0.05 *vs.* control (one-way ANOVA followed by *post-hoc* Bonferroni multiple comparison test).

Figure 8. Effect of 5-FU on activated macrophage infiltration in rat ileal and colonic tissues. Animals were treated with Vehicle+Saline (0.5 and 2.5 mL,

respectively), Vehicle+5-FU (5-FU: 150 mg kg^{-1} day^{-1}, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}), WIN+Saline (WIN: 0.5 mg kg^{-1} day^{-1}, 4 days, ip) or 5-FU+WIN. Histological samples were embedded in paraffin and stained with anti-CD163 antibody. A, B: representative images of ileal and colonic tissues from 5-FU treated rats showing activated macrophage infiltration (encircled); scale bar= 50 μm. A', B': quantitative analysis of activated macrophage infiltration. Bars show mean number ± SEM of macrophages per field 40x. Each group consisted of 4-6 rats and at least 5 fields of view per animal were evaluated. **p<0.01, ***p<0.001 *vs.* control (Student's *t*-test with Welch's correction where appropriate).

FIGURE LEGENDS - SUPPLEMENTARY MATERIAL

Figure 1 – SUPPLEMENTARY MATERIAL. Experimental protocol. In this study, 4 experimental groups were used (*n* = 4-12, as shown in the figure). For 4 experimental days, male Wistar rats received an ip injection of vehicle (1.6 mL kg^{-1}) or the non-selective cannabinoid agonist WIN (0.5 mg kg^{-1} day^{-1}). On the first two days, 20 min after WIN injection, the rats received also saline (8.3 mL kg^{-1}) or the antitumoral drug 5-fluorouracil (5-FU, 150 mg kg^{-1} day^{-1}). Weight gain and food and water intake were recorded throughout the experiment. Radiographic analysis of gastrointestinal motility was performed on days 1 and 4 after intragastric contrast administration (2.5 mL barium sulfate, 2 g mL^{-1}). Histological analysis of gut wall structure was performed on day 4 in a parallel group of rats.

Figure 2 – SUPPLEMENTARY MATERIAL. Effects of WIN on upper gastrointestinal motor function measured invasively by the charcoal method. Rats were fasted overnight. Thereafter, they received an intraperitoneal (i.p.) injection of WIN at 0.5 mg kg^{-1} (n=6) or its vehicle (n=6, 0.5 mL). Twenty min after, they received 1 ml of a 10% (w v^{-1}) charcoal suspension in a 5% (w v^{-1}) gum Arabic solution via an orogastric cannula. After 20 min, the gastrointestinal tract was removed *en bloc*. Upper gastrointestinal transit, measured as the % of the small intestine travelled by charcoal front (A), and stomach weight (B) were recorded. Data represent mean ± SEM. **p*<0.05 *vs* control (Student's *t*-test).

Figure 3 – SUPPLEMENTARY MATERIAL. Effect of 5-FU administration on stomach size in the rat – day 1 *vs*. day 4. Gastrointestinal motor function was evaluated by radiological methods (see text). Rats received WIN (0.5 mg kg^{-1} day^{-1}, 4 days, ip) or its vehicle (Veh, 0.5 mL), followed by 5-FU (150 mg kg^{-1} day^{-1}, 2 days,

starting on day 1, ip, cumulative dose of 300 mg kg^{-1}) or saline (2.5 mL). Thus, the following 4 groups were used: Veh+Saline (*n* = 8); Veh+5-FU (*n* = 12); WIN+Saline (*n* = 4); WIN+5-FU (*n* = 8). On days 1 (20 min after the first dose of 5-FU or saline) and 4 (2 days after the second dose of 5-FU or saline, 20 min after the fourth dose of WIN or vehicle), barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered and X-rays obtained immediately after contrast (T0). The stomach size was morphometrically analyzed on both day 1 (solid bars) and day 4 (dotted or striped bars). Data represent mean ± SEM. **p*<0.05, ***p*<0.01 *vs* control (Student's *t*-test).

Figure 4 – SUPPLEMENTARY MATERIAL. Effect of 5-FU treatment on the rat stomach. Histological samples embedded in paraffin. Left (A, C): tissue samples from control animals treated with saline (8.3 mL kg^{-1}). Right (B, D): tissue samples from animals injected with 5-FU (150 mg kg^{-1} day^{-1}, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}). A-B: General view of the stomach fundus showing epithelial damage in the treated group. C-D: Stomach body; note gland damage in the treated group. Bar 100 μm.

Figure 5 – SUPPLEMENTARY MATERIAL. Effect of 5-FU treatment on goblet and enteroendocrine cells in the rat ileum. Ileal histological samples were embedded in paraffin. The number of goblet cells per villi was counted after PAS staining (A, A') and the number of enteroendocrine cells was counted after immunohistochemistry for chromogranin A (B, B'). A, B: Tissue samples from control animals treated with Vehicle+Saline (0.5 and 2.5 mL, respectively). A', B': Samples from animals treated with Vehicle+5-FU (5-FU: 150 mg kg^{-1} day^{-1}, 2 days, ip, cumulative dose of 300 mg kg^{-1}). Examples of enteroendocrine epithelial cells immunoreactive to chromogranin A are encircled in B and B'. Bar: 100 μm.

Figure 6 – SUPPLEMENTARY MATERIAL. Effect of 5-FU and WIN treatment on proliferating cells of the rat small intestinal mucosa. Histological samples embedded in paraffin and stained with the Ki67 antibody. A: Tissue sample from a control animal treated with Vehicle+Saline (0.5 and 2.5 mL, respectively). B: Sample from an animal treated with Vehicle+5-FU (5-FU: 150 mg kg^{-1} day^{-1}, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg^{-1}). C: Ileum from a rat that received WIN+Saline (WIN: 0.5 mg kg^{-1} day^{-1}, 4 days, ip). D: Sample from an animal injected with 5-FU+WIN. Bar: 100 μm.

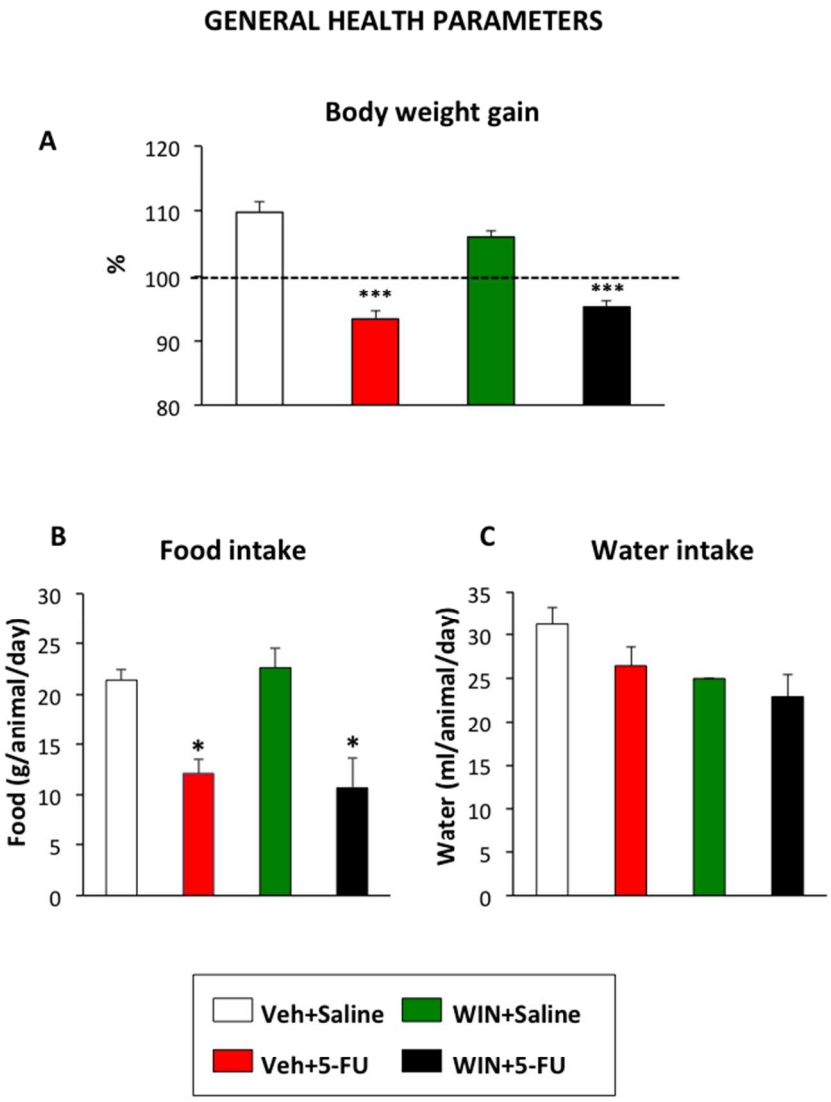

Figure 1. Effect of 5-FU on general health parameters in the rat. Rats received WIN (0.5 mg kg-1 day-1, 4 consecutive days, ip) or its vehicle (Veh, 0.5 mL), followed by 5-fluorouracil (5-FU, 150 mg kg-1 day-1, 2 consecutive days, starting on day 1, ip, cumulative dose of 300 mg kg-1) or saline (2.5 mL). Thus, the following 4 groups were used: Veh+Saline (control, n = 8); Veh+5-FU (n = 12); WIN+Saline (n = 4); WIN+5-FU (n = 8). Body weight gain (A), food intake (B) and water intake (C) were recorded at the end of the 4 experimental days. Data represent mean ± SEM. *p<0.05, ***p<0.001 vs control (one-way ANOVA followed by post-hoc Bonferroni multiple comparison test).

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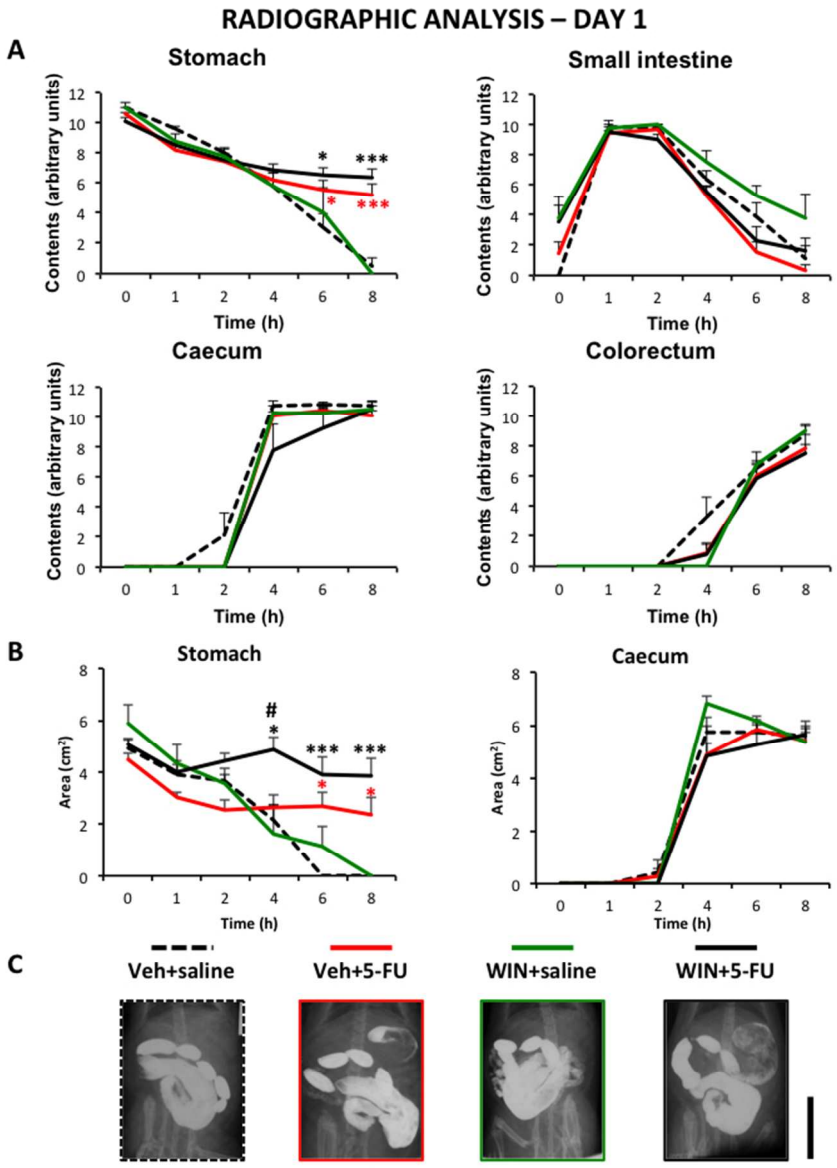

Figure 2. Effect of 5-FU administration on GI motor function in the rat - day 1. Gastrointestinal motor function was evaluated by radiological methods (see text). Rats received WIN (0.5 mg kg-1 day-1, 4 days, ip) or its vehicle (Veh, 0.5 mL), followed by 5-FU (150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1) or saline (2.5 mL). Thus, the following 4 groups were used: Veh+Saline (n = 8); Veh+5-FU (n = 12); WIN+Saline (n = 4); WIN+5-FU (n = 8). Twenty min after the first dose of 5-FU or saline, barium sulfate (2.5 mL, 2 g mL-1) was intragastrically administered and X-rays obtained 0-8 h after contrast. A: Semiquantitative analysis of motility in the stomach, small intestine, caecum and colorectum. B: Morphometric analysis of the stomach and caecum sizes. Data represent mean ± SEM. *p<0.05, ***p<0.001 vs control (two-way ANOVA followed by post-hoc Bonferroni multiple comparison test). C: representative images of animals of the 4 treatment groups, 8 h after contrast administration. Scale bar: 30 mm.

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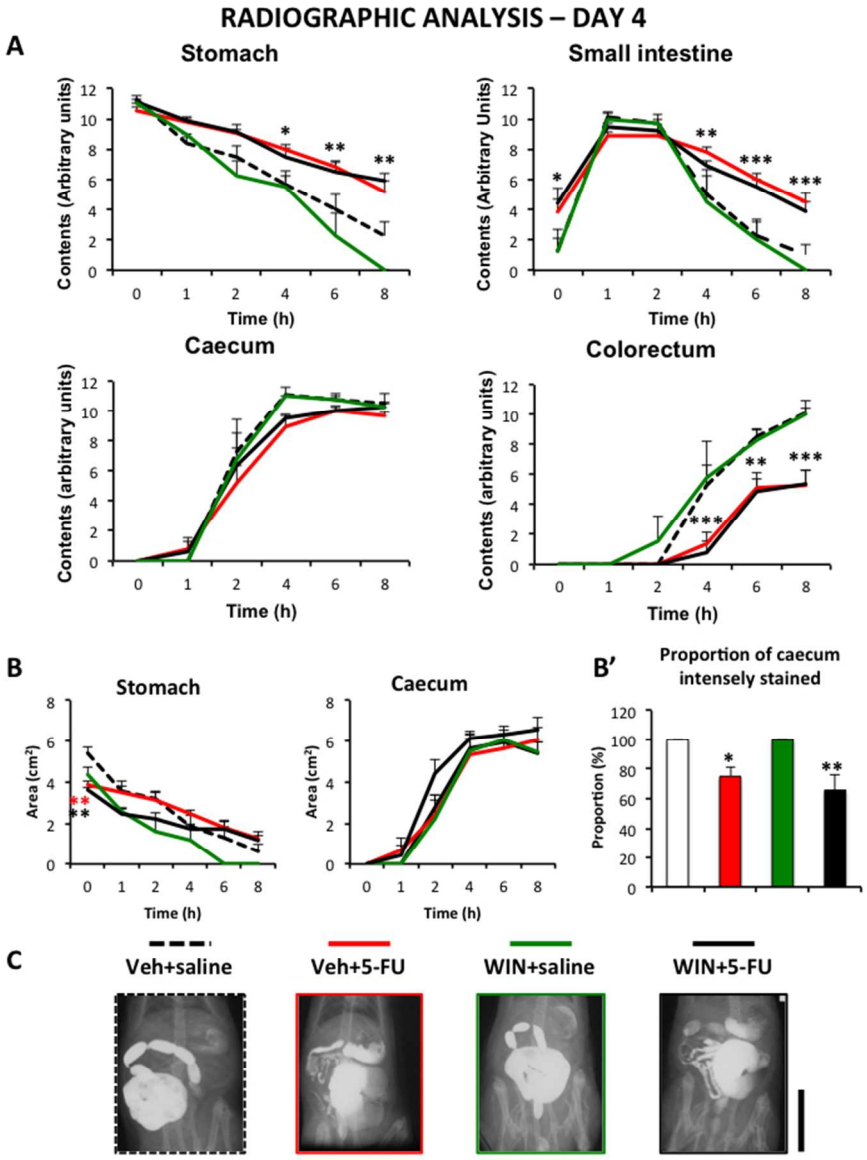

Figure 3. Effect of 5-FU administration on GI motor function in the rat - day 4. Gastrointestinal motor function was evaluated by radiological methods (see text). Rats received WIN (0.5 mg kg-1 day-1, 4 days, ip) or its vehicle (Veh, 0.5 mL), followed by 5-FU (150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1) or saline (2.5 mL). Thus, the following 4 groups were used: Veh+Saline (n = 8); Veh+5-FU (n = 12); WIN+Saline (n = 4); WIN+5-FU (n = 8). On day 4 (2 days after the second dose of 5-FU or saline), 20 min after the fourth dose of WIN or vehicle, barium sulfate (2.5 mL, 2 g mL-1) was intragastrically administered and X-rays obtained 0-8 h after contrast. A: Semiquantitative analysis of motility in the stomach, small intestine, caecum and colorectum. B: Morphometric analysis of the stomach and caecum sizes. B': Proportion of the caecum intensely stained with barium contrast 8 h after barium administration. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs control (one- or two-way ANOVA followed by post-hoc Bonferroni multiple comparison test; for more clarity, in A and B symbols are only shown for WIN+5-FU but the same statistical significance was found for Veh+5-FU). C: representative images of animals of the 4 treatment groups, 8 h after contrast administration. Scale bar: 30 mm.

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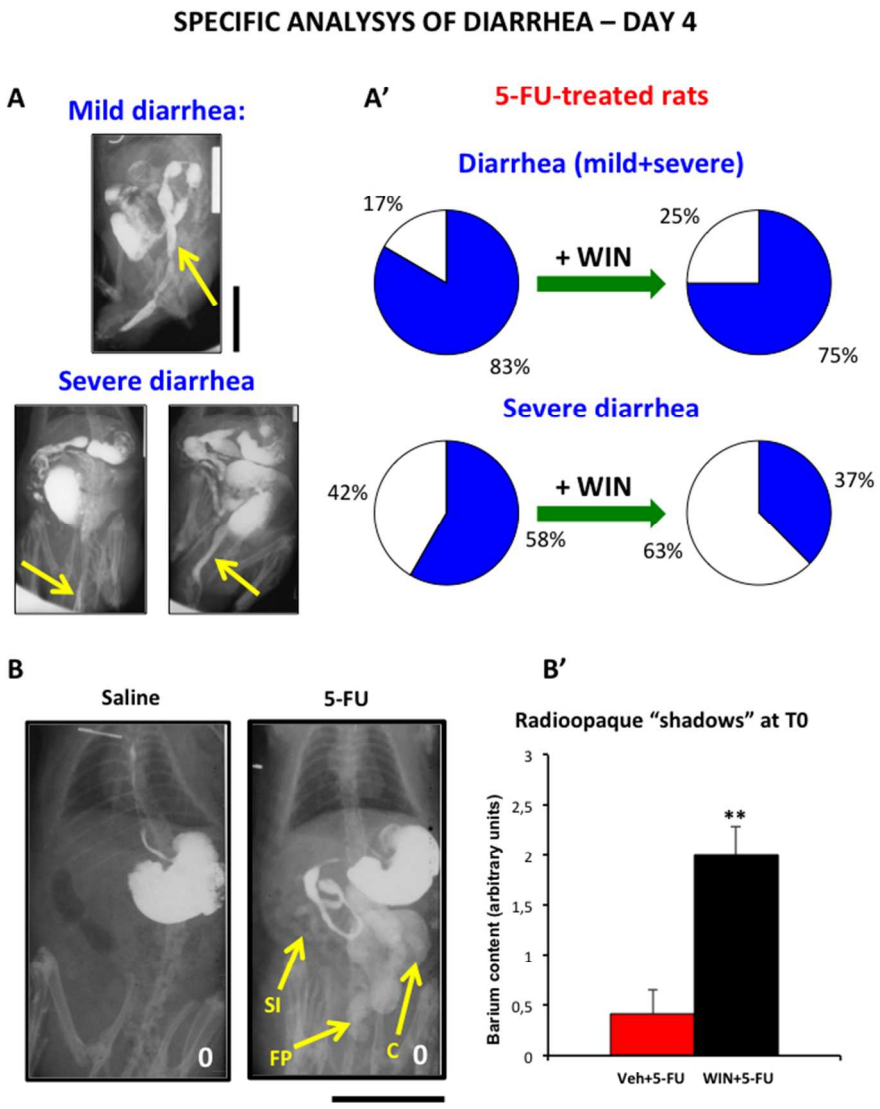

Figure 4. Specific radiographic analysis of 5-FU-induced diarrhea and effect of WIN. Rats received WIN (0.5 mg kg-1 day-1, 4 days, ip) or its vehicle (Veh, 0.5 ml), followed by 5-FU (150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1) or saline (2.5 ml). Thus, the following 4 groups were used: Veh+Saline (n = 8); Veh+5-FU (n = 12); WIN+Saline (n = 4); WIN+5-FU (n = 8). On day 1 (after the first dose of 5-FU) and 4 (2 days after the second dose of 5-FU or saline, 20 min after the fourth dose of WIN or vehicle), barium sulfate (2.5 mL, 2 g mL-1) was intragastrically administered and X-rays obtained 0-8 h after contrast. A: Representative images of mild (upper panel) and severe (lower panel) diarrhea; in mild diarrhea the colon seems to contain both liquid and fecal pellets, whereas in severe diarrhea only liquid is seen in the colon. A': % of rats showing diarrhea (mild+severe, upper panel) or only severe diarrhea (lower panel) on the X-rays taken on experimental day 4; only 5-FU-treated animals were considered (none of saline-treated animals showed diarrhea on X-rays); data were statistically evaluated by means of ∩2 (although p>0.05 in both cases, a tendency to a reduction of diarrhea, particularly severe diarrhea, was noted) B: Representative X-rays obtained from control (left panel) or 5-FU-treated animals (right panel) on

day 4, immediately after intragastric contrast administration. B': Quantitative analysis, immediately after intragastric administration (T0), of the intestinal barium given on day 1 still remaining within the gut on day 4 (radiopaque "shadows"); only 5-FU-treated rats were considered (none of saline-treated animals showed shadows on day 4 at T0), co-treated with either WIN or its vehicle; data represent mean ± SEM, **p<0.05 vs. Veh+5-FU (Student's t-test).

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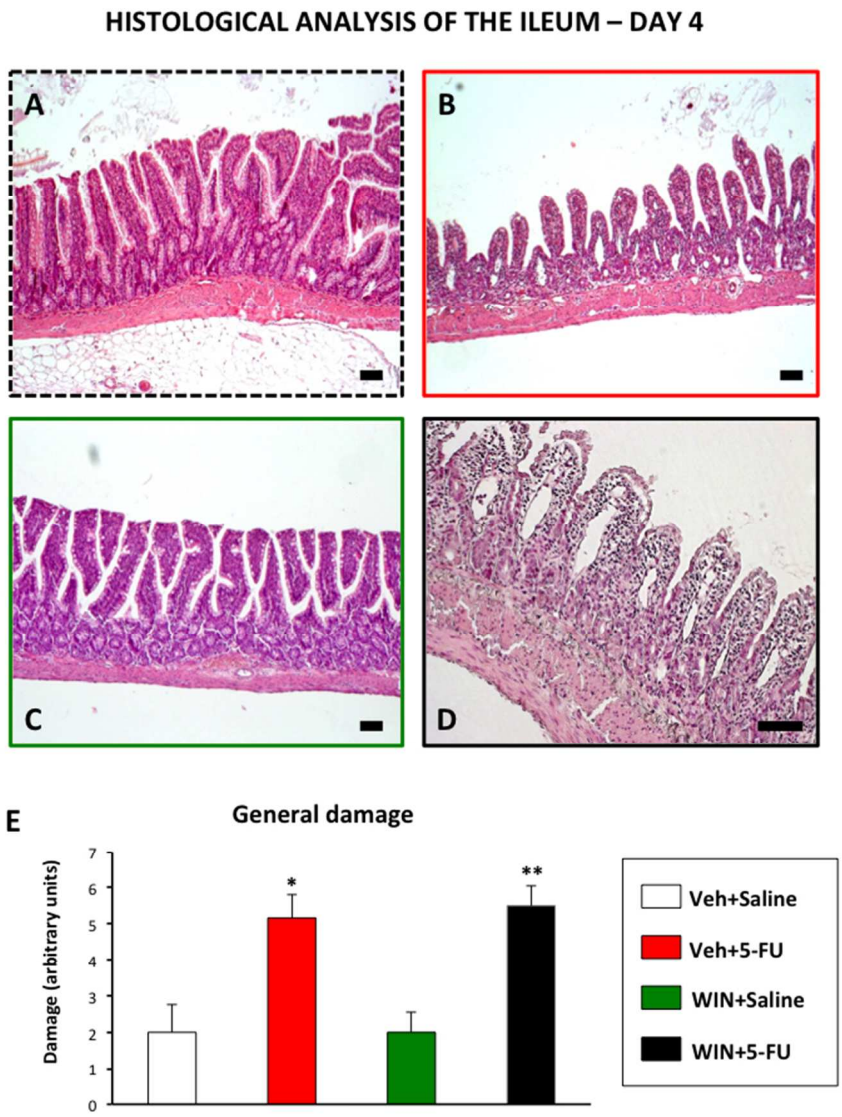

Figure 5. Effect of 5-FU on the general structure of the rat ileum. Histological samples were obtained on experimental day 4 and embedded in paraffin sections. A: Tissue sample from control animals treated with Vehicle+Saline (0.5 and 2.5 mL, respectively). B: Sample from an animal treated with Vehicle+5-FU (5-FU: 150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1). C: Ileum from a rat that received WIN+Saline (WIN: 0.5 mg kg-1 day-1, 4 days, ip). D: Sample from an animal injected with WIN+5-FU. Bar: 100 µm. (E) Quantitative analysis. Bars show mean values ± SEM for organ damage: control (white), vehicle+5-FU (red), WIN+Saline (green) and WIN+5-FU-treated animals (black). Each group consisted of 6 rats. *p<0.05, **p<0.01 vs. control (one-way ANOVA followed by post-hoc Bonferroni multiple comparison test).

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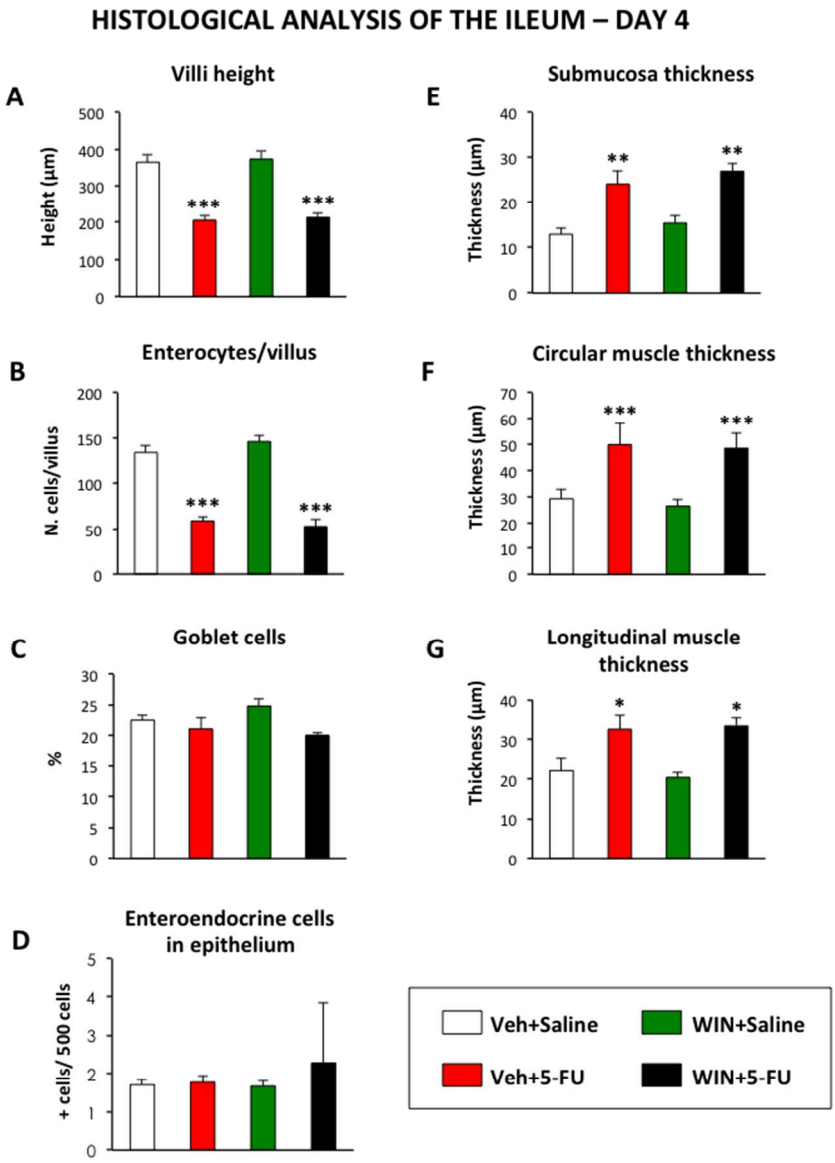

Figure 6. Quantitative analyses of the effect of 5-FU treatment on specific structural features of the rat ileum. Bars show mean values ± SEM for distinct parameters. A: Villi height. B: Number of enterocytes/villus. C: % goblet cells. D: Number of enteroendocrine epithelial cells. E: Submucosa thickness. F: Circular muscle thickness. G: Longitudinal muscle thickness. Animals were treated with Vehicle+Saline (0.5 and 2.5 mL, respectively, white), Vehicle+5-FU (5-FU: 150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1, red), WIN+Saline (WIN: 0.5 mg kg-1 day-1, 4 days, ip, green) or 5-FU+WIN (black). Each group consisted of 6 rats. *p<0.05, **p<0.01, ***p <0.001 vs. Vehicle+Saline (one-way ANOVA followed by post-hoc Bonferroni multiple comparison test).

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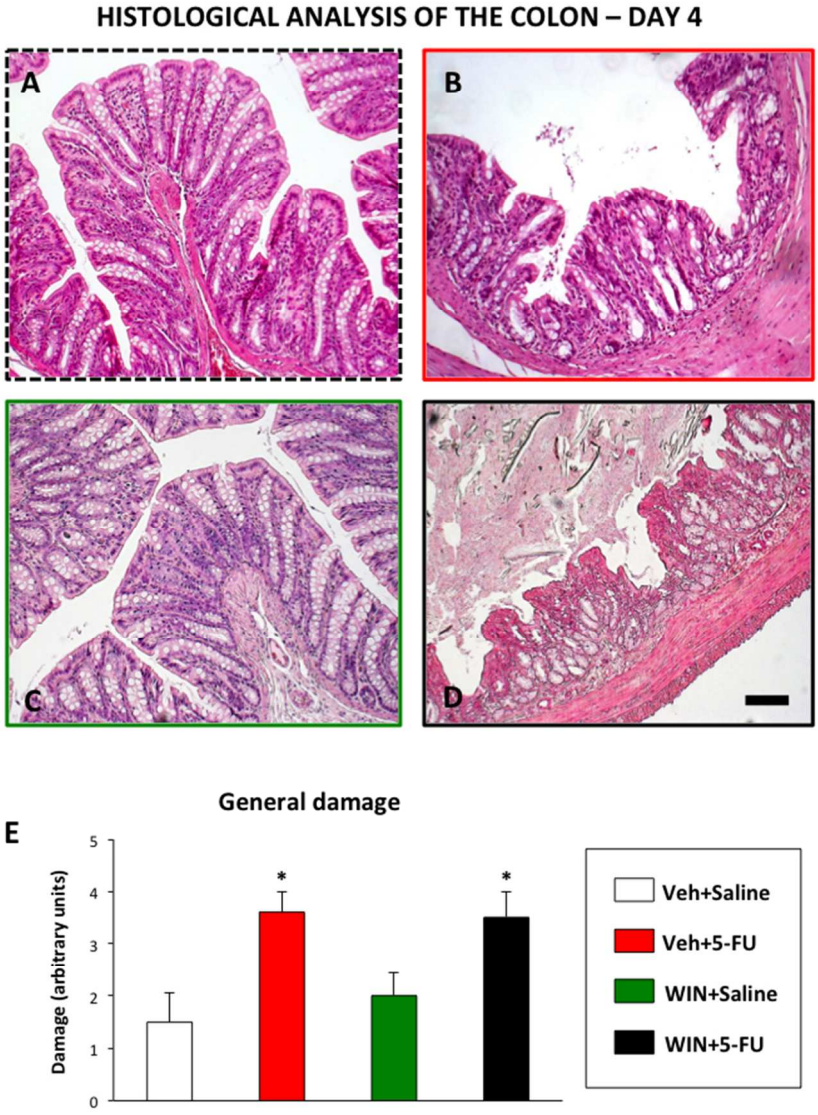

Figure 7. Effect of 5-FU treatment on the general structure of the rat colon. Histological samples embedded in paraffin sections. A: Tissue sample from a control animal treated with Vehicle+Saline (0.5 and 2.5 mL, respectively). B: Sample from an animal treated with Vehicle+5-FU (5-FU: 150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1). C: Colon from a rat that received WIN+Saline (WIN: 0.5 mg kg-1 day-1, 4 days, ip). D: Sample from an animal injected with 5-FU+WIN. Bar: 100 μm. E: Quantitative analysis. Bars show mean values ± SEM for organ damage; control (Vehicle+Saline, white), Vehicle+5-FU (red), WIN+Saline (green) and 5-FU+WIN-treated animals (black). Each group consisted of 6 rats. *p<0.05 vs. control (one-way ANOVA followed by post-hoc Bonferroni multiple comparison test).

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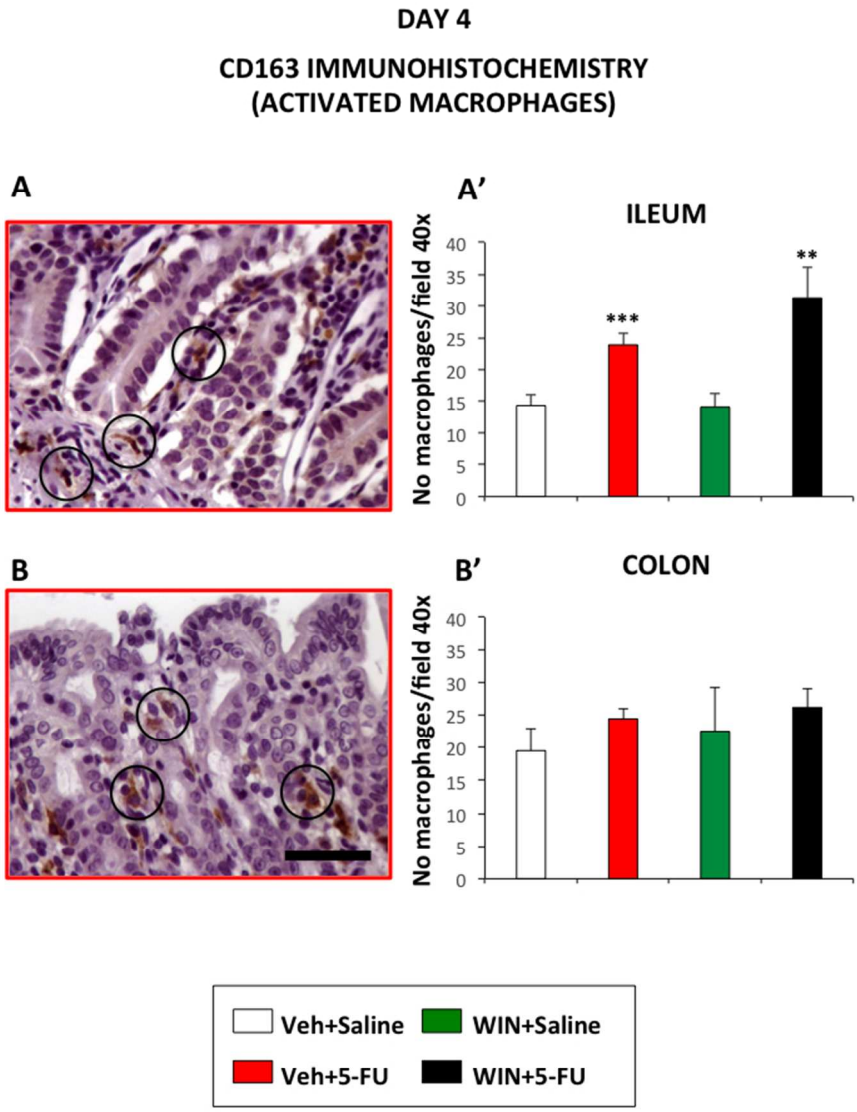

Figure 8. Effect of 5-FU on activated macrophage infiltration in rat ileal and colonic tissues. Animals were treated with Vehicle+Saline (0.5 and 2.5 mL, respectively), Vehicle+5-FU (5-FU: 150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1), WIN+Saline (WIN: 0.5 mg kg-1 day-1, 4 days, ip) or 5-FU+WIN. Histological samples were embedded in paraffin and stained with anti-CD163 antibody. A, B: representative images of ileal and colonic tissues from 5-FU treated rats showing activated macrophage infiltration (encircled); scale bar= 50 µm. A', B': quantitative analysis of activated macrophage infiltration. Bars show mean number ± SEM of macrophages per field 40x. Each group consisted of 4-6 rats and at least 5 fields of view per animal were evaluated. **p<0.01, ***p<0.001 vs. control (Student's t-test with Welch's correction where appropriate).

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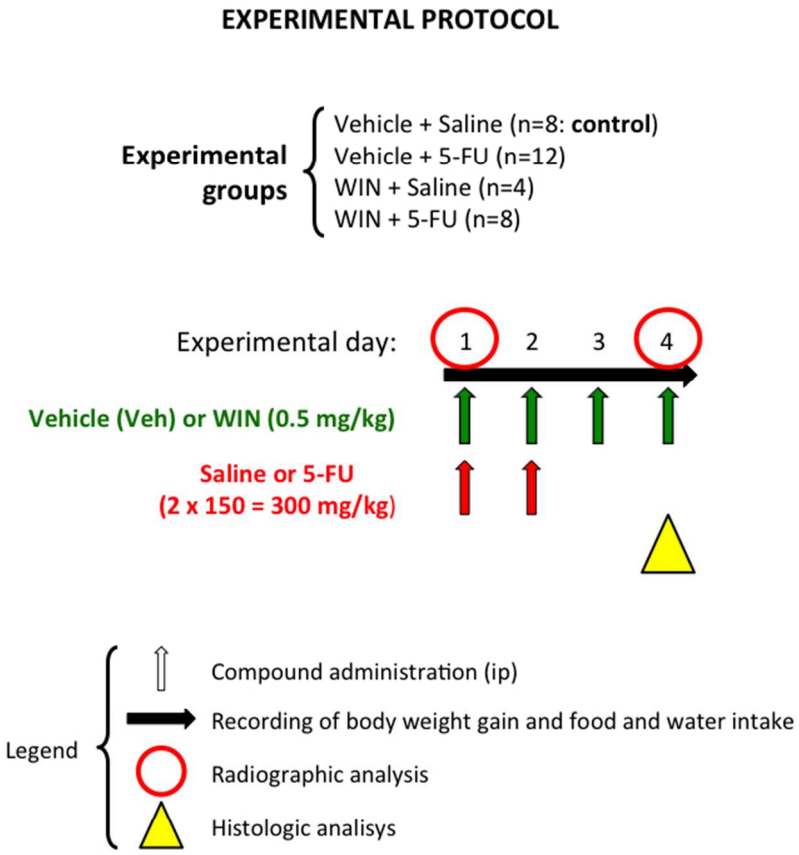

Figure 1 – SUPPLEMENTARY MATERIAL. Experimental protocol. In this study, 4 experimental groups were used (n = 4-12, as shown in the figure). For 4 experimental days, male Wistar rats received an ip injection of vehicle (1.6 mL kg-1) or the non-selective cannabinoid agonist WIN (0.5 mg kg-1 day-1). On the first two days, 20 min after WIN injection, the rats received also saline (8.3 mL kg-1) or the antitumoral drug 5-fluorouracil (5-FU, 150 mg kg-1 day-1). Weight gain and food and water intake were recorded through the experiment. Radiographic analysis of gastrointestinal motility was performed on days 1 and 4 after intragastric contrast administration (2.5 mL barium sulfate, 2 g mL-1). Histological analysis of gut wall structure was performed on day 4 in a parallel group of rats.

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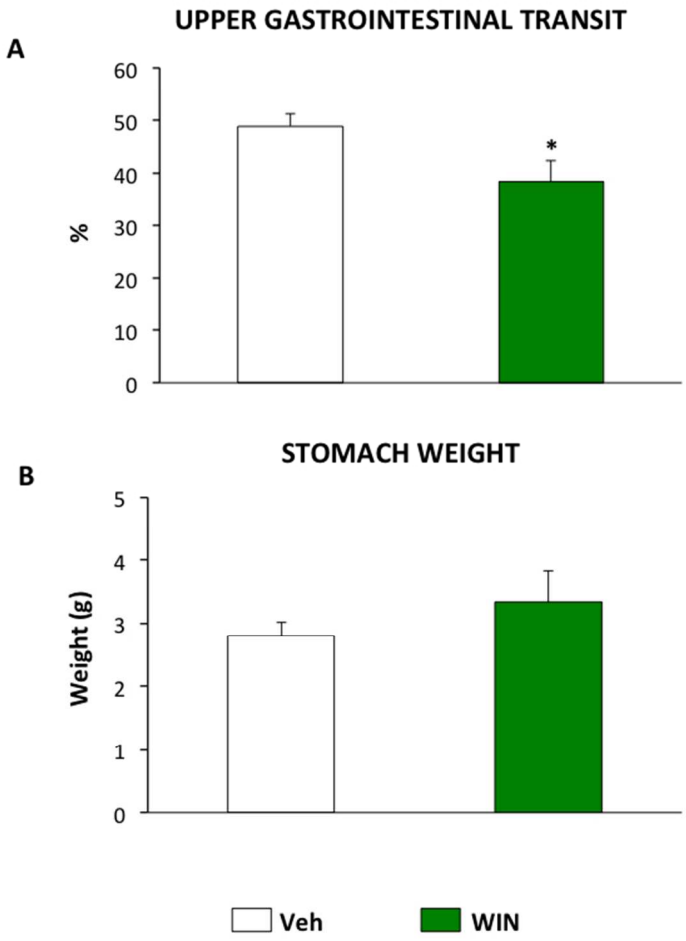

Figure 2 – SUPPLEMENTARY MATERIAL. Effects of WIN on upper gastrointestinal motor function measured invasively by the charcoal method. Rats were fasted overnight. Thereafter, they received an intraperitoneal (i.p.) injection of WIN at 0.5 mg kg-1 (n=6) or its vehicle (n=6, 0.5 mL). Twenty min after, they received 1 ml of a 10% (w v-1) charcoal suspension in a 5% (w v-1) gum Arabic solution via an orogastric cannula. After 20 min, the gastrointestinal tract was removed en bloc. Upper gastrointestinal transit, measured as the % of the small intestine travelled by charcoal front (A), and stomach weight (B) were recorded. Data represent mean ± SEM. *p<0.05 vs control (Student's t-test).

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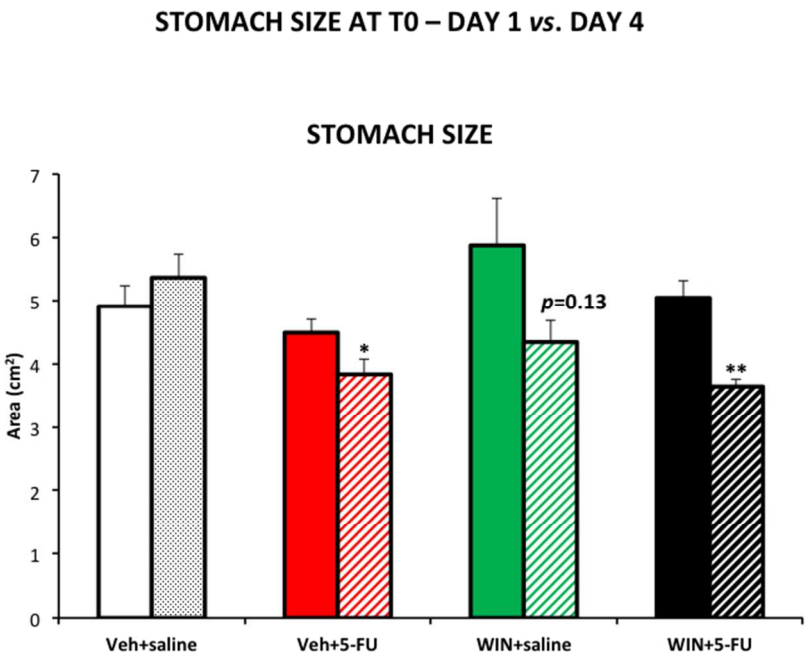

Figure 3 - SUPPLEMENTARY MATERIAL. Effect of 5-FU administration on stomach size in the rat - day 1 vs. day 4. Gastrointestinal motor function was evaluated by radiological methods (see text). Rats received WIN (0.5 mg kg-1 day-1, 4 days, ip) or its vehicle (Veh, 0.5 mL), followed by 5-FU (150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1) or saline (2.5 mL). Thus, the following 4 groups were used: Veh+Saline (n = 8); Veh+5-FU (n = 12); WIN+Saline (n = 4); WIN+5-FU (n = 8). On days 1 (20 min after the first dose of 5-FU or saline) and 4 (2 days after the second dose of 5-FU or saline, 20 min after the fourth dose of WIN or vehicle), barium sulfate (2.5 mL, 2 g mL-1) was intragastrically administered and X-rays obtained immediately after contrast (T0). The stomach size was morphometrically analyzed on both day 1 (solid bars) and day 4 (dotted or striped bars). Data represent mean ± SEM. *p<0.05, **p<0.01 vs control (Student's t-test).

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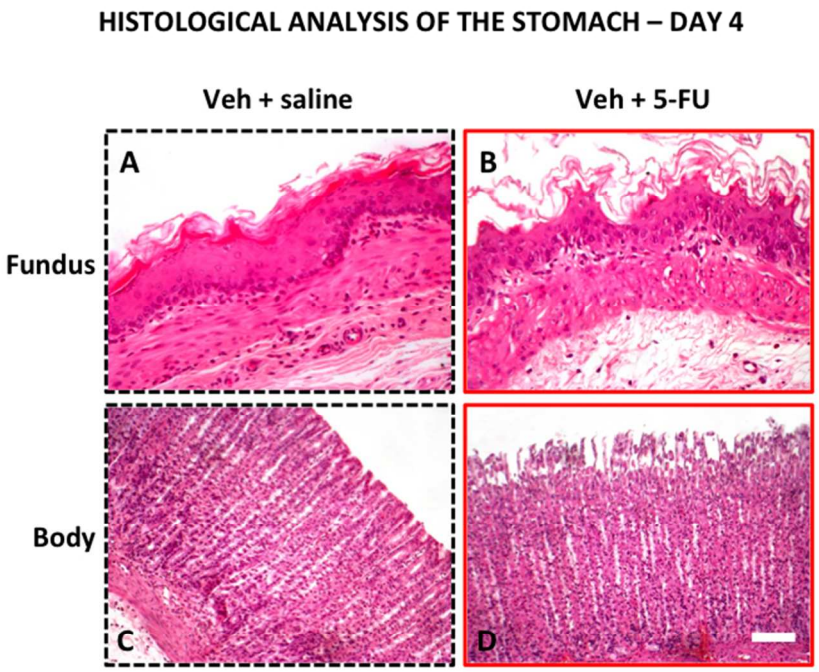

Figure 4 - SUPPLEMENTARY MATERIAL. Effect of 5-FU treatment on the rat stomach. Histological samples embedded in paraffin. Left (A, C): tissue samples from control animals treated with saline (8.3 mL kg-1). Right (B, D): tissue samples from animals injected with 5-FU (150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1). A-B: General view of the stomach fundus showing epithelial damage in the treated group. C-D: Stomach body; note gland damage in the treated group. Bar 100 µm.

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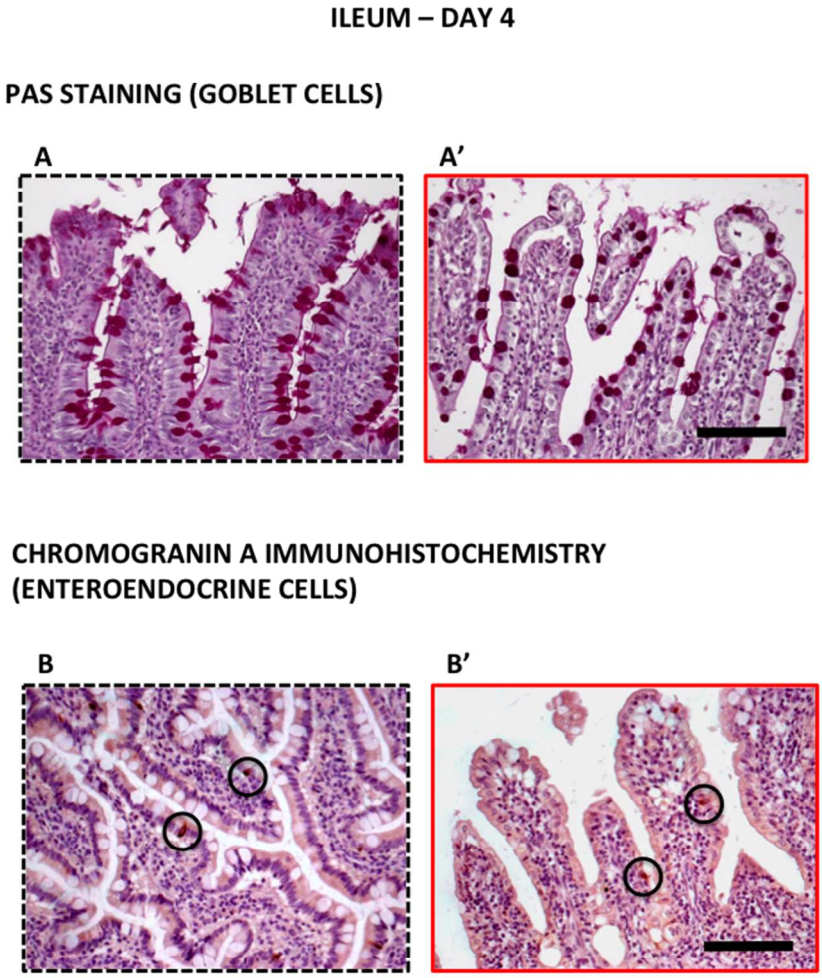

Figure 5 – SUPPLEMENTARY MATERIAL. Effect of 5-FU treatment on goblet and enteroendocrine cells in the rat ileum. Ileal histological samples were embedded in paraffin. The number of goblet cells per villi was counted after PAS staining (A, A') and the number of enteroendocrine cells was counted after immunohistochemistry for chromogranin A (B, B'). A, B: Tissue samples from control animals treated with Vehicle+Saline (0.5 and 2.5 mL, respectively). A', B': Samples from animals treated with Vehicle+5-FU (5-FU: 150 mg kg-1 day-1, 2 days, ip, cumulative dose of 300 mg kg-1). Examples of enteroendocrine epithelial cells immunoreactive to chromogranin A are encircled in B and B'. Bar: 100 µm.

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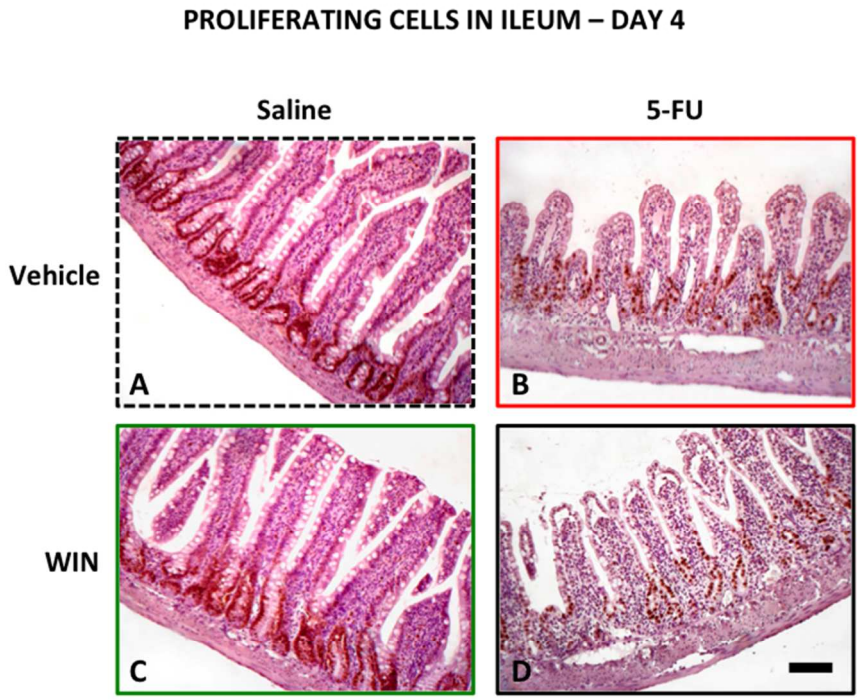

Figure 6 – SUPPLEMENTARY MATERIAL. Effect of 5-FU and WIN treatment on proliferating cells of the rat small intestinal mucosa. Histological samples embedded in paraffin and stained with the Ki67 antibody. A: Tissue sample from a control animal treated with Vehicle+Saline (0.5 and 2.5 mL, respectively). B: Sample from an animal treated with Vehicle+5-FU (5-FU: 150 mg kg-1 day-1, 2 days, starting on day 1, ip, cumulative dose of 300 mg kg-1). C: Ileum from a rat that received WIN+Saline (WIN: 0.5 mg kg-1 day-1, 4 days, ip). D: Sample from an animal injected with 5-FU+WIN. Bar: 100 µm.

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