

Research Article

Influence of H₂-receptor antagonists on intestinal mucositis induced by 5-fluorouracil in rats

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Abstract

AIM: Although histamine (H₂) receptor antagonists appear clinically beneficial; their effects on chemotherapy-induced mucositis remain poorly understood. Therefore, we compared the influences of cimetidine, famotidine, and lafutidine on intestinal mucositis induced by 5-fluorouracil (5-FU) in rats. Simultaneously, we assessed whether the antitumor efficacy was influenced by these treatments for mucositis.

METHODS: Rats were divided into five groups. The first group (control) was orally administered 0.5% carboxymethylcellulose once daily for 5 or 7 days. The second, third, fourth, and fifth groups were treated with 5-FU (30 or 50 mg/kg), 5-FU plus cimetidine (100 mg/kg), 5-FU plus famotidine (5 mg/kg), and 5-FU plus lafutidine (30 mg/kg), respectively. We compared the levels of antitumor activity and mucosal damage among these groups. Furthermore, the possible participation of capsaicin-sensitive afferent neurons was investigated.

RESULTS: 5-FU was shown to reduce weight gain and intestinal mucin accumulation. Oral administration of the H₂-receptor antagonists had no effect on the antitumor activity of 5-FU. Lafutidine, but neither cimetidine nor famotidine, prevented the 5-FU-induced side effects, including reduction of bodyweight gain, mucosal damage, and decrease in mucin content. These beneficial effects disappeared after capsaicin-sensitive afferent neurons were ablated.

CONCLUSION: Second-generation H₂-receptor antagonists may lead to a more effective prevention of chemotherapy-induced intestinal mucositis.

Key Words: Cancer chemotherapy, capsaicin-sensitive afferent neurons, 5-fluorouracil, intestinal mucositis, H₂-receptor antagonists, rat

1. Introduction

In recent years, intestinal mucositis has emerged as a clinically important and sometimes dose-limiting complication of cancer chemotherapy [1-5]. Although the pathogenesis of mucositis is more complex, in its simplest terms, chemotherapy causes direct epithelium damage [1,2]. This is supported by evidence that

treatment with 5-fluorouracil (5-FU) can cause DNA strand breaks, vacuolation, and degeneration of basal epithelial cells in the intestinal mucosa of rats [1]. These findings suggest that the reduction of chemotherapy-induced intestinal injury could also reduce the growth-inhibitory ability of anti-cancer agents. In addition, despite continuing research into

drugs to prevent or reduce the severity of mucositis [6,7], there is a lack of information regarding whether therapeutic interventions in mucositis affect the efficacy of anti-cancer agents.

The clinical practice guidelines recommend using either a proton-pump inhibitor (omeprazole) or an H₂-receptor antagonist (ranitidine) for the prophylaxis of epigastric pain after treatment with cyclophosphamide, methotrexate, and 5-FU or with combination of 5-FU and folic acid [8]. This is consistent with evidence from randomized trials in Europe revealing that omeprazole and ranitidine had potential ability in preventing chemotherapy-induced gastroduodenal injury [3,8-10]. In Japan, the H₂-receptor antagonists famotidine and lafutidine are prophylactically administered to patients during chemotherapy even in the absence of supporting evidence from randomized controlled trials. Using a rat model, we previously reported the effectiveness of lafutidine on the intestinal mucus barrier against both oral administration of 5-FU and intravenous injection of cisplatin [11,12]. Although we demonstrated the lafutidine prevented cisplatin-induced alterations in rat intestinal mucus without affecting cell turnover, it is unclear whether this H₂-receptor antagonist reduces the growth-inhibitory ability of oral administration of 5-FU.

The first aim of this study to investigate the effects of cimetidine, famotidine, and lafutidine on the antitumor activity of oral administration of 5-FU in Yoshida sarcoma-bearing rats. The Yoshida sarcoma is one transplantable allograft tumor model utilized in the study of antitumor activity. Second, we compared the efficacy of these H₂-receptor antagonists against 5-FU-induced rat intestinal mucosal injury and evaluated their effects on mucin accumulation in different areas of the intestinal tract. As lafutidine was reported to reverse the 5-FU-induced gastric ulcer healing delays mediated by capsaicin-sensitive neurons [13], we assessed the participation of these neurons in the mechanism underlying H₂-receptor antagonists' protective effects.

2. Materials and methods

2.1. Experiment I

2.1.1. Experimental animals and tumor inoculation

The antitumor activity of 5-FU was evaluated in Yoshida sarcoma-bearing rats. We purchased male Donryu rats weighing 165.4–187.4 g (Charles River Laboratories Japan Inc.) and intraperitoneally transferred ascitic tumor cells (Yoshida sarcoma). Cells were then collected for experimentation 4 days after

inoculation, and 2×10^5 Yoshida sarcoma cells were subcutaneously implanted into the right axillas of each rat. We then randomized the animals into five groups (seven rats per group) on the basis of the body weight after implantation (i.e., day 0). The rats were weighed at day 0, 2, 4, 6, and 8.

2.1.2. Drug preparation and administration

We orally administered 5-FU by gavage (30 mg/kg) once daily for 7 days. The H₂-receptor antagonist treatment was by cimetidine (Sigma-Aldrich Corp., St. Louis, USA), famotidine (Sigma-Aldrich Corp.), and lafutidine (Taiho Pharm. Co. Ltd., Tokyo, Japan), which were administered at dosages of 100 mg/kg, 5 mg/kg, and 30 mg/kg, respectively. All drugs were suspended in 0.5% carboxymethylcellulose solution and immediately prepared before use. Each H₂-receptor antagonist was orally administered 30 min before 5-FU administration.

In the first group (control), animals received 0.5% carboxymethylcellulose instead of 5-FU and H₂-receptor antagonists. The second, third, fourth, and fifth groups were treated with 5-FU, 5-FU plus cimetidine, 5-FU plus famotidine, and 5-FU plus lafutidine, respectively.

2.1.3. Evaluation of antitumor activity

On day 8 after tumor inoculation, rats were weighed and sacrificed, and the tumor weights measured. The body weight change (BWC; g or %) and tumor growth inhibition (TGI; %) were calculated as follows: BWC (g) = mean BW on Day 8 – mean BW on Day 0; BWC (%) = [(mean BW on Day 8 – mean BW on Day 0)/(mean BW on Day 0)] × 100; and TGI (%) = [1 – (mean tumor weight of treated group/mean tumor weight of control group)] × 100.

2.1.4. Statistical analysis

The difference in the mean values among the groups was analyzed by one-way analysis of variance with Dunnett's test. A p value of <0.05 was considered to indicate statistical significance.

2.2. Experiment II

2.2.1 Experimental animals and drug administration

Seven-week-old male Wistar rats (CLEA-Japan, Tokyo, Japan) were then used to compare the efficacy of H₂-receptor antagonists in the treatment of 5-FU-induced intestinal mucosal injury. These animals were housed in our animal care facility for 1–2 weeks while their bodyweight stabilized. The animals were housed

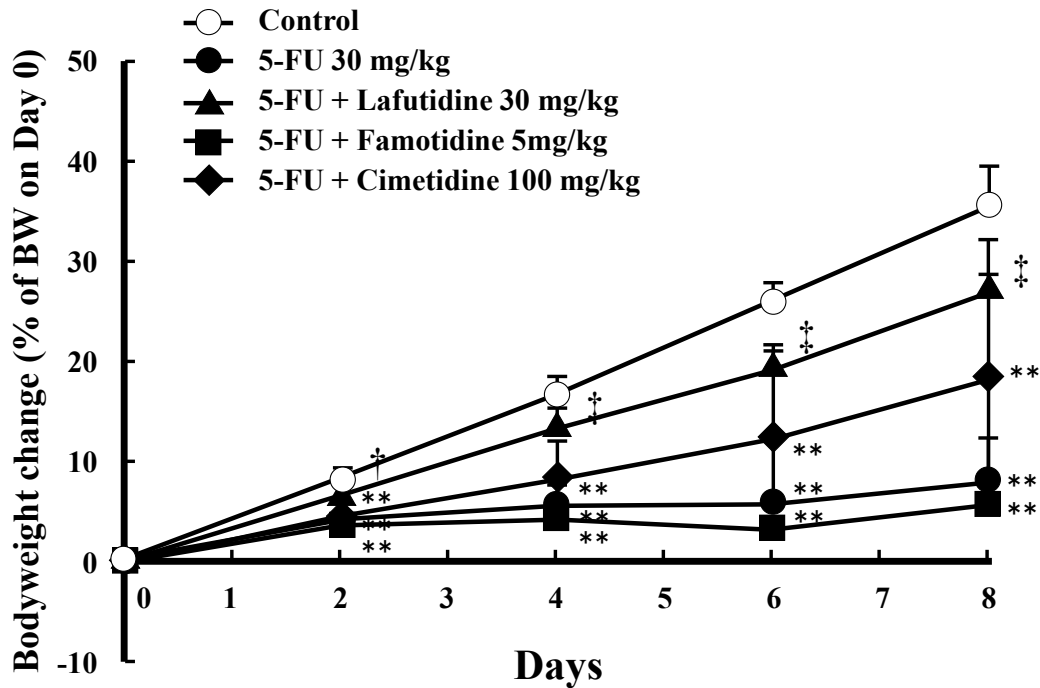


Figure 1. Effects of oral administration of 5-fluorouracil (5-FU), with and without H₂-receptor antagonists, on bodyweight change in Yoshida sarcoma-bearing rats. Each point and vertical bar represent the mean \pm SD of seven experiments. **: $P < 0.01$ vs. control group, †: $P < 0.05$, ‡: $P < 0.01$ vs. 5-FU group by Tukey's comparison test.

in individual cages with raised mesh bottoms and in a temperature- and humidity-controlled environment with a 12-h dark–light cycle (dark cycle from 6:00 pm to 6:00 am). During treatment, rats were given food and water *ad libitum*.

Rats were divided into five groups (four or six rats per group). We orally administered 5-FU (50 mg/kg) once daily for 5 days. Three H₂-receptor antagonists were administered at the same dosages of experiment I. This study was conducted according to the guidelines of the Animal Laboratory Center of Kitasato University School of Medicine.

2.2.2. Histology and immunohistochemistry

At 24 h after final drug administration, animals were weighed, sacrificed, and their proximal and distal small intestines (corresponding to the jejunum and ileum, respectively) were removed. Tissue specimens were immediately fixed for 3 h in freshly prepared Carnoy's solution, following a previously described method [14]. After fixation, the materials were dehydrated using ethanol, cleared in xylene, and embedded in paraffin.

From these specimens, 3- μ m paraffin sections were prepared for immunostaining with our original anti-mucin monoclonal antibody, PGM34. Regarding PGM34 established by us, it was recently demonstrated that the epitope of this monoclonal antibody was a specific sulfated oligosaccharide of the mucin molecule; the antibody stains the goblet cells of the small intestine of the rat [15]. Villus height in the epithelium of the jejunum and ileum was measured in four rats per group. The villus height was measured at three sites of three high-power fields (totally nine sites) in each rat, and the mean value and standard deviation were calculated.

2.2.3. Extraction and measurement of mucin

Specimens from each tissue were lyophilized and powdered for mucin extraction using a previously described method [12]. Each sample was suspended in 50 mM Tris-HCl, pH 7.2, containing 2% Triton X-100 (Triton-Tris buffer), before being homogenized and then incubated at 37°C for 1 h. After centrifugation at 8000 \times g for 30 min at 4°C, the supernatant was

Table 1. Effects of H₂-receptor antagonists on anti-tumor activity of 5-FU in Yoshida sarcoma-bearing rats.

	No. of animals	No. with diarrhea	No. of deaths	TW ^{a)} (g, mean ± SD)	TGI ^{b)} (%)	Bodyweight change ^{c)} (g, mean) (%; mean ± SD)	
Control	7	0	0	2.5478 ± 0.3679	-	62.3	35.4 ± 3.9
5-FU	7	0	0	1.3964 ± 0.4197**	45.2	14.0	7.9 ± 10.7**
Cimetidine+5-FU	7	0	0	1.5059 ± 0.2901**	40.9	32.4	18.1 ± 10.8**
Famotidine+5-FU	7	0	0	1.4518 ± 0.3720**	43.0	9.8	5.5 ± 6.8**
Lafutidine+5-FU	7	0	0	1.4718 ± 0.3428**	42.2	47.1	26.8 ± 5.2 ‡

** : P<0.01 by Dunnett's test as compared to the control group.

‡ : P<0.01 by Dunnett's test as compared to the 5-FU group.

a) : Tumor weight (TW) on Day 8

b) : Tumor growth inhibition rate (TGI) on Day 8 on the basis of TW was calculated according to the following formula:

$$\text{TGI (\%)} = [1 - (\text{mean tumor weight of the treated group}) / (\text{mean tumor weight of the control group})] \times 100$$

c) : Bodyweight (BW) change (g; mean or %; mean ± SD) on Day 8 was calculated according to the following formula:

$$\text{BWC (g)} = (\text{BW on Day 8}) - (\text{BW on Day 0}), \text{BWC (\%)} = [(\text{BW on Day 8}) - (\text{BW on Day 0})] / (\text{BW on Day 0}) \times 100$$

collected, and an aliquot was applied to a Bio-Gel A-1.5-m column, and eluted with the Triton-Tris buffer. The void volume fraction monitored by hexose measurement was collected as mucin. The hexose content in this fraction was measured by the phenol-sulfuric acid method using galactose as the standard. Mucin content (i.e., the void-volume-fraction hexose value) is expressed as micrograms of hexose per rat.

2.2.4. Defunctionalization of capsaicin-sensitive afferent neurons

The ablation of capsaicin-sensitive afferent neurons was chemically performed by the subcutaneous injection of capsaicin once daily for 3 consecutive days (total dose: 100 mg/kg) two weeks before the experiment, according to a previously described method [16]. All capsaicin injections were administered under ether anesthesia, and the rats were intramuscularly pre-treated with terbutaline (0.1 mg/kg) and aminophylline (10 mg/kg) to prevent respiratory impairment. The effectiveness of the treatment was tested by examining the protective wiping movements of the eye.

2.2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, USA). The difference in the mean values among the groups was analyzed by one-way analysis of variance with Tukey's test. A p value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Bodyweight change

The bodyweight changes of Yoshida sarcoma-bearing rats in each experimental group are summarized in Figure 1. Over 8 days, weight gain was observed in the control group, whereas the weight gain was reduced by approximately 30% in animals orally administered 5-FU at a dosage of 30 mg/kg once daily for 7 consecutive days. As shown in Figure 1, the bodyweight changes of both the 5-FU plus famotidine and 5-FU plus cimetidine groups were significantly decreased compared to that of the control group. Lafutidine treatment prevented the reduction of bodyweight gain compared to the 5-FU group. Similar patterns were observed in rats administered 5-FU at a dosage of 50 mg/kg once daily for 5 days with or without each H₂-receptor antagonist (data not shown).

Table 2. Influence of H₂-receptor antagonists on mucin accumulation in the 5-FU-induced intestinal mucosal damage

	n	Hexose Value	
		Jejunum	Ileum
Control	6	4015.2 ± 150.2	5198.9 ± 111.6
5-FU	6	1064.9 ± 118.2	1854.1 ± 275.5
Cimetidine+5-FU	4	1569.6 ± 104.9	2013.1 ± 221.8
Famotidine+5-FU	4	928.4 ± 40.8	2047.7 ± 258.5
Lafutidine+5-FU	6	3137.6 ± 114.1	4081.5 ± 156.4

*: P<0.05, **: P<0.01

Fr-1 hexose values corresponding to mucin content are expressed as micrograms of hexose per rat and represent means ± SE.

3.2. Effects of the H₂-receptor antagonists on the antitumor activity of 5-FU

As summarized in Table 1, oral 5-FU at a dosage of 30 mg/kg/day significantly inhibited Yoshida sarcoma growth. However, neither lafutidine, famotidine, nor cimetidine influenced the anti-cancer activity of 5-FU.

3.3. Changes in immunoreactivity and mucin content of the small intestinal mucosa

Figure 2 shows the morphological changes in the small-intestinal mucosa after treatment. In the control rats, immunohistochemical reactivity for PGM34 could be detected in the goblet cells as well as in the surface mucus gel layer of the jejunum and ileum (Figures 2A and F). As shown in Figures 2B and G, treatment with 5-FU caused a marked decrease in both villus height and the number of PGM34-positive goblet cells. In the animals treated with a combination of 5-FU and lafutidine, significant observable damage could rarely be observed in the jejunal or ileal mucosal sections (Figures 2E and J); however, neither cimetidine (Figures 2C and H) nor famotidine (Figures 2D and I) prevented the 5-FU-induced intestinal mucosal damage.

Table 2 shows a comparison of the effects of the H₂-receptor antagonists on the small-intestinal mucin content in the presence of 5-FU-induced mucosal

damage. A decrease in the mucin content of the jejunum and ileum was observed after treatment with 5-FU (27% and 36% of the control mucin content, respectively), but pretreatment with lafutidine significantly inhibited these changes (to 78% and 79% of the control mucin levels, respectively). No significant change could be detected in the mucin content in the small intestines of rats treated in combination with 5-FU and either cimetidine or famotidine.

3.4. Effect of sensory deafferentation (capsaicin pretreatment) on the immunoreactivity and mucin content of the small-intestinal mucosa in rats treated with 5-FU

Figure 3 shows the morphological and biochemical changes related to 5-FU treatment with and without lafutidine in the small-intestinal mucosa after sensory deafferentation. After chemical ablation of capsaicin-sensitive afferent neurons, lafutidine could not prevent the 5-FU-induced mucosal damage in the jejunum and ileum (Figures 3A and B).

4. Discussion

We found significant differences in the efficacy of the H₂-receptor antagonists on intestinal mucositis induced by 5-FU. As we previously demonstrated [17], oral

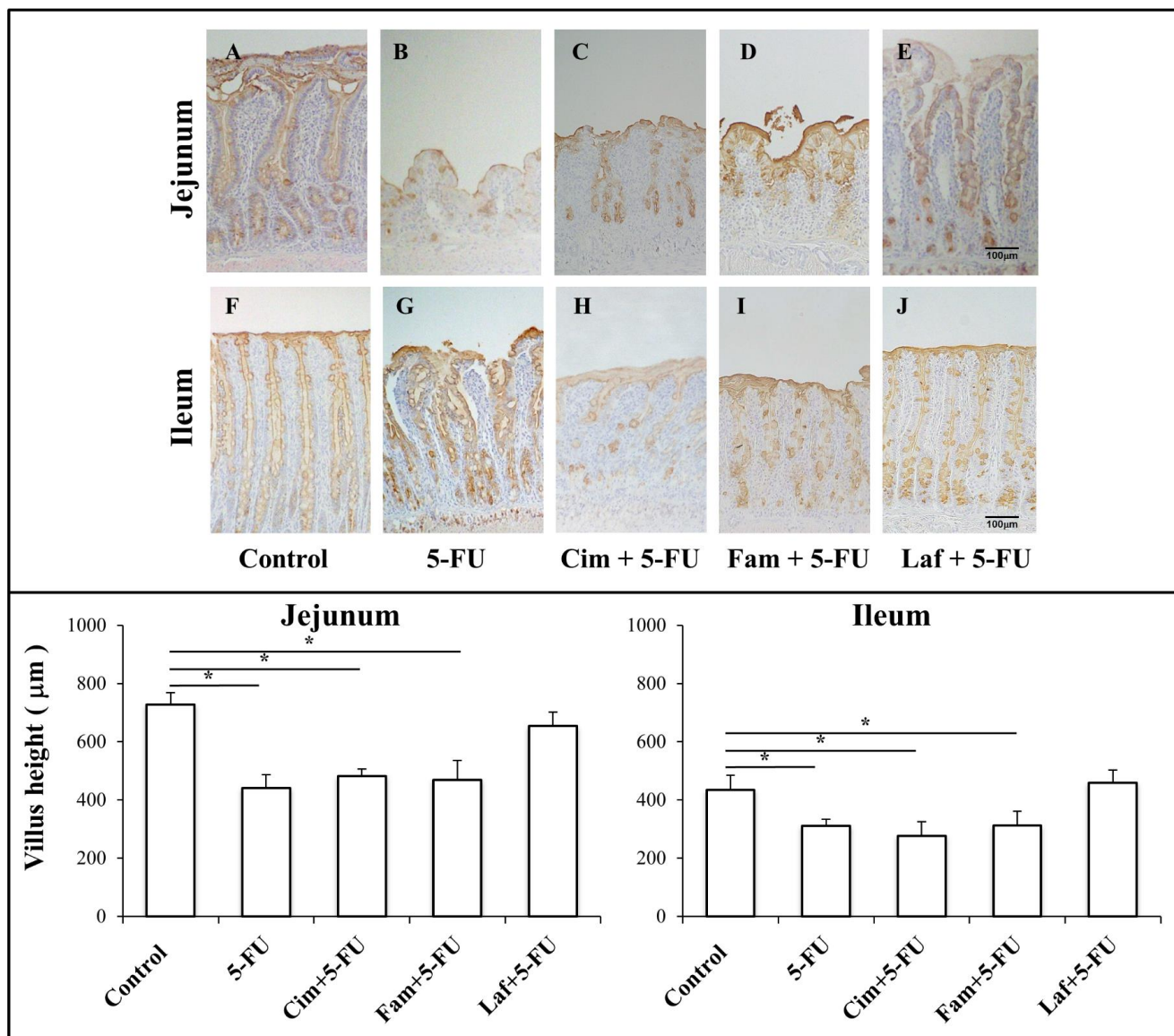


Figure 2. Morphological changes in the small intestinal mucosa. Mucus cells stained with anti-mucin monoclonal antibody PGM34 (Upper) and villus height was measured (Lower). Small-bowel tissues were obtained from control rats (A, F), rats treated with 5-fluorouracil (5-FU) alone (B, G), rats treated with cimetidine (Cim) + 5-FU (C, H), rats treated with famotidine (Fam) + 5-FU (D, I), and rats treated with lafutidine (Laf) + 5-FU (E, J). Data are shown as means \pm SD. *Differences were statistically significant ($P < 0.05$).

administration of 5-FU caused marked decreases in both the number of mucus cells and the mucin content in the small intestine of the rat, the jejunum in particular. In this study, cimetidine and famotidine failed to alleviate the changes in both the morphological defects and mucin contents in the intestinal mucosae of rats treated with 5-FU. Moreover, we previously reported a preventive effect of proton

pump inhibitors, such as omeprazole and lansoprazole, on the 5-FU-induced alteration of mucus in the gastric mucosa but not intestinal mucosa [11]. These findings suggest the distinct protective mechanisms in the gastric and intestinal mucus cells against mucosal injury caused by 5-FU. Here we report a preventive effect of lafutidine on the 5-FU-induced alteration of intestinal mucus in rats. Although the protective

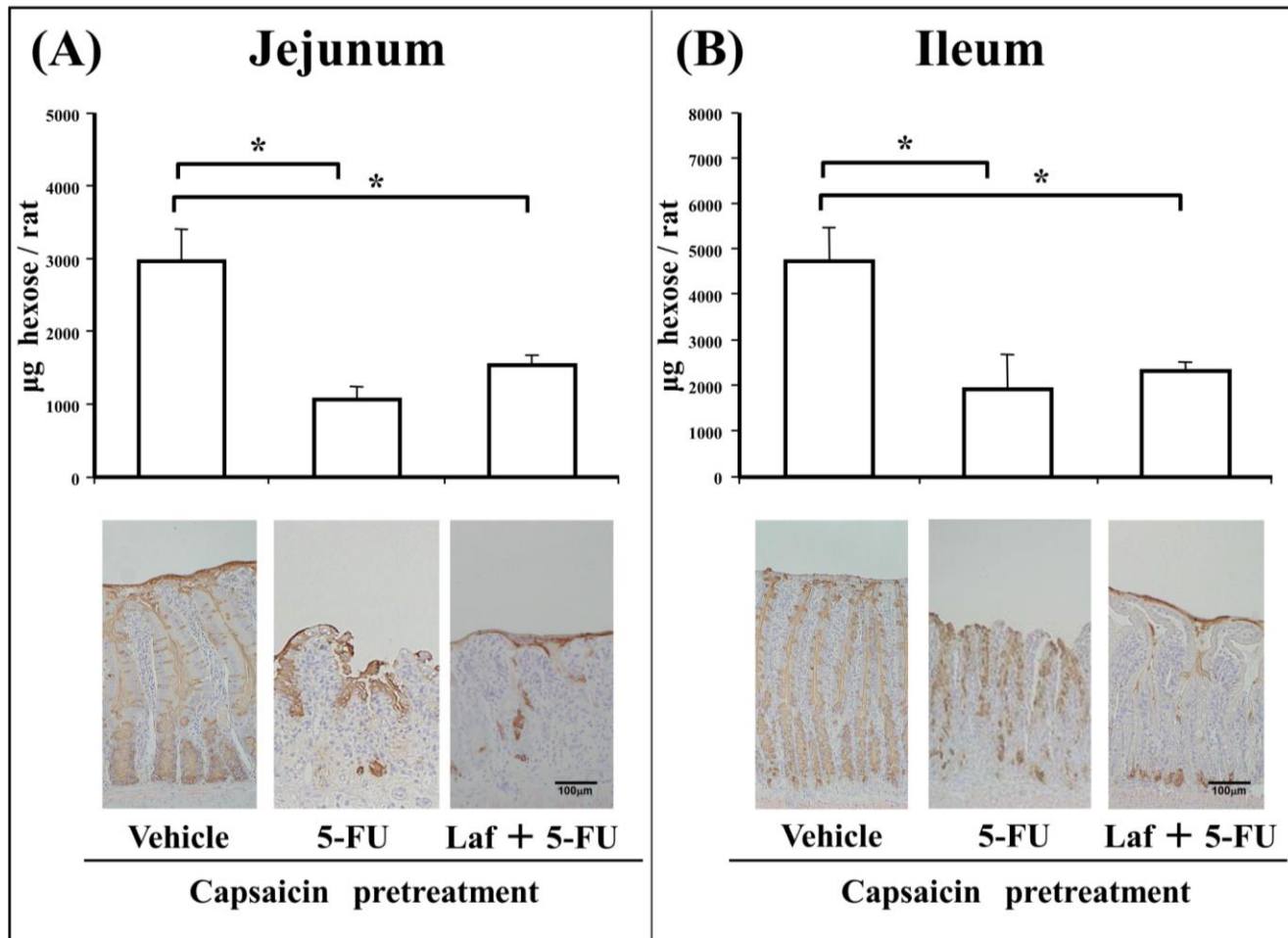


Figure 3. Effect of lafutidine on mucin accumulation and immunostaining of the jejunal (A) and ileal (B) mucosal damage induced by 5-fluorouracil (5-FU) following sensory deafferentation. The sensory deafferentation was created by three consecutive subcutaneous injections of capsaicin (total 100 mg/kg) 2 weeks before the experiment. Then, 5-FU was administered orally once daily for 5 days. Lafutidine (Laf; 30 mg/kg) was orally administered 30 min before the 5-FU administration. The void-volume-fraction hexose values corresponding to mucin content are expressed as micrograms of hexose per rat and presented as means \pm SD. $n = 6$ (each group), $*P < 0.05$. Immunostaining of PGM34 could be detected in the goblet cells in the small-intestinal mucosa.

property of intestinal mucus has received limited attention compared with that of gastric mucus [18], our results demonstrate that an important prophylactic mechanism, independent of H_2 -receptor antagonistic property, participates in the effect of lafutidine on 5-FU-induced intestinal mucosal injury.

The most striking finding in this study was that the preventive effects of lafutidine against 5-FU-induced intestinal mucosal damage were significantly attenuated by sensory deafferentation following capsaicin pretreatment. Capsaicin-sensitive sensory

neurons play an important role in maintaining the integrity of gastric mucosa [19]. Murashima et al. [13] reported that lafutidine reversed the 5-FU-induced gastric ulcer-healing delays mediated by capsaicin-sensitive neurons. Second-generation H_2 -receptor antagonists, such as lafutidine, have a unique component structurally differing from the conventional H_2 -receptor antagonists, which facilitate capsaicin-sensitive neurons and exert gastroprotective effects [20]. These neurons are widely distributed throughout the intestinal tract in mammals [21]. Recently, a study

demonstrated a similar role of these neurons in the intestinal mucosa [22]. Altogether, these findings indicate that the capsaicin-sensitive neurons play an essential role in protecting the intestinal and gastric mucosa by lafutidine. Activation of capsaicin-sensitive sensory neurons could lead to improvements in the effectiveness of prevention against 5-FU-induced intestinal mucositis.

We found marked alterations of tumor growth in Yoshida sarcoma-bearing rats after orally administering 5-FU at a dosage of 30 mg/kg once daily for 7 consecutive days. Our results are consistent with the previous finding that 5-FU, which was administered according to the same protocol, caused statistically significant antitumor activity [23]. In that study, the dose response relationship exhibited not only a median effective dose of 29 mg/kg but also a further marked reduction in tumor weight at a dosage of 45 mg/kg [23]. Previous literature reported that the H₂-receptor antagonists may affect drug absorption by increasing gastric pH or they may impair hepatic or renal drug clearance by one of the several mechanisms, including altered cytochrome P450 hepatic drug metabolism [24-26]. Therefore, it is possible that the attenuation of 5-FU-induced mucositis may be attributed to a reduction in its growth-inhibitory activity. We demonstrated that the antitumor activity of 5-FU was influenced by none of the H₂-receptor antagonist used in this study. These findings indicate that lafutidine prevented 5-FU-induced alterations in rat intestinal mucus without affecting the cell turnover.

We recently reported significant changes in the mucus barrier of the rat during cisplatin-induced intestinal mucositis [12]. In that study, lafutidine, but not famotidine, inhibited cisplatin-induced reduction in the intestinal mucin content and bodyweight [12]. In this study, unlike lafutidine, neither cimetidine nor famotidine improved the changes in the intestinal mucin content or bodyweight gain in rats treated with 5-FU. First-generation H₂-receptor antagonists, such as cimetidine and famotidine, have been reported to

reduce the production and secretion of rat intestinal mucin [20]. In contrast, second-generation H₂-receptor antagonists, such as lafutidine, were shown to directly stimulate mucus cells [20]. The effect of lafutidine against anticancer drug-induced intestinal damage may be attributed to the increased mucus production by the goblet cells that remained viable after chemotherapy. The active secretion of mucin could result in the recovery of bodyweight. Although further studies are required to clarify the precise mechanism underlying intestinal mucosal injury caused by anticancer drugs, second-generation H₂-receptor antagonists, such as lafutidine, could lead to improvements in the effectiveness of the prevention against chemotherapy-induced adverse effects.

In conclusion, we present two important research findings. First, oral administration of the H₂-receptor antagonists used in this study did not influence the antitumor efficacy of 5-FU. However, only lafutidine ameliorated the reduction in bodyweight gain induced by 5-FU treatment. Second, lafutidine appeared to be protective against 5-FU-induced intestinal mucosal injury by reversing the decreased mucin accumulation. These effects involved the function of capsaicin-sensitive sensory neurons, raising the possibility of more effective prevention of chemotherapy-induced intestinal mucositis.

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Abbreviations

BMC, body weight change;
5-FU, 5- fluorouracil;
TGI, tumor growth inhibition.

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