1. Title page

Title:

Mucosal protective agents prevent exacerbation of NSAID-induced small intestinal lesions caused by antisecretory drugs in rats

Authors:

Hiroshi Satoh, Kikuko Amagase, Koji Takeuchi

Affiliation:

Department of Pharmacology & Experimental Therapeutics, Division of Pathological Science, Kyoto Pharmaceutical University, Kyoto, Japan
1. Running title page

a) Running title

Prevention of intestinal ulcer by mucosal protective drugs

b) Corresponding author

Hiroshi Satoh, DVM, PhD

Department of Pharmacology & Experimental Therapeutics, Division of Pathological Science, Kyoto Pharmaceutical University,

Misasagi, Yamashina, Kyoto 607-8414, Japan

Phone: +81-75-595-4680

Fax: +81-75-595-4774

e-mail: h_satoh@mb.kyoto-phu.ac.jp

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d) **Nonstandard abbreviations used in this paper**

ATR, atropine; CE, capsule endoscopy; DIC, diclofenac Na; H₂-RA, histamine 
H₂-receptor antagonist; IND, indomethacin; IRS, irsogladine; LI, lesion index; 
MPA, mucosal protective agent; MIS, misoprostol; MUC, mucin; OPZ, 
omeprazole; RAN, ranitidine; REB, rebamipide; VEH, vehicle

e) **Recommended section**

Gastrointestinal
ABSTRACT

Antisecretory drugs such as histamine H₂-receptor antagonists and proton pump inhibitors are commonly used for the treatment of upper gastrointestinal mucosal lesions induced by NSAIDs. However, it has recently been reported that these drugs exacerbate NSAID-induced small intestinal lesions in rats. Unfortunately, there are few effective agents for the treatment of this complication. We examined the effects of mucosal protective agents (MPAs), misoprostol, irsogladine and rebamipide, and mucin of porcine stomach on diclofenac-induced intestinal lesions and the exacerbation of the lesions by ranitidine or omeprazole. The effects of the drugs on intestinal motility and mucus distribution/content were also examined. Male Wistar rats (180-220 g) were used. Each drug was administered orally under fed conditions. Diclofenac (1-10 mg/kg) produced multiple lesions in the small intestine dose-dependently. Both ranitidine (30) and omeprazole (100) significantly increased the intestinal lesions induced by low doses (3 and 6) of diclofenac. All of misoprostol (0.03-0.3), irsogladine (3-30) and rebamipide (30-300) as well as mucin (30-300) inhibited the formation of intestinal lesions caused by a high dose (10) of...
diclofenac alone and prevented the exacerbation of diclofenac-induced lesions
by antisecretory drugs. Diclofenac (10) markedly increased the intestinal motility
and decreased the mucosal mucus, and the decrease of mucus was significantly
inhibited by the MPAs. These results indicate the usefulness of the MPAs for the
treatment of intestinal lesions induced by NSAIDs alone or by co-administration
with antisecretory drugs, and suggest that mucus plays an important role in the
protection of intestinal mucosa by the MPAs.
Introduction

Recent progress in endoscopic techniques, such as capsule endoscopy (CE) and double-balloon (push) endoscopy, has revealed that nonsteroidal anti-inflammatory drugs (NSAIDs) often cause mucosal lesions not only in the stomach and duodenum but also in the lower small intestine in humans (Graham et al., 2005; Maiden et al., 2005; Goldstein et al., 2005; Matsumoto et al., 2008). Anti-secretory drugs such as proton pump inhibitors (PPIs) and histamine H2-receptor antagonists (H2-RAs) are commonly used for the treatment of upper gastrointestinal (GI) mucosal lesions induced by NSAIDs (Leandro et al., 2001; Peura, 2004; Yeomans et al., 2006; Scarpignato and Hunt, 2010; Sugano et al., 2011). These drugs have been thought to be ineffective in treating NSAID-induced small intestinal lesions because acid does not seem to be involved in the formation of intestinal lesions. However, recently, it has been reported that the antisecretory drugs exacerbated the small intestinal lesions induced by NSAIDs in rats via dysbiosis (Wallace et al., 2011) and intestinal hyper-motility (Satoh et al., 2012). Furthermore, there are few effective agents for the treatment of small intestinal lesions induced by NSAIDs. Therefore,
identification of effective therapies for the treatment of NSAID-induced small intestinal lesions remains an urgent priority (Satoh & Takeuchi, 2012).

It is well known that GI side effects by NSAIDs are caused primarily by a decrease in the levels of prostaglandins (PGs) in the GI mucosa. It has therefore been proposed that compensation for the reduction in mucosal PGs by the administration of exogenous PGs may be able to minimize NSAID-induced GI side effects. Misoprostol (MIS), a PGE1 derivative, is only one compound available commercially among many derivatives of PGs that have been synthesized. The likelihood that MIS would be effective in protecting the small intestine was suggested by Bjarnason et al. (1989) and recent clinical studies using CE (Watanabe et al., 2009; Fujimori et al., 2009). However, it is well known that MIS sometimes causes diarrhea and abdominal pain as side effects, as reported by Watanabe et al. (2009), which represents a significant impediment, especially for long-term treatment.

On the other hand, compounds with different (i.e., non-PG) chemical structures but similar mucosal protective activity to PGs (mucosal protective agents, MPAs) have been developed in Japan, and some of the MPAs, irsogladine (IRS) (Ueda et al., 1984a, 1984b) and rebamipide (REB) (Uchida et
al., 1985; Yamasaki et al., 1987), are now used as anti-ulcer drugs for the treatment of gastric ulcer and gastritis as gastro-protective agents in Japan and other Asian countries (Arakawa et al., 2005; Naito et al., 2010; Hiraishi et al., 2010; Akagi et al., 2013). It has been reported that IRS protects the gastric mucosa by enhancing mucosal integrity through facilitation of gap junctional intracellular communications (Ueda et al., 1995) and suppression of superoxide production by increasing c-AMP level via inhibition of phosphodiesterase (Kyoi et al., 2004). REB is known to protect the gastric mucosa by acting as a free radical scavenger (Yoshikawa et al., 1993; Sakurai et al., 1995). Furthermore, it has been reported that both IRS (Kamei et al., 2008) and REB (Mizoguchi et al., 2001) prevented indomethacin (IND)-induced small intestinal lesions in rats.

In the present study, we examined the effects of both IRS and REB on 1) the formation of small intestinal lesions induced by diclofenac sodium (DIC), an NSAID commonly used in humans, and 2) the exacerbation of the lesions by antisecretory drugs, ranitidine (RAN) and omeprazole (OPZ), in rats. In addition, the results were compared with those of MIS, a well-known MPA, and mucin (MUC) from porcine gastric mucosa. The possible mechanism of the protection by IRS and REB on the small intestine was examined from the perspectives of
intestinal motility and intestinal mucus (PAS-positive substance) distribution. The results suggest that the MPAs can protect the intestinal mucosa against NSAIDs with or without antisecretory drugs, and that mucus plays at least a partial role in the protection of intestinal mucosa by the MPAs.
Materials and Methods

**Ethics approval.** Experimental protocols were approved by the Animal Research Committee in Kyoto Pharmaceutical University, Kyoto, Japan.

**Animals.** Seven-week-old male Wistar rats (Shimizu Laboratories, Shizuoka, Japan) weighing 180-220 g were used. The animals were given rat chow pellets (MF, Oriental-Yeast, Tokyo, Japan) and had free access to water during the experimental periods, unless otherwise specified.

**Drugs.** The following drugs and chemicals were used: atropine (ATR), carboxymethyl cellulose, diclofenac Na (DIC), diethylether, omeprazole (OPZ), ranitidine (RAN), NaHCO₃ and urethane (Wako, Osaka, Japan). Drugs for oral or subcutaneous administration were suspended in a 1% carboxymethyl cellulose solution. OPZ was suspended in 1% carboxymethyl cellulose containing 1% NaHCO₃ for oral administration. The drugs were prepared just prior to the experiments and administered in a volume of 0.2 ml/100 g body wt. The same volume of a solution containing 1% carboxymethyl cellulose was used as a vehicle (VEH).

**Induction of small intestinal lesions.** The animals were not fasted in this
study. DIC (1-30 mg/kg) was administered p.o. and the animals were sacrificed by diethylether overdose 24 h after DIC administration. The small intestines were spread out on paper, opened along the anti-mesenteric side and the contents were removed. The length (mm) of the individual lesions was measured under a dissecting microscope with a 1-mm square grid eyepiece (x 10), and the sum of the lengths of all the lesions in each intestine was used as the lesion index (LI). The person measuring the lesions did not know the treatments given to the animals.

**Experimental design.**

Effect on small intestinal lesions induced by DIC alone. In this study, a high dose of DIC (10 mg/kg, p.o.) was used, and MPAs or MUC were administered orally 30 min before DIC. The intestinal lesions were examined 24 h after DIC treatment.

Effects on exacerbation of DIC-induced intestinal lesions by RAN or OPZ. RAN (30 mg/kg) or OPZ (100 mg/kg) was administered orally 30 min before DIC (3 or 6 mg/kg, p.o.), and each of MPAs, MUC and ATR was given orally 30 min before RAN. The intestinal lesions were examined 24 h after DIC administration.
Measurement of intestinal motility. Intestinal motility was determined using a miniature balloon according to the method described in our previous paper (Takeuchi et al., 2002). In brief, rats were anesthetized with urethane (1.25 g/kg, i.p.) and a midline incision was performed to expose the small intestine. A thin, water-filled balloon, made from silicone rubber and attached to a polyethylene catheter, was then introduced into the ileum via a small incision made about 10 cm oral to the ileocecal junction. The volume in the balloon was adjusted to give an initial resting pressure of about 10-15 cm H2O, which was insufficient to cause active distension of the intestinal wall. After allowing the preparation to rest for 30 min, intestinal motility was monitored on a personal computer (Dynabook, Satellite A20, Toshiba, Tokyo, Japan) as intra-luminal pressure changes using a pressure transducer and a DC Strain Amplifier (AS2101, NEC, Tokyo, Japan). After basal motor activity had stabilized (≥1 h after the operation), the effects of the drugs were examined.

Histological examinations. DIC (10 mg/kg) was administered orally in fed rats, and 3 h later the lower part of the ileum (between 15 cm and 30 cm oral to the ileocecal junction) was removed after bleeding of whole blood from the abdominal aorta under deep anesthesia with diethyl ether. Then, the contents
were washed out with physiological saline, the preparations were infused with Carnoy solution and the whole preparation was fixed in Carnoy solution for 24 h. MPAs were administered 30 min before DIC, and the lower ileum was removed 3 h after DIC treatment. In another experiment, RAN (30 mg/kg) or OPZ (100 mg/kg) was administered orally in fed rats, and 3 h later the ileal preparations were removed and fixed in Carnoy solution.

Staining with PAS. Serial sections (4 μm) of Carnoy-fixed, paraffin-embedded samples of each tissue were mounted on slides. According to conventional methods, periodic acid-Schiff (PAS) staining was performed.

Measurement of PAS-positive area in the mucosa. Photographs of the ileal mucosa stained with PAS in the rats given DIC with or without MPAs, or RAN and OPZ, were taken under a microscope (x10). PAS-positive area in the mucosa was measured using Image J 1.4 (National Institutes of Health, Bethesda, MD) according to the method described in our previous paper (Yoda et al., 2010).

Statistics. All data are expressed as mean ± SEM. Differences between groups were analyzed by Student’s t-test for paired group comparisons, or analysis of variance (Dunnett’s multiple range test) if more than 2 variables were considered,
with the significance level set at $P<0.05$. The $ID_{50}$ values and their 95% confidence intervals (CI) were calculated by logistic regression analysis.
Results

Effects of MPAs and mucin on the small intestinal lesions induced by DIC in fed rats

Ulcerogenic effect of DIC on the small intestinal mucosa. DIC was administered orally under fed conditions, and the intestinal lesions were examined 24 h later. There are few reports that VEH alone caused lesions in the small intestine. However, in the present study, in the rats given VEH alone, small lesions were observed in the small intestine, and the LI was 8.1 ± 1.8 mm (n=8). DIC (1–10 mg/kg) produced multiple lesions in the middle and lower parts of the small intestine in a dose-dependent manner, but the LIs in the groups given 1 and 3 mg/kg DIC were not significantly different from that in the group given VEH (Fig. 1). The LIs in the rats given 6 and 10 mg/kg DIC were 42.6 ± 6.5 mm (n=8, P< 0.001 vs. VEH) and 101.5 ± 11.0 mm (n=8, P< 0.001 vs. VEH), respectively.

Effect of MPAs and mucin on intestinal lesions induced by DIC alone.

Effect of MPAs. As shown in Table 1 (Exp. 1-3), the formation of the intestinal lesions caused by a high dose of DIC (10 mg/kg) was prevented by MIS (0.01–0.3 mg/kg), IRS (1–30 mg/kg) and REB (10–300 mg/kg) in a
dose-dependent manner. The $ID_{50}$ values (CI) were 0.08 (0.05-0.14) mg/kg (n=7), 12.4 (6.8-33.6) mg/kg (n=7) and 283.4 (128.2-5436.3) mg/kg (n=7), respectively. The effect of high doses of each drug was significant (Table 1).

**Effect of MUC.** MUC (10–300 mg/kg) inhibited the formation of intestinal lesions dose-dependently. The $ID_{50}$ value (CI) was 200.4 (125.0-508.5) mg/kg (n=8). The effects of doses of 30 mg/kg and above were significant (P< 0.05 and 0.001 vs. VEH) (Table 1, Exp. 4).

**Effect of RAN on DIC-induced intestinal lesions.** In this study, a low dose of DIC (3 mg/kg, p.o.) was used. The LI in the group given VEH alone was 10.4 ± 6.0 mm (n=8). Both DIC (3 mg/kg, p.o.) and RAN (30 mg/kg, p.o.) alone slightly increased the intestinal lesions, but the LIs were not significantly larger than that of the VEH group. However, the drugs administered together produced marked lesions in the intestine, and the LI was 82.0 ± 7.2 mm (n=8, P< 0.001 vs. VEH, DIC and RAN alone) (Fig. 2). In another experiment, RAN (3–100 mg/kg, p.o.) increased DIC-induced intestinal lesions from a dose of 3 mg/kg in a dose-dependent manner, but significant effects were only observed in the groups given 30 and 100 mg/kg RAN.

**Effects of MPAs, MUC and ATR on exacerbation of DIC-induced
intestinal lesions by RAN.

**Effect of MPAs.** RAN (30 mg/kg, p.o.) markedly increased the lesions induced by DIC (3 mg/kg, p.o.), and the increase was prevented by MIS (0.03-0.3 mg/kg), IRS (3-30 mg/kg) and REB (30-300 mg/kg) dose-dependently (Figs. 3A-C). The high doses of each drug almost completely inhibited the increase of the lesions, and the effects were significant (Figs. 3A-C).

**Effect of MUC.** MUC (30-300 mg/kg) prevented the increase of the lesions dose-dependently, and the LI in the group given 300 mg/kg MUC was significantly (P< 0.05) smaller than that of the VEH group (Fig. 3D). The effect of MUC seemed to be weaker than those of the MPAs.

**Effect of ATR.** ATR (1-10 mg/kg) prevented the increase of the lesions dose-dependently, and the LIs in the groups given 1, 3 and 10 mg/kg ATR were significantly (P< 0.05, 0.001 and 0.001) smaller than that of the VEH group (Fig. 3E). The increase of the lesions was not always completely prevented by ATR, even at a high dose of 10 mg/kg.

**Effect of OPZ on DIC-induced intestinal lesions.** OPZ at 100 mg/kg was administered orally 30 min before DIC (3 and 6 mg/kg, p.o.), and the intestinal lesions were examined 24 h after DIC administration. As shown in Fig. 4, OPZ
mildly increased the lesions induced by a low dose of DIC (3 mg/kg), but the
effect was not significant. DIC (6 mg/kg) produced moderate lesions in the small
intestine, and the lesions were significantly (P<0.001 vs. DIC alone) increased
by pretreatment with OPZ. In another experiment, OPZ (10–300 mg/kg, p.o.)
increased the lesions induced by DIC (6 mg/kg, p.o.) in a dose-dependent
manner, but significant effects were observed only in the groups given 100 and
300 mg/kg OPZ. On the basis of these results, in the following studies, both a
moderate dose (6 mg/kg) of DIC and a high dose (100 mg/kg) of OPZ were
used.

Effects of MPAs and MUC on exacerbation of DIC-induced intestinal
lesions by OPZ.

Effect of MPAs. OPZ (100 mg/kg, p.o.) markedly increased the lesions
induced by DIC (6 mg/kg, p.o.), and the increase was prevented by MIS
(0.03-0.3 mg/kg), IRS (3-30 mg/kg) and REB (30-300 mg/kg) dose-dependently
(Fig. 5A-C). The effects of high doses of each drug were significant (Fig. 5A-C).

Effect of MUC. MUC (30-300 mg/kg) prevented the increase of the lesions
dose-dependently, and the LIs in the groups given 100 and 300 mg/kg MUC
were significantly (P< 0.01 and 0.001) smaller than that of the VEH group (Fig.
5D). The effect of MUC seemed to be milder than those of the MPAs.

**Effect of drugs on motility of the small intestine.** In urethane-anesthetized rats, the ileum usually did not show spontaneous strong contractions. Each drug was administered subcutaneously every 2 h. The effects on the motility were examined in 3-5 rats for each drug, and similar results were obtained, although the pattern of the response (onset and amplitude of contractile response) for each drug was somewhat different among the rats. The typical responses to each drug are shown in Fig. 6. DIC (3 mg/kg, s.c.) gradually increased motility, starting about 1 h post-treatment (Fig. 6a). The motor-stimulating effect of DIC increased with the dose (10 and 30 mg/kg), and the onset of the effect seemed to be more rapid at higher doses (Fig. 6a). The motor-stimulating effect of DIC (10 mg/kg) was markedly enhanced by RAN (30 mg/kg) (Fig. 6b, c). On the other hand, as shown in Fig. 6d, OPZ (100 mg/kg) did not affect the motility, although RAN markedly enhanced the motility in this rat. Both MIS (0.1 mg/kg) and ATR (10 mg/kg) decreased or abolished the increased motility caused by DIC (Fig. 6e, h). Neither IRS (10 mg/kg) nor REB (100 mg/kg) affected the increased motility induced by DIC (10 mg/kg), although ATR almost abolished the motility in these rats (Fig. 6f, g).
Effect of drugs on mucus distribution in the mucosa. DIC (10 mg/kg) was administered orally in fed rats, and 3 h later the lower ileum was removed. The MPAs were administered 30 min before DIC. Fig. 8 shows typical photos of the ileal mucosa stained with PAS from each group. In the group given VEH, PAS-positive substances were clearly observed in the goblet cells and over the surface and along the glands of the intestinal mucosa (Fig. 7A). DIC (10 mg/kg) apparently reduced the amount of PAS-positive substance in the goblet cells as well as in the glands, without causing any other obvious histological changes in the mucosa (Fig. 7B). The reduction in PAS staining caused by DIC was attenuated when MIS (0.1 mg/kg), IRS (10 mg/kg) and REB (100 mg/kg) were administered before DIC treatment (Figs. 7C-E). Fig. 8 shows the results of analysis using Image J 1.4 on the contents of PAS-positive substance in the mucosa. The PAS-positive area in the group given VEH was $3.7 \pm 0.5\%$ (n=6). DIC (10 mg/kg) significantly decreased the area to $1.7 \pm 0.1\%$ (n=6, P<0.01 vs. VEH). All of MIS (0.1 mg/kg), IRS (10 mg/kg) and REB (100 mg/kg) significantly (P<0.01 and 0.05) prevented the decrease of the PAS-positive area by DIC.

In another experiment, either RAN (30 mg/kg) or OPZ (100 mg/kg) was administered orally to fed rats, and 3 h later the distribution of PAS-positive
substance in the mucosa was examined. RAN, but not OPZ, mildly decreased the PAS-positive area in the mucosa.
Although anti-secretory drugs such as PPIs and H₂-RAs are commonly used for the treatment of upper GI mucosal lesions induced by NSAIDs, it has been reported that the antisecretory drugs exacerbated the small intestinal lesions induced by NSAIDs in rats (Wallace et al., 2011; Satoh et al., 2012). This was supported by the present study, that is, both RAN and OPZ markedly exacerbated the small intestinal lesions induced by DIC. However, at present, there are few effective agents for the treatment of small intestinal lesions induced by NSAIDs. In the present study, we examined the effects of the MPAs (MIS, IRS and REB) on the small intestinal lesions induced by DIC in fed rats. The MPAs prevented the formation of intestinal lesions induced by high-dose DIC alone, and also inhibited the exacerbation of lower-dose DIC-induced intestinal lesions by RAN or OPZ. These results strongly suggest the usefulness of the MPAs for the treatment of small intestinal lesions induced by NSAIDs with or without antisecretory drugs.

We examined the effects of RAN and OPZ on the formation of intestinal lesions caused by lower doses (3 and 6 mg/kg) of DIC because DIC is
commonly used in patients at a lower dose, namely, 25 mg/person, t.i.d. (about 1.5 mg/kg/day). In this study, 3 mg/kg DIC produced mild lesions in the small intestine in fed rats, although the LI was not significantly different from that of the VEH group. However, when DIC was preceded by RAN, it produced marked lesions in the small intestine. The effect of RAN on lesion formation was observed dose-dependently from 3 mg/kg, and significant effects were observed at doses of 30 mg/kg and above. On the other hand, RAN (30 mg/kg) did not show any significant ulcerogenic effect on the small intestinal mucosa by itself. These results indicate that H₂-RAs aggravate mild lesions in the small intestine caused by NSAIDs, suggesting a possible risk of co-administration of NSAIDs and H₂-RAs, even if the NSAID itself seems to be safe in terms of the GI mucosa.

In the present study, the intestinal motility was increased by DIC dose-dependently and the motility was further enhanced by RAN. Furthermore, all of the increased motility by DIC, enhancement of the motility by RAN and the exacerbation of DIC-induced intestinal lesions by RAN were inhibited by treatment with ATR. These results support the previous findings that H₂-RAs exacerbated NSAID-induced intestinal lesions by increasing the intestinal motility.
motility via the cholinergic nervous system (Satoh et al., 2012). However, there are few reports of the effect of NSAIDs and RAN on small intestinal motility in humans, which will be useful for elucidating pathophysiology of intestinal lesions induced by NSAIDs. This will be a subject for a future research. In addition, in the present study, RAN mildly decreased the PAS-positive area in the mucosa. Therefore, it is possible that the observation that RAN depleted mucus distribution/content may explain, at least in part, the exacerbation of intestinal lesions by RAN.

On the other hand, OPZ even at a high dose of 100 mg/kg did not affect the intestinal lesions induced by a low dose (3 mg/kg) of DIC, but markedly increased the lesions induced by a moderate dose (6 mg/kg) of DIC. The effect of OPZ was significant at higher doses of 100 and 300 mg/kg. In addition, OPZ neither affected the intestinal hyper-motility induced by DIC nor decreased the PAS-positive area in the mucosa. These results suggest that OPZ exacerbates DIC-induced intestinal lesions via a different mode of action from that of RAN.

Wallace et al. (2011) reported that 9 days of treatment with OPZ caused a significant reduction of Actinobacteria and *Bifidobacteria* spp. in the jejunum in rats, and suggested that PPIs exacerbated NSAID-induced intestinal injury.
through dysbiosis. As discussed later, we suggested that undigested solid components of food play an important role in NSAID-induced intestinal lesions (Satoh et al., 2009, 2010; Satoh, 2010). It is conceivable that strong and sustained inhibition of gastric acid secretion by antisecretory drugs may exacerbate NSAID-induced intestinal lesions via dysbiosis and/or increasing the amount of undigested solid components of food in the small intestine due to indigestion in the stomach.

Although a deficiency of endogenous PGs due to cyclooxygenase inhibition is the most important base for the ulcerogenic responses to NSAIDs, several different factors related to the pathogenesis of NSAID-induced intestinal ulcers have been suggested (Takeuchi et al., 2010; Wallace et al., 2013). Among them, we have indicated the importance of hyper-motility of the small intestine (Takeuchi et al., 2002), decrease of mucus secretion (Kunikata et al., 2002) and undigested solid components of food (Satoh et al., 2009, 2010), especially in the early stage of lesion formation in the small intestine induced by NSAIDs. Under conditions where the amount of surface mucus is decreased by NSAIDs, it is possible that undigested solid components of food may cause physical damage to the mucosal surface when the intestine is strongly contracted by NSAIDs, and
accelerate the invasion of intestinal bacteria and irritants (bile acids, etc.) into the mucosa. Therefore, in the present study, we examined the effects of MPAs and antisecretory drugs on the intestinal motility and distribution/content of PAS-positive substance (mucus) in the intestinal mucosa.

As mentioned above, DIC increased the intestinal motility dose-dependently in terms of both grade and duration. In addition, DIC markedly decreased PAS-positive substances in the goblet cells as well as in the glands of the intestinal mucosa without causing any other obvious histological changes in the mucosa at 3 h after oral administration. MIS markedly inhibited the motility increased by DIC and significantly prevented the decrease of PAS-positive substance in the mucosa. The effects of MIS can be easily explained by the compensation of PGs reduced by DIC because MIS is a derivative of PGE₁. On the other hand, both IRS and REB, different from MIS, did not affect the intestinal hyper-motility caused by DIC, but significantly reversed the decrease of PAS-positive substance in the mucosa. It has been reported that both IRS and REB increased mucus secretion in the GI tract in rats (Kamei et al., 2008; Ishihara et al., 1992) and stomach in humans (Iijima et al., 2009), and the results of the present study are consistent with these findings. In the present study, we
further examined the effects of MUC from porcine stomach on the intestinal lesions, and found that porcine MUC moderately prevented the formation of intestinal lesions caused by a high dose of DIC alone as well as the exacerbation of low-dose DIC-induced lesions by antisecretory drugs, probably by compensating for a decrease in barrier function caused by NSAIDs. From these results, it is suggested that mucus plays at least a partial role in the protective effects of MIS, IRS and REB on the intestinal mucosa.

To reduce the adverse effects of NSAIDs on the GI tract without affecting their pharmacological activities, novel GI-sparing NSAIDs have been developed, that is, NSAIDs chemically coupled with mucosal protective moieties such as nitric oxide and hydrogen sulfide (Wallace et al., 2003, 2010), and formation of an NSAID mixed with phosphatidylcholine, a mucosal protective moiety (Lichtenberger et al., 2009, Cryer et al., 2011). In addition to these promising developments, in the present study, we found that both IRS and REB as well as MIS could prevent the intestinal lesions caused by DIC with or without antisecretory drugs. Furthermore, several recent pilot studies using CE have revealed that both IRS (Kuramoto et al., 2013) and REB (Miwa et al., 2008; Mizukami et al., 2012), as well as MIS (Watanabe et al., 2009, Fujimori et al.,
2009), are effective in limiting the small intestinal side effects of aspirin and other NSAIDs including DIC. Taking all the present findings and the clinical findings in healthy volunteers together, it is concluded that both IRS and REB could be used as new candidate drugs for the treatment of intestinal lesions induced by NSAIDs with and without antisecretory drugs.

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Authorship Contributions

Participated in research design: Satoh, Takeuchi

Conducted experiments: Satoh, Amagase

Performed data analysis: Satoh, Amagase

Wrote or contributed to the writing of the manuscript: Satoh, Takeuchi
References


Footnotes

a) Financial support: We did not have any financial support for this study.

We also have no conflicts of interest.

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Hiroshi Satoh, DVM, Ph.D.

Department of Pharmacology & Experimental Therapeutics,

Division of Pathological Science, Kyoto Pharmaceutical University,

Misasagi, Yamashina, Kyoto 607-8414, Japan

e-mail: h_satoh@mb.kyoto-phu.ac.jp

d) Numbered footnotes: None
Figure Legends

**Fig. 1.** Ulcerogenic effect of DIC on small intestinal lesions in rats. DIC (1-10 mg/kg) was administered orally without fasting, and intestinal lesions were measured 24 h after DIC administration. (A) Dose-related response of ulcerogenic effect of DIC on the small intestine. Data represent mean ± SEM (n=7). ***: P< 0.001 vs. VEH (Dunnett’s test). (B) Macroscopic observation of small intestinal lesions caused by 10 mg/kg DIC. Each arrow shows the mucosal lesions.

**Fig. 2.** Effect of RAN on small intestinal lesions induced by DIC in rats. DIC (3 mg/kg) was administered orally without fasting, and intestinal lesions were measured 24 h after DIC administration. RAN (30 mg/kg) was given orally 30 min before DIC. Data represent mean ± SEM (n=7). ***: P< 0.001 vs. VEH (Dunnett’s test), ###: P< 0.001 (Student’s t-test). The dose (mg/kg) is listed in parentheses.

**Fig. 3.** Effect of drugs on the enhancing effect of RAN on DIC-induced small
intestinal lesions. RAN (30 mg/kg, p.o.) was administered 30 min before DIC (3 mg/kg, p.o.), and intestinal lesions were measured 24 h after DIC administration. Drugs were administered orally 30 min before RAN (a: MIS, b: IRS, c: REB, d: MUC, e: ATR). Data represent mean ± SEM (n=7). *: P< 0.05, **: P< 0.01, ***: P< 0.001 vs. VEH (Dunnett’s test), ##: P< 0.01, ###: P< 0.001 (Student’s t-test). The dose (mg/kg) is listed in parentheses.

**Fig. 4.** Effect of OPZ on small intestinal lesions induced by DIC in rats. DIC (3 and 6 mg/kg) was administered orally without fasting, and intestinal lesions were measured 24 h after DIC administration. OPZ (100 mg/kg) was given orally 30 min before DIC. Data represent mean ± SEM (n=7). *: P< 0.05, ***: P< 0.001 vs. VEH (Dunnett’s test), ###: P< 0.001 (Student’s t-test), (NS): not significant. The dose (mg/kg) is listed in parentheses.

**Fig. 5.** Effect of drugs on the enhancing effect of OPZ on DIC-induced small intestinal lesions. OPZ (100 mg/kg, p.o.) was administered 30 min before DIC (6 mg/kg, p.o.), and intestinal lesions were measured 24 h after DIC administration. Drugs were administered 30 min before OPZ (a: MIS, b: IRS, c: REB, d: MUC).
Data represent mean ± SEM (n=7). **: P< 0.01, ***: P< 0.001 vs. VEH (Dunnett’s test), ###: P< 0.001 (Student’s t-test). The dose (mg/kg) is listed in parentheses.

**Fig. 6.** Effect of drugs on DIC-induced intestinal motility in anesthetized rats.

Each drug was administered subcutaneously every 2 h. DIC (a, 3, 10 and 30 mg/kg) was administered every 2 h. Each of VEH (b), RAN (c, 30 mg/kg), OPZ (d, 100 mg/kg), MIS (e, 0.1 mg/kg), IRS (f, 10 mg/kg) and ATR (g, 10 mg/kg) was administered 2 h after DIC (10 mg/kg, s.c.). The dose (mg/kg) is listed in parentheses.

**Fig. 7.** Effect of MPAs on the distribution of PAS-positive substance in the ileal mucosa in rats (PAS staining). DIC (10 mg/kg) was administered orally without fasting. MIS (0.1 mg/kg), IRS (10 mg/kg) and REB (100 mg/kg) were administered orally 30 min before DIC. The lower ileum was removed 3 h after DIC treatment. a–e: Typical photographs of the mucosa in each group.

**Fig. 8.** Effect of MPAs on the distribution of PAS-positive substance in the ileal mucosa in rats (Image J analysis). Data represent mean ± SEM values (% of
PAS-positive area, n=6). *: P < 0.05, **: P < 0.01 vs. VEH (Dunnett’s test), ###: P < 0.01 (Student’s t-test). The dose (mg/kg) is listed in parentheses.
Table 1. Effect of MPAs and MUC on small intestinal lesions induced by DIC in rats. DIC (10 mg/kg, p.o.) was administered without fasting. MPAs and MUC were administered orally 30 min before DIC. Intestinal lesions were measured 24 h after DIC administration. Data represent mean ± SEM (n=7 or 8).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg, p.o.)</th>
<th>No. of rats</th>
<th>Lesion index (mm)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>Exp. 1 (MIS)</td>
<td></td>
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<tr>
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<td>7</td>
<td>120.3 ± 13.6</td>
<td>-</td>
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<tr>
<td></td>
<td>0.01</td>
<td>7</td>
<td>93.6 ± 8.5</td>
<td>22.2</td>
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<tr>
<td></td>
<td>0.03</td>
<td>7</td>
<td>80.1 ± 8.4†</td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>7</td>
<td>58.6 ± 10.6***</td>
<td>51.3</td>
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<tr>
<td></td>
<td>0.3</td>
<td>7</td>
<td>32.1 ± 3.4***</td>
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<td>Exp. 2 (IRS)</td>
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<td>-</td>
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<td>1</td>
<td>7</td>
<td>59.4 ± 6.0</td>
<td>18.3</td>
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<tr>
<td></td>
<td>3</td>
<td>7</td>
<td>46.3 ± 3.9†</td>
<td>36.3</td>
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<tr>
<td></td>
<td>10</td>
<td>7</td>
<td>37.0 ± 4.6**</td>
<td>49.1</td>
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<td>30</td>
<td>7</td>
<td>30.1 ± 4.8***</td>
<td>58.6</td>
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Exp. 3 (REB)

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<tr>
<td>VEH</td>
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<td>100.6 ± 10.8</td>
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<td></td>
<td>10</td>
<td>7</td>
<td>80.9 ± 10.5</td>
<td>19.6</td>
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<tr>
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<td>30</td>
<td>7</td>
<td>86.6 ± 6.1</td>
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<td>100</td>
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<td>63.4 ± 10.8</td>
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<td>300</td>
<td>7</td>
<td>48.7 ± 6.5*</td>
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Exp. 4 (MUC)

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<tr>
<td>VEH</td>
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<td>131.6 ± 6.4</td>
<td>-</td>
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<tr>
<td></td>
<td>30</td>
<td>8</td>
<td>103.4 ± 6.6*</td>
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<td>100</td>
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<td>68.6 ± 6.9***</td>
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<tr>
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<td>300</td>
<td>8</td>
<td>61.6 ± 3.5***</td>
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*: P<0.05, **: P<0.01, ***: P<0.001 vs. VEH (Dunnett’s test)
Figure 1

A) Bar graph showing the lesion index (mm) at different DIC doses (mg/kg, p.o.).

B) Image of the lesion with arrows indicating the area of interest.
(Figure 2)
A

(Figure 3A)
(Figure 3B)
(Figure 3C)
(Figure 3D)
Figure 3E

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<thead>
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<th>Group</th>
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<td>VEH</td>
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<td>VEH</td>
<td>90</td>
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<td>1 mg/kg ATR, p.o.</td>
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<tr>
<td>3 mg/kg ATR, p.o.</td>
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<tr>
<td>10 mg/kg ATR, p.o.</td>
<td>10</td>
</tr>
</tbody>
</table>

(Figure 3E)
(Figure 4)
(Figure 5A)
(Figure 5B)
(Figure 5C)
(Figure 5D)
(Figure 7)
(Figure 8)