INTRODUCTION

Egualen Na₃ (sodium 3-ethyl-7-isopropyl-1-azulenesulfonate 1/3 hydrate) (sodium), a more stable azulene derivative than sodium guaiazulene-3-sulfonate (Fig. 1), is known to exhibit antiulcer activity against a variety of experimental gastric lesions in rats (1-3) and is used as an antiulcer drug for the treatment of gastritis and gastric ulcers (4, 5). Although previous studies suggested the mucosal protective action of this drug, which is due to an increase in mucosal blood flow (3), the inhibition of endothelial injury (6), and thromboxane (TX) A₂ antagonistic action (7), the actual mechanisms involved remain unknown.

We recently found that gastric lesions generated by ischemia/reperfusion (I/R), gastric bleeding induced by double antiplatelet therapy with aspirin (ASA) plus clopidogrel, and small intestinal damage generated by loxoprofen, are prevented by drugs that inhibit TXA₂ production or blockage of TXA₂ receptors, suggesting the pathogenic importance of this prostanoid in these models (8, 9). Because TXA₂ is known to potentiate the bradykinin-induced nociceptive response (10) and may be a mediator of epigastric pain (11), TXA₂ antagonistic action is considered crucial to antiulcer drugs. We also set up a model of gastric bleeding induced by double antiplatelet therapy with low-dose aspirin (ASA) plus clopidogrel, a P₂Y₁₂ receptor antagonist (12), and found mucosal protective drugs to be effective in preventing the bleeding (13, 14). Since recent studies have reported a risk of adverse gastric reactions in patients taking antiplatelet drugs with nonsteroidal anti-inflammatory drugs (NSAIDs) (15, 16), it is important to find drugs effective in this model. Furthermore, clinical studies using capsule endoscopes or double-balloon endoscopes have confirmed that NSAIDs damage the small intestine at a higher incidence than previously thought (17-19). Since no satisfactory means for the prevention and treatment of these lesions are currently available, except for the use of PG analogs (19), the identification of effective therapies for the treatment of NSAID-induced small intestinal damage remains an urgent priority. Thus, it is critical to examine whether egualen has beneficial influences on these lesions induced in the stomach and the small intestine, to provide further information for clinical use of this drug in the treatment of gastrointestinal diseases.

In the present study, we examined the effects of egualen against gastric lesions produced by I/R as well as double antiplatelet therapy with ASA plus clopidogrel, and small intestinal lesions generated by loxoprofen in rats and investigated the possible mechanisms involved in the protective action of this drug.

PROPHYLACTIC EFFECT OF EGUALEN SODIUM, A STABLE AZULENE DERIVATIVE, ON GASTROINTESTINAL DAMAGE INDUCED BY ISCHEMIA/REPERFUSION, DOUBLE ANTIPLATELET THERAPY AND LOXOPROFEN IN RATS

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We examined the effect of egualen, a stable azulene derivative, against gastric damage induced by ischemia/reperfusion (I/R), gastric bleeding induced by double antiplatelet therapy with aspirin (ASA) plus clopidogrel, and small intestinal damage generated by loxoprofen, and investigated the possible mechanisms involved in its protective action. Male C57BL/6 mice or SD rats were used under urethane anesthesia (gastric lesions) or in a conscious (intestinal lesions) state. I/R-induced gastric injury was produced in mice by clamping the celiac artery for 30 min, followed by reperfusion for 60 min. Gastric bleeding was induced in rats by luminal perfusion with 25 mM ASA+50 mM HCl for 2 hours in the presence of clopidogrel (30 mg/kg). To produce small intestinal lesions the rats were given loxoprofen (60 mg/kg) p.o. and killed 24 hours later. Egualen was given i.d. 60 min before I/R or ASA perfusion, while given p.o. twice 30 min before and 6 hours after loxoprofen. Egualen significantly prevented the I/R-induced gastric damage, and the effect was equivalent to that of seratrodast (TXA₂ antagonist). This agent also significantly suppressed gastric bleeding induced by ASA plus clopidogrel, similar to PGE₂. Likewise, egualen significantly prevented loxoprofen-induced damage in the small intestine, accompanied by an increase in the secretion of mucus and suppression of bacterial invasion as well as iNOS expression. These results suggest that egualen has a prophylactic effect against various lesions in the gastrointestinal mucosa, probably through its characteristic pharmacological properties, such as TXA₂ antagonistic action, local mucosal protection, and stimulation of mucus secretion.

Key word: egualen, stable azulene derivative, gastrointestinal damage, gastric bleeding, ischemia/reperfusion, aspirin, nonsteroidal antiinflammatory drugs, clopidogrel, double antithrombotic therapy
MATERIALS AND METHODS

Animals

Male C57BL/6 mice (3 months old; SLC, Shizuoka, Japan) and Sprague-Dawley rats (200–260 g; Nippon Charles River, Shizuoka, Japan) were acclimated to standard laboratory conditions (12:12 h light-dark cycle, temperature 22 ± 1°C). Experiments were carried out with or without fasting, using four to six animals in a conscious state, unless otherwise specified. All experimental procedures involving animals were approved by the Experimental Animal Research Committee of the Kyoto Pharmaceutical University.

Induction of ischemia and reperfusion-induced gastric lesions

Acute gastric mucosal lesions were produced by I/R in 18 h-fasted mice, according to a recently published method (8). Briefly, under urethane anesthesia (1.25 g/kg i.p.), the celiac artery was clamped with a small clamp (dispensable vascular clip, holding force 40 g; BEAR Medical Corporation, Chiba, Japan), and 30 min later reperfusion was achieved through removal of the clamp. Then, the animals were killed 60 min after the onset of reperfusion following ischemia for 30 min, and the stomach was excised, inflated by injecting 0.4 ml of 2% formalin for 10 min to fix the tissue walls, and opened along the greater curvature. Egalen (30 and 60 mg/kg), ozagrel (TXA2 synthase inhibitor: 200 mg/kg), or serotonin (TXA2 antagonist: 3–30 mg/kg) was administered p.o. 60 min before ischemia. The area (mm2) of hemorrhagic lesions developed in the stomach was measured under a dissecting microscope (Olympus, Tokyo, Japan) with a square grid (×10). The person measuring the lesions did not know the treatments given to the animals. In some cases, the gastric mucosa was examined for damage under a light microscope following I/R with or without egualen (60 mg/kg). The animals were killed 60 min after the onset of reperfusion following ischemia for 30 min, and the stomach excised. The tissue samples were then immersed in 10% neutralized formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E).

Determination of myeloperoxidase (MPO) activity

MPO activity in the gastric mucosa was measured after I/R treatment in mice, according to a modified version of the method of Krawisz et al. (20). At 60 min after I/R-treatment, the animals were sacrificed by the withdrawal of blood from the heart through perfusion with saline, and the stomach was excised. After rinsing of the tissue with cold saline, the mucosa was scraped with glass slides, weighed, and homogenized in a 50 mM phosphate buffer containing 0.5% hexadecyl-trimethylammonium bromide (HTAB; pH 6.0; Sigma). The homogenized samples were subjected to freezing and thawing three times, and centrifuged at 2,000 g for 10 min at 4°C. MPO activity in the gastric mucosa was obtained from the slope of the reaction curve, recorded on a microplate reader (VERSAmax; Molecular Devices, CA) based on the following equation: Specific activity (µmol H2O2/ min/mg protein) = (OD/min)/OD/µmol H2O2.

An experimental system for effectively evaluating gastric ulcerogenic and bleeding responses to antiplatelet therapy was set up in 18 h-fasted rats under urethane anesthesia, according to a previously published paper (13). Briefly, two catheters were inserted into the rat stomach, one from an incision in the esophagus and another through the pylorus via an incision in the duodenum, and the stomach was then perfused with saline at a rate of 0.4 ml/min using an infusion pump, and the perfusate was collected every 5 min for determination of hemoglobin concentrations. After an equilibration period with saline perfusion for 2 hours, the stomach was perfused with 25 mM ASA plus 50 mM HCl for another 60 min. Clopidogrel (30 mg/kg) was given p.o. 24 hours before the perfusion. The doses of ASA and clopidogrel were determined based on our previous studies (13, 14). Gastric bleeding was evaluated as the hemoglobin concentration in the perfusate. At the end of the experiment (60 min after the onset of ASA+HCl perfusion) the animals were killed for examination of the gastric mucosa. The stomach was excised, treated with 2% formalin for fixation of the tissue walls, and then opened along the greater curvature, and the mucosa was examined for damage under a dissecting microscope (×10). The area (mm2) of macroscopically visible lesions was measured, summed for each tissue, and used as a lesion score. The person measuring the lesions did not know the treatments given to the animals. Egualen (10 and 30 mg/kg) or PGE2 (1 mg/kg) was given i.d. 60 min or i.v. 10 min before the ASA perfusion, respectively.

Determination of hemoglobin concentrations

Gastric bleeding was determined by an increase in the hemoglobin concentration of the gastric perfusate (13, 14). To this end the gastric perfusate was collected every 15 min during the experiment where the stomach was perfused with saline or 25 mM ASA in the presence of clopidogrel pretreatment (30 mg/kg). The concentration of hemoglobin was assessed on a Hitachi spectrophotometer (U-2000, Ibaraki, Japan), according to standard curves made by adding various amounts of rat hemoglobin (Sigma Chemicals, St. Louis, MO) to saline or 25 mM ASA solutions. When rat hemoglobin was dissolved in saline of pH 3.5, maximal absorption occurred at 386 nm. This wavelength was used for the estimation of hemoglobin in the gastric perfusates. Data were analyzed using the Short Softmax Program and the results were presented as micrograms per milliliter or micrograms per 90 min.

Induction of small intestinal damage by loxoprofen

Rats without fasting were administered loxoprofen (60 mg/kg) p.o. and killed 24 hours later under deep ether anesthesia (22). The small intestine was excised, treated with 2% formalin for 10 min to fix the tissue walls, and opened along the anti-mesenteric attachment. The area of macroscopically visible damage (mm2) was measured under a dissecting microscope with square grids (×10), summed per tissue, and used as a lesion score. To highlight hemorrhagic lesions, a 1% Evans blue solution was administered i.v. in a volume of 1 ml/animal 30 min before killing. The person measuring the lesions did not know the treatment given to the animals. Egualen was given p.o. twice daily (3–30 mg/kg: 9:30 AM and 8:30 PM) for 3 days before the administration of loxoprofen or acutely twice (10–100 mg/kg) 30 min before and 6 hours after loxoprofen. In some cases, the small intestine was examined with a light microscope following the administration of loxoprofen (60 mg/kg) with or without egualen (100 mg/kg). The animals were killed 24 hours after the loxoprofen treatment, and

Determination of myeloperoxidase (MPO) activity

MPO activity in the gastric mucosa was measured after I/R treatment in mice, according to a modified version of the method of Krawisz et al. (20). At 60 min after I/R-treatment, the animals were sacrificed by the withdrawal of blood from the heart through perfusion with saline, and the stomach was excised. After rinsing of the tissue with cold saline, the mucosa was scraped with glass slides, weighed, and homogenized in a 50 mM phosphate buffer containing 0.5% hexadecyl-trimethylammonium bromide (HTAB; pH 6.0; Sigma). The homogenized samples were subjected to freezing and thawing three times, and centrifuged at 2,000 g for 10 min at 4°C. MPO activity in the supernatant was determined by adding 50 µl of the supernatant to 50 µl of 10 mM phosphate buffer (pH 6.0) and 50 µl of 1.5 M o-dianisidine hydrochloride (Sigma) containing 0.0005% (v/v) hydrogen peroxide. The changes in absorbance at 450 nm were recorded on a microplate reader (VERSAmax; Molecular Devices, CA) based on the following equation: Specific activity (µmol H2O2/ min/mg protein) = (OD/min)/OD/µmol H2O2 × mg protein). Egalen (30 and 60 mg/kg), ozagrel (TXA2 synthase inhibitor: 200 mg/kg) or serotonin (TXA2 antagonist: 3–30 mg/kg) was administered p.o. 60 min before ischemia.
the small intestine was excised. The tissue samples were then immersed in 10% neutralized formalin, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E).

**Determination of mucus secretion**

The amount of mucus secreted in the small intestine was determined by periodic acid-Schiff (PAS) staining. Three hours after the administration of loxoprofen (60 mg/kg, p.o.), the animals were killed under deep diethyl ether anesthesia, and the small intestines were removed. The removed tissues were fixed in Carnoy’s fluid (ethanol: acetic acid: chloroform = 6:1:3) for 24 hours, embedded in paraffin, and sectioned at a thickness of 8 µm. PAS staining was performed according to the conventional method. Egualen (100 mg/kg) was given p.o. with or without co-administration of loxoprofen. In the combined administration, this agent was given p.o. 30 min before the administration of loxoprofen.

**Determination of enterobacterial counts**

Enterobacteria were enumerated according to a method described by Deitch et al. (23). Six hours after loxoprofen treatment (60 mg/kg, p.o.), the animals were killed under deep ether anesthesia, and the small intestines were removed. After each intestine was rinsed with sterile saline, the mucosa was scraped with glass slides, weighed, and homogenized in 1 ml of sterile phosphate-buffered saline (PBS) per 100 mg of wet tissue. Aliquots of the homogenate were placed on blood agar and Gifuco anaerobic medium agar (Nissui, Tokyo, Japan). Blood agar plates were incubated at 37°C for 24 hours under aerobic conditions (BBL Gas Pack Pouch Anaerobic System; BD Biosciences, San Jose, CA). Plates containing 10 to 300 colony-forming units (CFU) were examined for numbers of enterobacteria, and the data were expressed as log CFU per gram of tissue. Egualen (100 mg/kg) was given p.o. 30 min before the administration of loxoprofen.

**Determination of iNOS mRNA expression**

The expression of iNOS mRNA in the small intestinal mucosa was measured by reverse transcription-polymerase chain reaction (RT-PCR) (24). The animals were killed under deep ether anesthesia 6 hours after the administration of loxoprofen (60 mg/kg, p.o.), and the small intestines were removed and stored at −80°C prior to use. Egualen (100 mg/kg) was given p.o. 30 min before the administration of loxoprofen. Total RNA was extracted from tissue samples using Sepasol RNA I (Nacalai Tesque, Kyoto, Japan). The total RNA was reverse-transcribed with a first strand cDNA synthesis kit (ReverTra Ace alpha, TOYOBO, Osaka, Japan). The sequences of the sense and antisense primers for rat iNOS and GAPDH and each product size are shown in Table 1.

**Preparation of drugs**

The drugs used were urethane (Tokyo Kasei, Tokyo, Japan), egualen Na (Ajinomoto Pharm Co., Kawasaki, Japan), ozagrel, prostaglandin E2 (Cayman Chemical, Ann Arbor, MI), seratrodast (LKT Laboratories, St. Paul, MN), clopidogrel (Sanofi-Aventis, Tokyo, Japan), and loxoprofen (Sigma Chemicals, St. Louis, MO). Loxoprofen was suspended in a hydroxypropylcellulose (HPC) solution (Wako Pure Chemicals, Osaka, Japan). Other agents were dissolved in saline. Each agent was prepared immediately before use and administered p.o. in a volume of 0.1 ml/20 g body weight or 1 ml/200 g body weight, respectively, in mice or rats, and i.d. or i.v. in a volume of 1 ml/200 g body weight or 0.1 ml/100 g body weight, respectively, in rats. Control animals received the vehicle in the same volume and via the same route.

**Statistical analysis**

Data are presented as the mean±S.E. for 5–7 mice or 4–13 rats per group. Statistical analyses were performed using a one-way analysis of variance (ANOVA) and Student’s t-test or Dunnett’s multiple comparison test where appropriate, and values of P<0.05 were considered significant.

**RESULTS**

**Effect of egualen on ischemia/reperfusion-induced gastric damage in mice**

Laparotomy without clamping of the gastric artery (sham operation) did not produce any damage in the mouse gastric

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Sequences</th>
<th>PCR Product</th>
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<tbody>
<tr>
<td>iNOS</td>
<td>780 bp</td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-CGGTTTACACGTCTTGTGAAG-3'</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-CAGGTGTTCCTCCCAGGTAG-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
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<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5'-GAAAGGGAAGCTTACTGGCATGCC-3'</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5'-TGAGGTCCACCACCTGGTCTG-3'</td>
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mucosa. In the animals subjected to I/R treatment (30 min of ischemia followed by reperfusion for 60 min), however, multiple hemorrhagic lesions were observed in the gastric mucosa, the lesion score being 9.6±1.9~10.3±1.1 mm² (Fig. 2). Pretreatment of the animals with ozagrel (200 mg/kg, p.o.), a TXA₂ synthase inhibitor, significantly prevented the I/R-induced development of gastric lesions, the inhibition being 57.1%. Likewise, the severity of these lesions was dose-dependently reduced by prior administration of seratrodast (3–30 mg/kg), a TXA₂ antagonist, and the effect was significant at 10 mg/kg or greater. Equalen (30 and 60 mg/kg) also dose-dependently and significantly mitigated the severity of the I/R-induced gastric damage, and the effect at 60 mg/kg was as potent as that of seratrodast at 30 mg/kg, the inhibition being 64.1%. In sham-operated animals without I/R treatment, no damage was detected even by histological observation (Fig. 3A). By contrast, severe damage was observed histologically in the stomach after I/R treatment; most of the damage was restricted to the surface epithelium, but some damage occurred deep in the mucosa (Fig. 3B). When the animals were pretreated with equalen (60 mg/kg, p.o.), the severity of the histological damage was markedly reduced, and only slight damage was observed in the surface epithelium (Fig. 3C).

**Myeloperoxidase (MPO) activity**

Gastric mucosal MPO activity in sham-operated mice was less than 0.02 µmol H₂O₂/min/mg protein. The MPO activity was markedly increased after I/R, the values being 0.044±0.007 µmol H₂O₂/min/mg protein. This response was significantly abrogated by the prior administration of equalen (60 mg/kg, p.o.), the inhibition being 51.5%. Both ozagrel (200 mg/kg, p.o.) and seratrodast (30 mg/kg) also suppressed the increase of MPO activity induced by I/R, the inhibition being 66.7% and 66.9%, respectively.

**Effect of equalen on gastric bleeding and ulcerogenic responses to acidified ASA with or without clopidogrel pretreatment**

Perfusion of the rat stomach with acidified ASA (25 mM in 50 mM HCl) produced few lesions, but the ulcerogenic response to acidified ASA was aggravated by pretreatment with clopidogrel (30 mg/kg). These treatments caused a time-dependent increase in the hemoglobin concentration, resulting in many hemorrhagic lesions in the stomach (Figs. 4 and 5A). PGE₂ (1 mg/kg, i.v.) markedly reduced gastric bleeding and hemorrhagic damage induced by acidified ASA in the presence of clopidogrel; the total hemoglobin output and hemorrhagic lesions were both reduced to about 20% of that in the vehicle-treated animals. Equalen (10 and 30 mg/kg, i.d.) dose-dependently reduced gastric bleeding and ulcerogenic responses to acidified ASA in the presence of clopidogrel, and the effects were significant at 30 mg/kg. Notably, prior administration of equalen at 30 mg/kg also decreased the severity of lesions under such conditions almost as effectively as PGE₂ at 1 mg/kg, the inhibition being 62.1%. As shown in Fig. 5B and Fig. 5C, the severity of gastric lesions produced by 25 mM ASA plus clopidogrel was markedly reduced by pretreatment with equalen (30 mg/kg), converting the lesions from hemorrhagic to non-hemorrhagic.

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Fig. 1. Chemical structure of equalen Na (sodium 3-ethyl-7-isopropyl-1-azulesmesulfonate 1/3 hydrate).

![Image](image1.png)

**Fig. 2.** Effects of equalen, ozagrel, and seratrodast on gastric lesions induced by I/R in mice. Under urethane anesthesia, the celiac artery was clamped. Reperfusion followed 30 min later with removal of the clamp, and then, the stomach was excised 60 min later. Equalen (3 and 60 mg/kg), ozagrel (200 mg/kg), or seratrodast (3–30 mg/kg) was given p.o. 30 min before the onset of ischemia. Data are presented as the mean±S.E. for 5–7 mice. *Significant difference from control, at P<0.05.
Orally administered loxoprofen (60 mg/kg) in normally fed rats produced multiple hemorrhagic lesions in the small intestine, the lesion score being 223.2±25.1 mm² (Fig. 6). When the animals were pretreated with egualen (3–30 mg/kg) given p.o. twice daily for 3 days prior to loxoprofen treatment, the development of these intestinal lesions was prevented in a dose-dependent manner, and a significant effect was observed at 30 mg/kg, the inhibition being 64.3%. Likewise, this drug exhibited a prophylactic effect when administered acutely twice 30 min before and 6 hours after the

**Effect of egualen on loxoprofen-induced small intestinal damage**

Orally administered loxoprofen (60 mg/kg) in normally fed rats produced multiple hemorrhagic lesions in the small intestine, the lesion score being 223.2±25.1 mm² (Fig. 6). When the animals were pretreated with egualen (3–30 mg/kg) given p.o. twice daily for 3 days prior to loxoprofen treatment, the development of these intestinal lesions was prevented in a dose-dependent manner, and a significant effect was observed at 30 mg/kg, the inhibition being 64.3%. Likewise, this drug exhibited a prophylactic effect when administered acutely twice 30 min before and 6 hours after the
administration of loxoprofen. In this case, egualen (10–100 mg/kg) also dose-dependently reduced the severity of the small intestinal lesions generated by loxoprofen, although the effective dose was slightly higher than that of the repeated administrations for 3 days. In particular, this drug at 100 mg/kg significantly suppressed the occurrence of these lesions in response to loxoprofen; the inhibition being 68.7%. Histologically, the lesions produced by loxoprofen were deep in the mucosa, almost reaching the muscularis mucosae (Fig. 7), while the severity of the damage was markedly lessen in rats pretreated with egualen (100 mg/kg).

Effect of egualen on various events induced in the small intestinal mucosa by loxoprofen treatment

It was found that egualen prevented the occurrence of damage in the small intestine after loxoprofen treatment when the animals

Fig. 6. Effect of egualen on loxoprofen-induced small intestinal damage in rats. Animals were given loxoprofen (60 mg/kg) p.o. and killed 24 hours later. Egualen (10–100 mg/kg) was given p.o. twice 30 min before and 6 hours after the administration of loxoprofen or twice daily (9:30 AM and 8:30 PM) for 3 days prior to loxoprofen treatment. Data are presented as the mean±S.E. for 4–13 rats. ÆSignificant difference from control, at P<0.05.
were even acutely pretreated 30 min before loxoprofen treatment, and this effect at 100 mg/kg was significant and reproducible. To further investigate the functional mechanism responsible for the prophylactic action of egualen, we examined the effects of egualen at 100 mg/kg on various events that are considered critical in the pathogenesis of NSAID-induced enteropathy (24).

Enterobacterial invasion

Aerobic and anaerobic bacterial counts in the normal intestinal mucosa were 6.11±0.30 and 6.49±0.34 log CFU/g tissue, respectively. Those following the administration of loxoprofen (60 mg/kg) were about 70–90 times greater after 6 hours, being 8.02±0.47 and 8.04±0.40 log CFU/g tissue, respectively. Pretreatment with egualen (100 mg/kg) given p.o. 30 min before the administration of loxoprofen significantly suppressed the enhanced invasion of enterobacteria following loxoprofen treatment, the number of bacteria being 6.76±0.26 and 6.88±0.22 log CFU/g tissue, respectively.

Mucosal expression of iNOS mRNA

Expression of iNOS mRNA was not detected in the normal intestine, yet markedly upregulated in the mucosa when
Fig. 9. Effect of loxoprofen on mucus secretion (PAS staining) in the rat small intestine, with or without loxoprofen treatment. The animals were given loxoprofen (60 mg/kg) or egualen (100 mg/kg) p.o. and killed 3 hours later for examination of PAS staining. In the combined administration, egualen was given p.o. 30 min before loxoprofen. Figures show: (A) normal; (B) loxoprofen alone; (C) egualen plus loxoprofen; (D) egualen alone. (PAS; ×100). Note that loxoprofen markedly decreased PAS-positive materials in the mucosa, but this response was apparently prevented by prior administration of egualen.

Fig. 10. Factors involved in the development of loxoprofen-induced small intestinal damage, and the influences of egualen on these processes. Loxoprofen causes functional changes such as an increase in intestinal motility and a decrease in mucus secretion, followed by enterobacterial invasion in the mucosa. Endotoxin released from enterobacteria upregulates iNOS expression and NO production as well as inflammation, which results in damage to the small intestine. Egualen increases mucus secretion and hampers the mucosal invasion of enterobacteria, and by so doing suppresses the upregulation of iNOS expression, eventually leading to protection against damage in the small intestine.
examined 6 hours after the administration of loxoprofen (60 mg/kg) (Fig. 8). The upregulation of iNOS expression caused by loxoprofen was mitigated by prior administration of egualen (100 mg/kg).

Mucus secretion

In the normal intestinal mucosa, PAS-positive substances were clearly observed over the surface epithelial cells and along the glands (Fig. 9A). Loxoprofen (60 mg/kg) apparently reduced the amount of PAS-positive substances on the epithelial cells as well as in the glands (Fig. 9B). However, when the animals were pretreated with egualen (100 mg/kg, p.o.) before the loxoprofen treatment, the decrease in PAS staining was prevented and the amount of PAS-positive materials in the mucosa was largely restored (Fig. 9C). In addition, egualen alone also increased the amount of PAS-positive substances in the mucosa when compared to the control mucosa (Fig. 9D).

DISCUSSION

Egualen, a stable azulene derivative, has been used to treat gastritis and gastric ulcers (4, 5). Although previous studies have showed a prophylactic effect of this drug in various models of damage in the stomach and duodenum (1-3, 6), no study had evaluated the effectiveness of egualen on small intestinal damage. In the present study, we examined the effect of egualen on NSAID-induced enteropathy as well as gastric injury induced by I/R or dual antiplatelet therapy, and found that the drug effectively suppressed the occurrence of damage.

First, we examined the effect of egualen on the development of gastric damage under I/R conditions. Previous study showed that I/R-induced gastric damage was worsened by pretreatment with indomethacin and rofecoxib (a selective cyclooxygenase (COX)-2 inhibitor) but not SC-560 (a selective COX-1 inhibitor), and this aggravation was abrogated by COX-2 inhibitor) but not SC-560 (a selective COX-1 inhibitor), and this aggravation was abrogated by co-administration of iloprost (a prostacyclin (PGI2) analogue), suggesting a role for COX-2/PGI2 in mucosal defense under such conditions (8, 26). Furthermore, we have also shown that the severity of these lesions was reduced by pretreatment with ozagrel or seratorodast, similar to iloprost, suggesting the involvement of COX-1/TXA2 in the pathogenesis of I/R-induced gastric damage, in addition to COX-2/PGI2 (9). As expected, the present study showed that egualen significantly prevented the development of gastric damage under I/R conditions, similar to ozagrel and seratorodast. We also confirmed the neutrophil infiltration in the stomach during I/R-treatment, as represented by a marked increase of MPO activity (27), and further observed that egualen attenuated the increase of MPO activity following I/R treatment. Because egualen is known to have a TXA2 antagonistic action (7), the present results further supported the pathogenic importance of TXA2 in I/R-induced gastric damage.

Recent clinical studies showed that the risk of gastric bleeding is increased by the concomitant use of antiplatelet drugs with NSAIDs or low-dose ASA in the presence of exogenous or endogenous acid in the rat stomach (15, 16, 28). Clopidogrel is an antiplatelet drug that specifically and irreversibly inhibits the P2Y12 subtype of the adenosine diphosphate receptor, which is important to the aggregation of platelets and cross-linking by the protein fibrin (29). The present study confirmed in rats that pretreatment with clopidogrel aggravated gastric bleeding in response to acidified ASA, and eventually increased the severity of the acidified ASA-induced hemorrhagic damage in the stomach. Ritchie et al. (30) proposed three factors essential for the generation of gastric lesions; 1) gastric barrier disruption, 2) luminal acid, and 3) gastric mucosal ischemia. ASA is known to disrupt the gastric mucosal barrier, damage the stomach in the presence of acid, and attenuate the gastric hyperemic response to acid back-diffusion following barrier disruption by suppressing PG production (31). Thus, the model we used fulfills the conditions necessary to generate damage in the stomach. In the present study, egualen dose-dependently and significantly mitigated gastric bleeding and ulcerogenic responses to acidified ASA plus clopidogrel and the effect was almost equivalent to that of PGE2. Similar effects have been observed with several other agents used as mucosal protective drugs in the treatment of gastric ulcers (13, 14, 28). The mechanism by which egualen prevented gastric bleeding and damage under these conditions remains unknown. However, because egualen binds to the gastric mucosa via a non-specific hydrophobic interaction to form a complex that is less vulnerable to ASA irritation and because this drug exhibits gastric hyperemic and anti-inflammatory effects (1-3, 6, 7), it is assumed that egualen prevents gastric bleeding and lesions under such conditions, probably by its local mucosal protective action and anti-inflammatory action. At present, the clinical effectiveness of mucosal protective drugs against gastric bleeding associated with dual antiplatelet therapy remains unknown. Further animal and clinical studies are needed to clarify these points.

Most important in the present study is the finding that egualen, given acutely or subacutely for 3 days before, prevented the development of small intestinal damage following the administration of loxoprofen, a NSAID frequently used in Asian countries. NSAIDs cause intestinal ulceration in humans and laboratory animals after short-term and long-term administration (32, 33). In particular, recent clinical studies using capsule endoscopes or double-balloon endoscopes confirmed that NSAIDs damage the small intestine at a higher incidence than previously thought (34). Several factors have been implicated in the pathogenesis of NSAID-induced small intestinal ulceration, including bacterial flora, bile acid, and hypermotility, in addition to PG deficiency (24, 25, 35). Since egualen did not affect the decreased PGE2 content in the presence of loxoprofen (data not shown), it is assumed to act downstream of the events resulting from the PG deficiency caused by loxoprofen and eventually prevent the development of small intestinal lesions.

NSAID-induced intestinal damage is prevented by pretreatment with antibiotics such as ampicillin (24, 25), suggesting a key pathogenic role for enterobacteria in this model. The importance of bacterial was also demonstrated in the pathogenesis of not only the small intestinal damage but also colonic damage induced by NSAIDs (36). Boughton-Smith et al. (37) reported that bacterial endotoxin enhanced intestinal permeability through up-regulation of iNOS expression and overproduction of NO in the mucosa. This was further supported by the findings that indomethacin increased iNOS activity and NO production, preceding the onset of intestinal damage, and that aminoguanidine prevented the intestinal ulcerogenic response by suppressing NO production due to iNOS (24). As expected, we observed that loxoprofen caused bacterial invasion in the mucosa, followed by the up-regulation of iNOS expression and MPO activity, and these responses were suppressed by egualen. These findings suggest that the protective effect of egualen against loxoprofen-induced intestinal damage is functionally associated with the down-regulation of iNOS expression resulting from the suppression of bacterial invasion. The mechanism by which enterobacteria invade the mucosa remains unknown, yet previous studies suggest that a decrease in mucus secretion and an increase of mucosal permeability may contribute to this process after indomethacin treatment (22, 38, 39). Since mucus plays a crucial role in innate host defenses against
intestinal pathogens and irritants, it is possible that a decrease in mucus secretion weakens the intestinal barrier, resulting in bacterial invasion.

In the present study, we found that the amount of PAP-positive materials in the small intestine was markedly reduced after loxoprofen treatment, but this response was restored by prior administration of egualen. Furthermore, egualen by itself apparently increased the amount of PAP-positive substances in the mucosa. It is assumed that egualen stimulates mucus secretion, thereby increasing the mucus gel’s thickness and hampering bacterial invasion following the administration of loxoprofen. Thus, the present results together suggest that egualen protects the small intestine against loxoprofen-induced damage, and this effect may be functionally associated with an increase in the secretion of mucus, resulting in suppression of bacterial invasion and iNOS expression, the major pathogenic events in NSAID-induced small intestinal ulceration (Fig. 10).

Given the findings of the present study, we conclude that egualen has a prophylactic effect against gastric damage induced by I/R and gastric bleeding induced by double antiplatelet therapy with ASA plus clopidogrel as well as small intestinal damage generated by loxoprofen, probably through its characteristic pharmacological properties, such as TXA2 antagonistic action, local mucosal protection, and increase in mucosal blood flow as well as mucus secretion (2, 3, 7).

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REFERENCES

24. Takeuchi K, Yokota A, Taniguchi M, Takahira Y, Tanaka A. Factors involved in up-regulation of inducible nitric oxide synthase in rat small intestine following administration of...


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