Original Article

Rebamipide protects small intestinal mucosal injuries caused by indomethacin by modulating intestinal microbiota and the gene expression in intestinal mucosa in a rat model

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The effect of rebamipide, a mucosal protective drug, on small intestinal mucosal injury caused by indomethacin was examined using a rat model. Indomethacin administration (10 mg/kg, p.o.) induced intestinal mucosal injury was accompanied by an increase in the numbers of intestinal bacteria particularly *Enterobacteriaceae* in the jejunum and ileum. Rebamipide (30 and 100 mg/kg, p.o., given 5 times) was shown to inhibit the indomethacin-induced small intestinal mucosal injury and decreased the number of *Enterococcaceae* and *Enterobacteriaceae* in the jejunal mucosa to normal levels. It was also shown that the detection rate of segmented filamentous bacteria was increased by rebamipide. PCR array analysis of genes related to inflammation, oxidative stress and wound healing showed that indomethacin induced upregulation and downregulation of 14 and 3 genes, respectively in the rat jejunal mucosa by more than 5-fold compared to that of normal rats. Rebamipide suppressed the upregulated gene expression of TNFα and Duox2 in a dose-dependent manner. In conclusion, our study confirmed that disturbance of intestinal microbiota plays a crucial role in indomethacin-induced small intestinal mucosal injury, and suggests that rebamipide could be used as prophylaxis against non-steroidal anti-inflammatory drugs -induced gastrointestinal mucosal injury, by modulating microbiota and suppressing mucosal inflammation in the small intestine.

***Key Words*:** **non-steroidal anti-inflammatory drugs, small intestine, oxidative stress, rebamipide, intestinal flora**

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in clinical medicine, and their use is reported to be associated with a broad spectrum of adverse reactions in the liver, kidney, skin and gastrointestinal tract.[1] NSAIDs have toxic effects in the small and large intestine as well as stomach.[2] The NSAID loxoprofen sodium was reported to induce erosions and ulcers along the mesenteric margin of the distal jejunum,[3] and it was also demonstrated that the number of both aerobic and anaerobic bacteria markedly increased at the sites of the lesions. Other similar studies show that NSAID induced small intestinal mucosal injury is closely associated with dysbiosis of the small intestinal microbiota.[4,5]

Use of acid-suppressing drugs of the proton pump inhibitors (PPI) and histamine 2-receptor antagonist (H2) families has been associated with a 1.5- to 2-fold increased risk of community-acquired pneumonia.[6,7] According to a recent study,[8] 12% (71 out of 608 cases) of current PPI/H2 users had recurrent pneumonia, compared with 8% of nonusers, indicating that acid-suppressing drug use substantially increased the likelihood of recurrent pneumonia in high-risk elderly patients.

Rebamipide 2-{4-cholorobenzoylamino-3-[2(1*H*)-quinolinon-4-yl]} propionic acid, CAS 11911-87-6, is a gastric mucosal stabilizer and gastric mucosal prostaglandin inducer, and is used for treatment of gastric ulcers and gastritis.[9] It has been reported that rebamipide has various biological effects including increasing the production of mucus and prostaglandins, scavenging hydroxyl radicals, inhibition of neutrophil activation and suppression of gastric mucosal inflammation.[10–12] In addition to these effects, rebamipide has been reported to prevent NSAID-induced peptic ulcer in patients on long-term NSAID therapy.[13,14]

Wallace *et al.*[15] reported that PPIs (omeprazole and lansoprazole) significantly exacerbated naproxen- and celecoxib-induced intestinal ulceration and bleeding in rats. It was also indicated that omeprazole treatment resulted in significant increase in the number of aerobic bacteria and significant decrease in the number of Actinobacteria including *Bifidobacterium* spp. Colonization of germ-free mic with jejunal bacteria from PPI-treated rats increased the severity of NSAID-induced intestinal injury.

In the present study, the effects of rebamipide on small intestinal microbiota including aerobic and anaerobic bacteria were examined by both culture and real-time PCR techniques. In addition, the effect of rebamipide on the expression of genes in the jejunal mucosa after indomethacin administration was analyzed by quantitative real-time RT (reverse-transcription)-PCR assay.

Methods

Ethics statement. This study was carried out in accordance with Guidelines for Animal Care and Use in Otsuka Pharmaceutical Co., Ltd. And this study was approved by the Institutional Animal Care and Use Committee of Kyorin University School of Medicine (Approval No. 107-1, 107-2).

Animals. Specific-pathogen-free male Wistar/ST strain rats (6-weeks old) were purchased from Japan SLC Co. (Hamamatsu, Japan), and used after one week habituation. The animals were given CRF-1 (Oriental Yeast Co., Ltd., Osaka, Japan) and tap water *ad libitum*.

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Indomethacin-induced small intestinal injury. To induce small intestinal injury, indomethacin 10 mg/kg suspended in 0.5% carboxylmethylcellulose sodium salt (CMC) was orally administered in a volume of 4 ml/kg to non-fasted animals that were sacrificed 24 h later. Under the isoflurane gas anesthesia, 1 ml of blood was obtained from the abdominal vein and hematologic analysis was performed using an automated hematology analyzer (Sysmex XT-2000iV, Sysmex Co., Kobe, Japan). The animals were killed by cutting the abdominal aorta. The small intestine was removed and cut opened along the anti-mesenteric border, and gently rinsed with saline to remove fecal contents. The small intestine was divided into 4 segments named proximal, mid and distal jejunum and ileum. Image were captured by a digital camera for macroscopic evaluation of the mucosal lesion area. This was calculated using computer-assisted image analyzer software (WinROOF ver. 5, Mitani Corporation, Fukui, Japan) and summed per small intestine. The examiner was unaware of animal treatment.

Experimental groups. The rats were allocated to five experimental groups using stratified random sampling on the basis of weight on the experiment day. Rebamipide (30 and 100 mg/kg, Otsuka Pharmaceutical Co., Ltd.) was orally administered five times, 24 h, 16 h and 1 h prior to, and 3 h and 7 h after indomethacin treatment. Ampicillin (800 mg/kg, Sigma-Aldrich Co., St. Louis, MO) was orally administered twice, 24 h and 1 h before indomethacin treatment. Multiple oral administrations of rebamipide were given to ensure adequate intestinal mucosal distribution, due to the relatively short half-life of the drug.[9] In the control group, 0.5% CMC was orally administered according to the same dosing regimen as the rebamipide groups. Untreated animals were also prepared as normal group. Each group consisted of 10 animals except the normal group, which contained 6 animals.

Examination of microbiota in the small intestinal mucosa. Immediately after capturing the macroscopic image, the intestinal mucosa was obtained by scraping each specimen with a glass slide and weighed. A small amount of the mucosa was frozen in liquid nitrogen and stored at −80°C for mRNA extraction.

The mucosal samples were added in a 9 fold volume of anaerobic dilution buffer and homogenized by a homogenizer. The diluted mucosal samples were divided into several tubes and stored at −80°C until use for culture and extraction of DNA.

DNA extraction from mucosal samples. The total DNA of the microbiota contained in the mucosa of each rat was extracted from diluted mucosal samples using the QIAamp DNA Stool kit (QIAGEN Valencia, CA). Two-hundred µl of each sample was added to 1.4 ml of buffer ASL (QIAGEN). Consequent steps were performed according to manufacture documentation. Finally, a total of 100 µl of DNA solution was obtained.

Analysis of viable bacterial flora in small intestine. Small intestinal mucosal samples from three jejunal and ileal sites were homogenized and 10-fold diluted in anaerobic diluent (KH_2PO_4 4.5 g, Na_2HPO_4, 6.0 g, L-cysteine hydrochloride 0.5 g, Tween 80 0.5 g and agar 0.5 g in 1 L of distilled water),[14] and stored at −80°C for later analysis.

The total viable aerobic and facultative anaerobic bacterial counts were determined by culture method on MacConkey agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and Brain Heart Infusion (BHI) agar (Difco, Detroit, MI) containing 5% horse blood as described previously.[16] The viable obligate anaerobe counts were cultured on Gifu Anaerobic Medium (GAM) agar (Nissui Pharmaceutical Co., Ltd.).

DNA extraction from standard bacterial strains. Standard strains of *Lactobacillus casei* (*L. casei*) ss. casei JCM 1134^T, *Enterococcus faecalis* (*E. faecalis*) ATCC 19433^T and *Escherishia coli* (*E. coli*) ATCC 25922 were used in this study. These microorganisms were obtained from the Japan Collection of Microorganisms, RIKEN BioResource Center (Saitama, Japan) (JCM/ATCC). *E. faecalis* ATCC 19433^T and *E. coli* were inoculated into Brain Heart Infusion broth (Difco) and cultured under aerobic conditions at 37°C. *L. casei* ss. *casei* JCM 1134^T was cultured anaerobically in GAM broth supplemented with 1% glucose at 37°C for 18 to 48 h. The bacterial samples were diluted and plated onto BHI or GAM agar. The plates were subsequently incubated at 37°C for 3 to 5 days in an incubator (SANYO Electric Co., Ltd., Tokyo, Japan) or anaerobic chamber (Hirasawa Works Inc., Tokyo, Japan), and cultural counts (in CFU) were determined in triplicate. Extraction of the standard DNA from the cultures (1 ml, $<5 \times 10^8$ CFU) of these bacteria was performed by using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). After centrifugation (21,000 × g for 2 min) of the cultures and removing the supernatant, each pellet was resuspended in 480 µl of 50 mmol/L EDTA. Then 120 µl of lysozyme (400 µg/ml for Gram negative bacteria or 1 mg/ml for Gram positive bacteria) was added to the suspension followed by incubation at 37°C for 60 min. According to the manufacturer's instruction, purified DNA was finally eluted by 100 µl of nuclease-free water. Segmented filamentous bacteria (SFB) was not culturable under *in vitro* condition. The SFB DNA (3 µg/ml, 8.4×10^4 bacterial number/ml) for this experiment was kindly provided by Dr. Koji Sawada, Yakult Central Institute.

Quantitative (qt) real-time PCR. qt-PCR amplification and detection was performed with an ABI PRISM 7500 sequence detection system (Applied Biosystems (ABI), Foster City, CA) as previously reported.[17] Each reaction mixture (10 µl) was prepared with 5 µl of SYBR Green I Mater mix (Takara Bio. Inc., Otsu, Japan), 1 µl of each of the specific primers (0.05 µmol/L) and 1 µl of 1× or 10× diluted template DNA. PCR was performed by one cycle of denaturing at 94°C for 5 min and annealing at 60, 58, 55 or 50°C for 20 s (Supplemental Table 1*)[18–21] and extension of the DNA chain at 72°C for 35 s, followed by final denaturing at 94°C for 15 s. The fluorescent products were detected at the last step of each cycle in the reactions.

According to the standard curves made by amplifying serial dilutions of a known quantity of amplicon, bacterial numbers in gastric samples were calculated based on the PCR kinetics of target genes in the sample.

In parallel, different amounts of chromosomal DNA of standard strain were used for PCR with each primer set. DNA extracted from *L. casei* ss. *casei* JCM 1134^T, *E. coli* ATCC25922, *E. faecalis* ATCC 19433^T and SFB were used as real-time PCR standards.

The standard ABI PRISM 7500 quantification software (Applied Biosystems) was used for data analysis. After amplification to distinguish the target product from non-specific annealing, the melting curve analysis was used for confirming the specificity of the PCR product. The melting curves were made by heating at a range of 60 to 95°C at a slow rate of 0.2°C/s, with continuous fluorescence collection. To confirm the specificity of PCR, the melting temperature (Tm) value of standard strains was determined, and the Tm obtained with standard Tm ± 1°C was considered to be identical to the target product.

As the genus *Lactobacillus* exhibited a wider range of PCR products between species, Tm ± 2°C was used for the above determination.

PCR array for the analysis of intestinal mucosal gene expression. Small intestinal mucosa from mid jejunum immersed in RNAlater (Ambion Inc., Austin, TX) was dissolved in Nucleic Acid Purification Lysis Solution (Applied Biosystems). Total RNA was purified by using the ABI PRISM 6100 Nucleic Acid PrepStation System (Applied Biosystems) according to the manufacturer's instructions. The RT2 Profiler PCR Array System was used to quantify mRNA expression with common rat cytokines and chemokines-, oxidative stress-, and wound healing-related genes (84 genes of each, Qiagen). The same quantity of RNA from each sample (10 samples per group except for 6 samples from the normal group) was mixed to make a pooled

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RNA up to a total of 100 ng, which was reverse-transcribed with the RT2 First Strand Kit (Qiagen). cDNA was mixed with the RT2 SYBR Green/ROX qPCR Master Mix (Qiagen) and amplified on an ABI 7500 Fast 96-well real-time PCR machine (Applied Biosystems) according to the manufacturer's protocol. Results were analyzed with the RT2 Profiler Array Data Analysis ver. 3.5 at the manufacturer's website (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

RT-PCR for the analysis of intestinal mucosal gene expression. Small intestinal mucosa immersed in RNAlater (Ambion) was dissolved in Nucleic Acid Purification Lysis Solution (Applied Biosystems). Total RNA was purified using the ABI PRISM 6100 Nucleic Acid PrepStation System (Applied Biosystems) according to manufacturer's instructions. cDNA was synthesized with MultiScribe Reverse Transcriptase (Applied Biosystems) and real-time PCR was performed using the Applied Biosystems 7500 Fast real-time PCR System. The reaction mixture was prepared according to the manufacturer's protocol using TaqMan® Gene Expression Primer & Probe (Applied Biosystems). The thermal cycling conditions were 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. The expression levels of mRNA for TNFα, NADP oxidase type 2 (Nox2), dual oxidase 1 (Duox1) and dual oxidase 2 (Duox2) were standardized to β-actin mRNA and the relative expression of each gene was quantified by the comparative Ct (ΔΔCt) method as ratios to the mean value for normal intestinal tissue.

Statistical analysis. The data were expressed as the mean ± SEM for all rats in each group. Statistical analysis was performed on the raw data using the two-tailed *t* test for the comparison between two groups. One-way analysis of variance followed by two-tailed Dunnett multiple comparison test was performed between control and rebamipide-treated groups. Differences with *p* values less than 0.05 were considered significant. The Student's *t* test for unpaired values was used to compare the differences between the three groups.

Results

Effects of rebamipide and ampicillin on small intestinal mucosal injury after indomethacin administration. Macroscopically, multiple mucosal injuries characterized by segmental round ulcerations extending along the mesenteric border of the jejunum (Fig. 1A, arrows) and ileum were observed 24 h after administration of indomethacin 10 mg/kg p.o. Intestinal lesion area in the control group (0.5% CMC, p.o.) was 175.6 ± 19.2 mm^2 (Fig. 1B). Rebamipide 30 and 100 mg/kg, p.o. significantly reduced the intestinal lesion area in a dose-dependent manner, by 41% ($p<0.01$) and 51% ($p<0.01$), respectively. Ampicillin 800 mg/kg, p.o. also significantly reduced the lesion area by 56% ($p<0.01$).

The most severe lesions were observed in the mid jejunum. (63.8 ± 19.2 mm^2) followed by distal jejunum (55.8 ± 10.1 mm^2), proximal jejunum (33.9 ± 20.1 mm^2) and ileum (22.1 ± 5.1 mm^2) (Fig. 2A–D). Rebamipide dose-dependently reduced the lesion at the sites of proximal, mid and distal jejunum, and the reduction rates at 100 mg/kg were 54% (not significant), 79% ($p<0.01$) and 45% ($p<0.05$), respectively. Ampicillin also significantly inhibited mucosal injury in the mid-jejunum (64%, $p<0.05$), distal jejunum (44%, $p<0.05$) and ileum (76%, $p<0.01$).

Suppression of indomethacin-induced small intestinal bleeding by rebamipide and ampicillin administration. Indomethacin-induced small intestinal injury is often accompanied by mucosal bleeding. In this study, severity of small intestinal bleeding was diagnosed by a blood test. As shown in Table 1, red blood cell number was significantly reduced by 27% ($p<0.01$), accompanied by significant reductions in hemoglobin level (27% reduction, $p<0.01$) and hematocrit ratio (22% reduction, $p<0.01$) 24 h after indomethacin administration. Anemia was inhibited by rebamipide 30 and 100 mg/kg in a dose-dependent manner, and was completely abolished by ampicillin 800 mg/kg. The indomethacin treatment also significantly reduced the platelet number by 35% ($p<0.01$) and the white blood cell number by 6% ($p<0.01$). Rebamipide 100 mg/kg and ampicillin recovered the platelet and the white blood cell numbers.

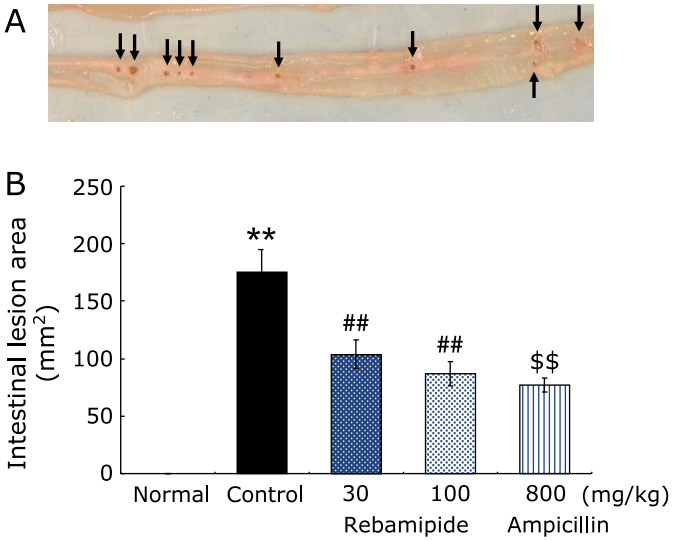

Fig. 1. Effect of rebamipide and ampicillin on indomethacin-induced small intestinal mucosal injury in rats. (A) Macroscopic appearance of the mucosal injuries (arrows) in the mid-jejunum 24 h after indomethacin 10 mg/kg p.o. (control, 0.5% CMC). (B) Rebamipide was orally administered five times, 24, 16 and 1 h prior to, and 3 and 7 h after indomethacin administration. Ampicillin was orally administered twice, 24 and 1 h prior to indomethacin administration. $^{**}p<0.01$ Normal vs Control by *t* test (two-tailed), $^{\#\#}p<0.01$ Rebamipide vs control by Dunnett test (two-tailed), $^{\$\$}p<0.01$ ampicillin vs control by *t* test (two-tailed).

Evaluation of total bacterial number by culture. The total viable number of aerobes, facultative anaerobes and obligate anaerobes were increased in the jejunum of control rats by oral administration of indomethacin (Supplemental Fig. 1 and 2*). Furthermore, the number of *Enterobacteriaceae* was markedly increased by 1,000–10,000 times in the jejunum of indomethacin-treated control rats compared with normal rats untreated with indomethacin (Supplemental Fig. 3*).

The numbers of total aerobes and facultative anaerobes in proximal and central jejunum were decreased by treatment with rebamipide 100 mg/kg (Supplemental Fig. 1a and b*), and the number of total obligate anaerobes was decreased in mid jejunum by treatment with rebamipide 100 mg/kg (Supplemental Fig. 2b*). However, the numbers of aerobes, facultative anaerobes and obligate anaerobes were not decreased by treatment with rebamipide in the distal jejunum and ileum (Supplemental Fig. 1c, 1d, 2c and 2d). The number of *Enterobacteriaceae* was significantly decreased by treatment with rebamipide 100 mg/kg in all 4 small intestinal sites (Supplemental Fig. 3*). In particular, the number of *Enterobacteriaceae* was markedly reduced by treatment with rebamipide 100 mg/kg in the proximal jejunum (Supplemental Fig. 3a*).

Ampicillin treatment extensively decreased the number of total aerobes, facultative anaerobes and obligate anaerobes compared with normal rats (Supplemental Figs. 1 and 2*). It was also shown that the number of *Enterobacteriaceae* in jejunum and ileum of the ampicillin-treated rats was significantly lower than that in control rats (Supplemental Fig. 3*).

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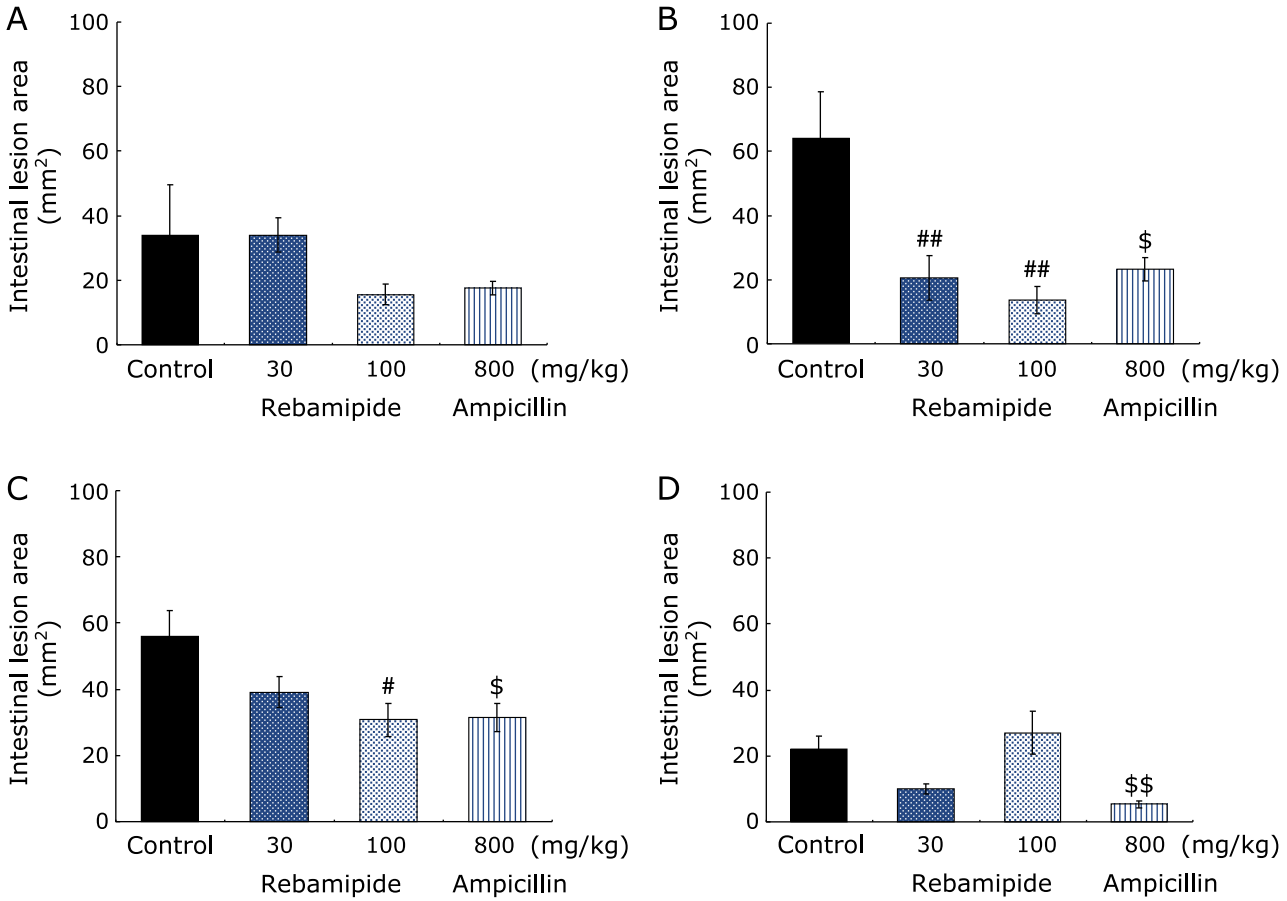

Fig. 2. Site-dependent-small intestinal mucosal injury caused by indomethacin administration and inhibition by rebamipide and ampicillin. (A) Proximal jejunum, (B) mid-jejunum, (C) distal jejunum, (D) ileum. $^{\#}p<0.05$, $^{\#\#}p<0.01$ rebamipide vs control by Dunnett test (two-tailed), $^{\$}p<0.05$, $^{\$\$}p<0.01$ ampicillin vs control by *t* test (two-tailed).

Table 1. Effect of rebamipide and ampicillin on various blood cell numbers 24 h after oral administration of indomethacin in the rat

Group	*n*	RBC (10^7/ml)	Hemoglobin (g/dl)	Hematocrit (%)	Platelet (10^7/ml)	WBC (10^5/ml)
Normal	6	735.5 ± 17.1	14.2 ± 0.3	44.3 ± 0.8	87.0 ± 5.7	101.2 ± 7.6
Control	10	539.3 ± 32.7**	10.4 ± 0.6**	34.5 ± 1.9**	56.3 ± 2.2**	95.0 ± 5.9**
Rebamipide 30 mg/kg	10	614.6 ± 33.6$^{\#}$	11.9 ± 0.6$^{\#}$	38.6 ± 1.9	63.5 ± 3.8	96.7 ± 9.4
Rebamipide 100 mg/kg	10	694.9 ± 17.2$^{\#\#}$	13.4 ± 0.3$^{\#\#}$	42.9 ± 0.9$^{\#\#}$	89.0 ± 5.5$^{\#\#}$	113.1 ± 9.9
Ampicillin 800 mg/kg	10	742.4 ± 28.3$^{\$\$}$	14.4 ± 0.5$^{\$\$}$	44.5 ± 1.6$^{\$\$}$	83.6 ± 9.2$^{\$\$}$	107.7 ± 9.6

The blood was collected at 24 h after the indomethacin 10 mg/kg p.o. administration. Rebamipide at the doses of 30 and 100 mg/kg were orally administered 5 times during the experiment at 24, 16 and 1 h before, and 3 and 7 h after treatment of indomethacin. Ampicillin with 800 mg/kg was orally administered 24 and 1 h prior to the indomethacin administration. The data are expressed as the mean ± SEM from 10 rats except the Normal group as 6 rats. **$p<0.01$ Normal vs Control (0.5% CMC-treated). $^{\#}p<0.05$, $^{\#\#}p<0.01$ Control vs Rebamipide groups. $^{\$\$}p<0.01$ Control vs Ampicillin. RBC, red blood cell; WBC, white blood cell.

Analysis of microbiota in mucosa of the small intestine by real-time PCR. For identification of microbiota, 10 bacterial strains cultured on MacConkey, BHI and GAM agar by aerobic or anaerobic culture were identified by 16S rRNA sequencing in normal rats. Eight strains of *Lactobacillus* sp., one strain of *Enterococcus* sp. (*E. faecalis*) and one strain of *Enterobacteriaceae* (*E. coli*) were detected (Supplemental Table 2*). We analyzed each member quantitatively by real-time PCR using 16S or 23S specific RNA gene targeted primers for the detection of *Lactobacillus* sp., *Enterococcus* sp., and *Enterobacteriaceae*. For the detection of non-culturable bacteria, quantitative real-time PCR using SFB (segmented filamentous bacteria) specific primers was performed.

The average numbers of jejunal and ileal bacteria of each rat group analyzed by real-time PCR are shown in Table 2. In normal rats, *Enterobacteriaceae* was dominant and *Lactobacillus* spp. was subdominant in proximal and mid-jejunum. The higher numbers ($10^{7.73–7.87}$/g mucosa) of *Lactobacillus* spp. were detected in distal jejunum and ileum of normal rats, and *Enterobacteriaceae* and SFB were subdominant in this area. In control rats treated with indomethacin, the numbers of *Enterococcaceae* and *Enterobacteriaceae* were significantly higher in mid-jejunum compared to normal rats. SFB were not detected in the mid-jejunum of any control rats, and the number of SFB in the distal jejunum was

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Table 2. Number of *Enterococcaceae*, *Enterobacteriaceae*, *Lactobaillus* and SFB in mucosa of jejunum and ileum of rats treated with indomethacin, rebamipide and ampicillin

Animal	Number of bacterial DNA (Log number/g mucosa)			
Group (number)	Jejunum: proximal	Jejunum: mid	Jejunum: distal	Ileum
Normal (6)				
Enterococcaceae	3.7 ± 0.2 (6)	2.8 ± 0.06 (6)	3.8 ± 0.03 (6)	4.2 ± 0.13 (6)
Enterobacteriaceae	7.12 ± 0.01 (5)	5.74 ± 0.04 (6)	6.62 ± 0.06 (5)	6.65 ± 0.09 (6)
Lactobacillus sp.	4.07 ± 0.08 (6)	4.67 ± 0.12 (5)	7.73 ± 0.05 (6)	7.87 ± 0.03 (6)
SFB	2.59 ± 0.11 (5)	3.14 ± 0.23 (6)	6.15 ± 0.18 (6)	6.55 ± 0.13 (6)
Control (10)				
Enterococcaceae	3.9 ± 0.08 (10)	4.0 ± 0.17 (10)**	4.2 ± 0.04 (10)*	4.2 ± 0.10 (10)
Enterobacteriaceae	7.13 ± 0.02 (8)	6.79 ± 0.13 (10)**	6.81 ± 0.22 (10)	6.46 ± 0.15 (10)
Lactobacillus sp.	4.50 ± 0.20 (10)	4.98 ± 0.34 (9)	7.92 ± 0.06 (10)	7.89 ± 0.10 (10)
SFB	2.90 ± 0.13 (5)	ND (0)	4.94 ± 0.10 (10)**	6.00 ± 0.18 (10)
Rebamipide 30 mg/kg (10)				
Enterococcaceae	3.7 ± 0.09 (10)	3.4 ± 0.16 (10)$^{\#}$	4.3 ± 0.11 (8)	4.3 ± 0.07 (10)
Enterobacteriaceae	7.08 ± 0.03 (9)	6.35 ± 0.17 (9)$^{\#}$	6.84 ± 0.08 (10)	7.03 ± 0.08 (10)
Lactobacillus sp.	4.73 ± 0.26 (9)	5.17 ± 0.15 (10)	7.90 ± 0.10 (10)	7.88 ± 0.08 (10)
SFB	3.21 ± 0.25 (7)	3.32 ± 0.24 (3)	5.42 ± 0.14 (10)	6.32 ± 0.28 (10)
Rebamipide 100 mg/kg (10)				
Enterococcaceae	3.3 ± 0.08 (9)$^{\#\#}$	2.9 ± 0.11 (10)$^{\#\#}$	4.1 ± 0.12 (8)	4.4 ± 0.06 (10)
Enterobacteriaceae	6.99 ± 0.03 (9)$^{\#\#}$	5.92 ± 0.12 (9)$^{\#\#}$	6.82 ± 0.04 (10)	6.61 ± 0.31 (10)
Lactobacillus sp.	3.83 ± 0.14 (9)$^{\#}$	5.14 ± 0.16 (10)	8.12 ± 0.10 (10)	8.06 ± 0.04 (10)
SFB	2.56 ± 0.10 (7)	2.98 ± 0.35 (3)	5.27 ± 0.09 (10)	6.08 ± 0.40 (10)
Ampicillin 800 mg/kg (10)				
Enterococcaceae	3.2 ± 0.05 (9)$^{\$\$}$	3.2 ± 0.11 (10)$^{\$\$}$	3.8 ± 0.05 (10)$^{\$\$}$	4.2 ± 0.03 (9)
Enterobacteriaceae	6.92 ± 0.01 (8)$^{\$\$}$	5.86 ± 0.08 (10)$^{\$\$}$	6.65 ± 0.04 (10)	7.10 ± 0.13 (10)$^{\$}$
Lactobacillus sp.	2.29 ± 0.16 (2)	1.92 (1)	3.82 ± 0.25 (10)	4.49 ± 0.45 (10)$^{\$\$}$
SFB	3.11 ± 0.09 (10)$^{\$\$}$	4.68 ± 0.10 (10)	7.57 ± 0.07 (9)$^{\$\$}$	7.71 ± 0.04 (10)

Bacterial numbers in small intestinal mucosal samples were calculated by comparing the PCR kinetics of target genes in the sample with standard curves made by amplifying serial dilutions of a known quantity of amplicon. The data are expressed as the mean ± SEM from 10 rats except the Normal group as 6 rats. When the number of bacterial was below the detection limit, the data was handled as missing value. SFB, short fragmented bacteria; ND, not detected. *p<0.05, **p<0.01 Normal vs Control (0.5% CMC-treated). $^{\#}p$<0.05, $^{\#\#}p$<0.01 Control vs Rebamipide groups. $^{\$}p$<0.05, $^{\$\$}p$<0.01 Control vs Ampicillin.

significantly lower in control rats than that in normal rats.

In rebamipide treated rats (30 mg/kg, 100 mg/kg), the numbers of *Enterobacteriaceae* in the proximal (100 mg/kg, p<0.05) and mid-jejunum were significantly lower (30 mg/kg, p<0.05; 100 mg/kg, p<0.01) than in control rats, indicating that rebamipide restored the numbers of *Enterobacteriaceae* increased by indomethacin to a similar level to normal rats. Similarly, the numbers of *Enterococcaceae* in proximal (Reb 100 mg/kg only) and mid-jejunum decreased to a similar level to normal rats. Although there was no significant difference in the number of SFB between rebamipide treated rats and normal rats, similar numbers ($10^{2.98–3.32}$/g mucosa) of SFB were detected in the mid-jejunum of 3 out of 10 rats. In the proximal jejunum, treatment with higher concentrations (100 mg/kg) of rebamipide decreased the number of *Enterobacteriaceae*, *Enterococcaceae* and *Lactobacillus* spp. compared to control rats.

In ampicillin treated rats, the numbers of *Enterococcaceae* in all sites of jejunum were significantly lower than those of control rats. Similarly, lower numbers of *Enterobacteriaceae* were detected in proximal and mid-jejunum. It was also shown that ampicillin treatment decreased the number of *Lactobacillus* spp. In the proximal and distal jejunum. Detection rates of SFB in ampicillin-treated rats was very low (2/10 in proximal jejunum; 1/10 in mid-jejunum) compared with normal rats.

Upregulated or downregulated genes in the jejunal mucosa after indomethacin administration and treatment with rebamipide and ampicillin. To obtain further insight into the mechanisms of reduction of indomethacin-induced intestinal mucosal injury by rebamipide, mRNA expression profiles of the the jejunal mucosa were compared between the experimental groups using rat RT2 profiler PCR arrays containing cytokine & chemokine-, oxidative stress-, and wound healing-related genes. As shown in Table 3, 14 genes were upregulated and 3 genes were downregulated more than 5-fold in indomethacin treated rats compared to normal rats. Quantitative real-time PCR revealed that the expression of TNFα and Duox2 were elevated by indomethacin to approximately 4- and 8-fold, respectively (Fig. 3). These elevated expression levels were significantly suppressed by ampicillin 800 mg/kg administration. TNFα expression was also significantly suppressed by rebamipide 100 mg/kg, and Duox2 expression was reduced by rebamipide in a dose-dependent manner.

Discussion

As reported previously, indomethacin induced multiple mucosal injuries including segmental ulceration in the small intestine in this study. By the analysis of small intestinal microbiota by aerobic and anaerobic cultures, it was demonstrated that indomethacin administration increased the numbers of intestinal bacteria particularly *Enterobacteriaceae* in the jejunum and ileum. In addition, by real-time PCR assay, we also detected increases in *Enterobacteriaceae*/*Enterococcaceae* and a decrease in SFB in the jejunal mucosa. Ampicillin treatment inhibited the injuries induced by indomethacin by regulating small intestinal microbiota as reported previously,[22,23] suggesting that NSAID-induced

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Table 3. Selected genes up-regulated more than 5 fold or less than 1/5 fold, comparing mid jejunum mucosa from the indomethacin-administered rat to that of normal, and effects of treatment with rebamipide 100 mg/kg

PCR Array	Gene ID	Gene or encoded protein	Fold change	Fold change by rebamipide
Cytokines & Chemokines	25296	Bmp4 bone morphogenetic protein 4	6.66	3.29 (0.49)
	361795	Ltb lymphotoxin beta (TNF superfamily, member 3)	8.23	10.35 (1.26)
	24835	TNFalfa	8.11	4.54 (0.56)
Oxidative stress	79107	Duox2 dual oxidase 2	6.27	1.60 (0.26)
	246245	Fmo2 flavin containing monooxygenase 2	0.19	0.36 (1.89)
	24599	Nos2 nitric oxide synthase 2, inducible	9.99	5.83 (0.58)
Wound healing	290905	Col4a1 collagen, type IV, alpha 1	5.35	1.24 (0.23)
	60379	Col5a3 collagen, type V, alpha 3	5.09	1.29 (0.25)
	29175	Ctsk cathepsin K	0.08	0.35 (4.38)
	25697	Ctsl1 cathepsin L1	6.39	1.09 (0.17)
	171551	Cxcl3 chemokine (C-X-C motif) ligand 3	7.46	1.29 (0.17)
	60665	Cxcl5 chemokine (C-X-C motif) ligand 5	6.65	1.02 (0.15)
	25313	Egf epidermal growth factor	0.17	0.22 (1.29)
	25584	F3 coagulation factor III (thromboplastin, tissue factor)	15.5	1.43 (0.09)
	25619	Plau plasminogen activator, urokinase	8.13	0.40 (0.05)
	24617	Serpine1 serpin peptidase inhibitor, clade E	27.98	1.62 (0.06)
	116510	Timp1 TIMP metallopeptidase inhibitor 1	8.47	0.56 (0.07)
	64566	Wnt5a wingless-type MMTV integration site family, member 5A	0.22	0.28 (1.27)

The mRNA expression profiles of mid jejunal mucosa were compared among the experimental groups using rat RT2 profiler PCR arrays containing cytokine & chemokine-, oxidative stress-, and wound healing-related 84 genes for each category. Up-regulated or down-regulated genes more than 5-fold change in indomethacin-administered rats were listed as compared with those of normal rats.

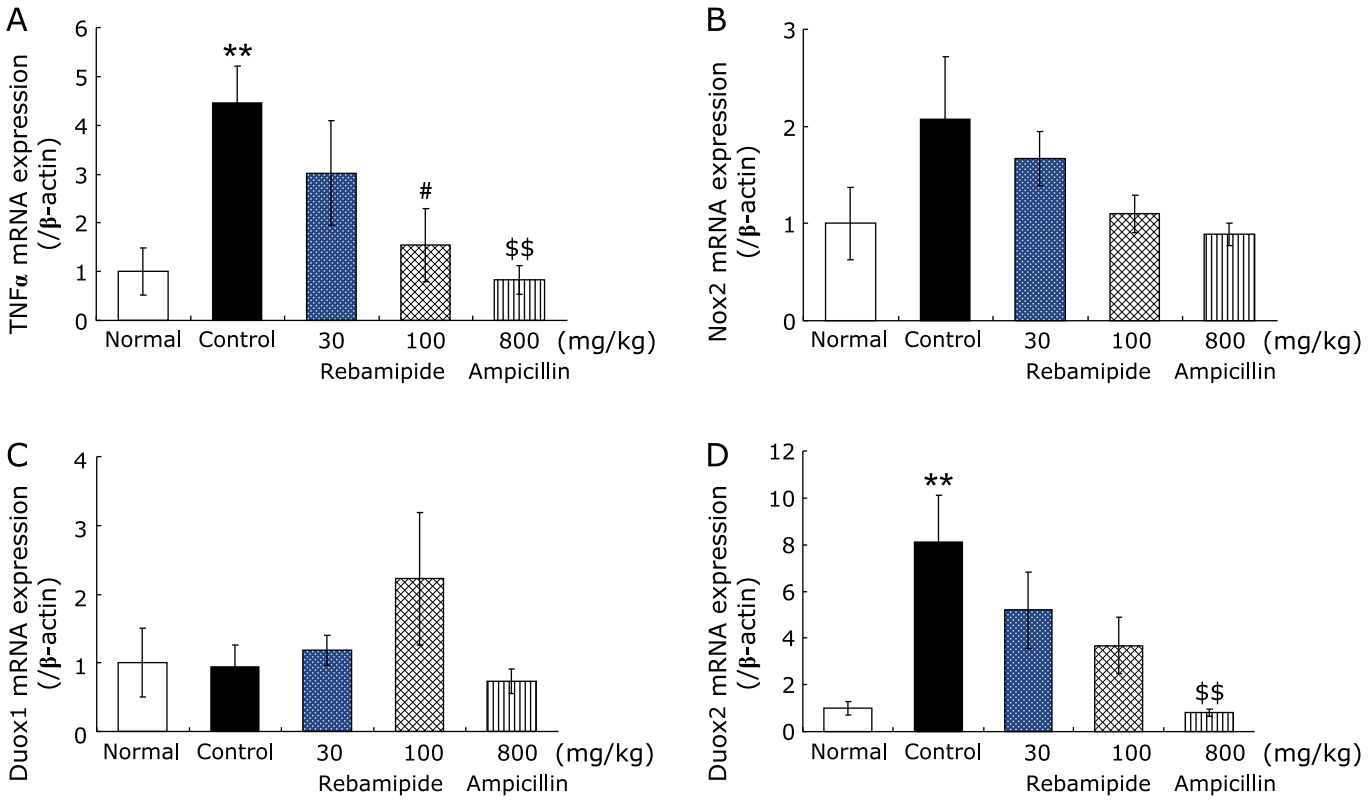

Fig. 3. Small intestinal mucosal expression of TNFα, iNOS, Duox1 and Duox2 after indomethacin administration and effects of rebamipide and ampicillin. (A) TNFα, (B) Nox2, (C) Duox1, (D) Duox2. $^{**}p<0.01$ normal vs control by *t* test (two-tailed), $^{\#}p<0.05$ rebamipide vs control by Dunnett test (two-tailed), $^{\$\$}p<0.01$ ampicillin vs control by *t* test (two-tailed).

mucosal injury is closely associated with the changes of intestinal microbiota.

In the present study, rebamipide was shown to inhibit indomethacin-induced small intestinal mucosal injury, particularly jejunal ulcers as reported previously.[23,24] Rebamipide treatment restored near-normal numbers of *Enterococcaceae* and *Enterobacteriaceae* in the jejunal mucosa. There have been many reports that intestinal microbiota contribute to the pathogenesis of NSAIDs-induced enteropathy.[4,5,25–28] In some studies, increase in specific bacteria such as *E. faecalis*, *Clostridium*, *Bacteroides* and *E. coli* in intestinal mucosa were detected following NSAIDs administration.[25–27] It was also reported that NSAIDs did not induce any intestinal mucosal injury in germ-free animals, clearly indicating the association of intestinal microbiota with NSAIDs-induced mucosal injury.

Recently, a study of terminal-restriction fragment length polymorphisms (T-RFLP) was performed for the evaluation of the changes in the microbiota in the ileum and cecum of indomethacin- and rebamipide-treated mice.[29] It was shown that rebamipide and indomethacin had no significant effects on the gut microbiota profiles in the ileum. However, the combination of rebamipide and indomethacin increased the orders *Bifidobacteriales* and *Lactobacillales*, the genera *Bacterioides* and *Prevotella* and the family *Clostridiaceae* in the ileum and cecum. In addition, it was also shown that the diversity of intestinal microbiota was significantly higher in the rats treated with both indomethacin and rebamipide than that in the mice treated with either indomethacin or rebamipide separately. The increase in *Clostridiaceae* was suggested to produce short-chain fatty acids such as butyrate, which are an energy source for gut epithelial cells and a potent anti-inflammatory factor. In a similar study to examine the effect of rebamipide on intestinal microbiota, Tanigawa *et al.*[30] reported that rebamipide increased the percentage of *Lactobacillales* and decreased the percentage of *Bacteroides* and *Clostridium* compared to those of indomethacin-treated controls using a mouse model. In these studies, the increase of *Lactobacillales* was commonly observed, but the different profiles of *Bacteroides* and *Clostridium* subcluster XIVa were observed by the treatment with rebamipide. Although the reason for the difference in the effect of rebamipide between these two studies is not determined, it seems to be due to the difference in the experimental protocols for the treatment by indomethacin and rebamipide. In the present study, quantification of the small intestinal bacteria was targeted on *Enterococcaceae*, *Lactobacillus* and *Enterobacteriaceae* by quantitative real-time PCR according to the results of small intestinal mucosal culture. The relative profile of small intestinal microbiota as indicated by Imaeda *et al.*[29] and Tanigawa *et al.*[30] was not clarified in this study, but we have shown that indomethacin treatment increased the numbers of both *Enterococcaceae* and *Enterobacteriaceae* and that the numbers of *Enterococcaceae* and *Enterobacteriacea* were recovered to near-normal levels by rebamipide treatment. It is possible that the difference in the results concerning the change in small intestinal microbiota between these studies[29,30] and ours may be due to the difference of analytical methodology and the animal species used. There have been previously no reports on the profile of small intestinal microbiota of rats, although Lacomber *et al.*[31] reported that 24 prokaryotic phyla were identified in the rat proximal colon by metagenomic sequencing and assembly and that Firmicutes, Bacteroidetes, Actinobacteria and Verrucomicrobia were the dominant phyla in the control rat consistent with the mammalian gut microbiome.

SFB has been reported to have a significant effect on the intestinal immune response.[32,33] SFB are Gram positive, spore-forming obligate anaerobes that have not yet been successfully cultured *in vitro*. They have been reported to promote the development of both intraepithelial lymphocytes and IgA-producing cells in the small intestine.[32] Recently, introduction of SFB into germ-free mice was shown to reinstate the lamina propria Th17 cell compartment and production of autoantibodies relating with the occurrence of arthritis.[33] In the present study, the number of SFB in the small intestinal mucosa was quantitatively determined. SFB were not detected at all in mid-jejunum of indomethacin treated rats and the number of SFB in the distal jejunum was significantly lower than that of normal rats. The detection rate of SFB was increased by rebamipide treatment. Similarly, the detection rate of SFB was increased and the number of SFB was increased by ampicillin treatment compared to control rats treated with indomethacin. These results suggest the possible action of rebamipide to regulate the function of SFB in jejunum similar to ampicillin.

As previously reported, TNFα-mRNA expression levels were markedly elevated by indomethacin administration in the rat jejunal mucosa.[34] We also found that 4 genes related to oxidative stress, including Duox2 and Nox2-mRNA, were upregulated. Quantitative analysis of mRNA expression using the RT-PCR technique revealed that TNFα- and Duox-mRNA expression levels were significantly increased. Duox2 is expressed throughout the digestive tract including the jejunum and ileum, and generates hydrogen peroxide.[35] These results suggest that the inflammatory response and oxidative stress play an important role in indomethacin-induced small intestinal mucosal injury. Rebamipide significantly suppressed the upregulation of TNFα-mRNA and showed a dose-dependent suppression of Duox2-mRNA. Recently, Tanigawa *et al.*[30] reported that rebamipide modulated small intestinal microbiota by upregulating the expression of α-defencin, which is an anti-microbial peptide, which prevented indomethacin-induced small intestinal injury in mice. We also found that rebamipide normalized the disturbance of rat small intestinal microbiota after indomethacin-induced small intestinal mucosal injury. Taken together, rebamipide may protect the small intestinal mucosa from indomethacin-induced injury by suppressing inflammation and oxidative stress, which is closely related to the normalization of small intestinal microbiota.

The possibility of development of a novel treatment for small intestinal mucosal injury has been reported by many investigators. The effect of probiotic treatment for patients with iron deficiency taking low-dose aspirin (100 mg/day) and omeprazole has been reported.[36] *L. casei* used for 3 months ($n = 13$) showed significant decreases in the number of mucosal breaks and capsule endoscopy score compared with the control group ($n = 12$). It was suggested that *L. casei* improved chronic inflammatory changes by inhibiting the expression of pro-inflammatory cytokines in the lamina propria mononuclear cells similar to what was previously postulated in the murine model.[37,38] The effect of geranylgeranylacetone (GGA), a mucosal protectant, on both the mucus content and loxoprofen sodium-induced lesions in the rat small intestine has also been evaluated.[39] It was shown that the mucin content increased dose-dependently after a single oral administration of GGA. It was also demonstrated that GGA significantly prevented the increase in aerobic and anaerobic bacteria in the jejunal mucosa following administration of loxoprofen sodium, suggesting that GGA inhibited enterobacterial invasion of the mucosa as a result of the increase in the mucosal barrier. Pharmacologic inhibition of intestinal bacterial β-D-glucuronidase by a bacteria-specific small-molecule inhibitor was also reported to protect the small intestinal mucosa from diclofenac, another NSAID, inducing ulceration.[40]

In this study, the inhibitory effect of rebamipide on indomethacin-induced small mucosal injury was demonstrated. The results obtained suggest that rebamipide regulates not only the small intestinal microbiota in particular decreasing the number of *Enterobacteriaceae* induced by indomethacin administration but also decreases the gene expression of TNFα and Duox2 upregulated by indomethacin treatment. In conclusion, rebamipide is possible prophylactic agent for NSAIDs-induced gastrointestinal mucosal injury.

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Conflict of Interest

Kazushi Sakurai, Masahumi Shibamori, Takako Nakashima, Naoya Uematsu, Takako Osaki and Satoshi Kurata declare no conflicts of interest. Shigeru Kamiya received a grant for medical research from Otsuka Pharmaceutical Co., Ltd., Japan.

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