Effect of CJ-42794, A Selective Antagonist of Prostaglandin E Receptor Subtype 4, on Ulcerogenic and Healing Responses in Rat Gastrointestinal Mucosa

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Running head: EP4 Antagonist on GI Mucosa

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Abbreviations:
nonsteroidal antiinflammatory drugs --------------------------- NSAIDs
prostaglandins ---------------------------------------------------------- PGs
cyclooxygenase --------------------------------------------------------- COX
prostaglandin E₂ -------------------------------------------------------- PGE₂
PGE receptor subtype 4----------------------------------------------- EP4 receptor
vascular endothelial derived growth factor --------------- VEGF
gastrointestinal -------------------------------------------------------- GI
Abstract

Recent study showed the involvement of prostaglandin E receptor subtype 4 (EP4) in hypersensitivity to inflammatory pain and suggested the EP4 receptor to be a potential target for the pharmacological treatment of inflammatory pain. We examined the effects of CJ-42794, a selective EP4 antagonist, on gastrointestinal ulcerogenic and healing responses in rats, in comparison with those of various cyclooxygenase (COX) inhibitors. CJ-42794 alone, given p.o., did not produce any damage in the gastrointestinal mucosa, similar to SC-560 (COX-1 inhibitor) or rofecoxib (COX-2 inhibitor), while indomethacin (nonselective COX inhibitor) caused gross lesions. Rofecoxib but not CJ-42794, however, damaged these tissues when co-administered with SC-560 and aggravated gastric lesions produced by aspirin. Indomethacin and SC-560 worsened the gastric ulcerogenic response to cold-restraint stress, yet neither CJ-42794 nor rofecoxib had any effect. Furthermore, indomethacin and SC-560 at lower doses damaged the stomach and small intestine of adjuvant arthritic rats. In arthritic rats, rofecoxib but not CJ-42794 provoked gastric ulceration, whereas CJ-42794 produced little damage in the small intestine. The repeated administration of CJ-42794 and rofecoxib as well as indomethacin impaired the healing of chronic gastric ulcers with a down-regulation of VEGF expression in the ulcerated mucosa. These results suggest that CJ-42794 does not cause any damage in the normal rat gastrointestinal mucosa and in the arthritic rat stomach and does not worsen the gastric ulcerogenic response to stress or aspirin in normal rats, although this agent slightly damages the small intestine of arthritic rats and impairs the healing of gastric ulcers.
Introduction

Nonsteroidal antiinflammatory drugs (NSAIDs) are frequently used to treat inflammatory pain. A major limitation to their use, however, is the damage they cause to the gastrointestinal (GI) tract, including the formation of gastric lesions, the potentiation of ulcerogenic responses to stress, and the impairment of gastric ulcer healing (Lanza, 1984; Wang et al., 1989; Konturek et al., 1990; Ukawa et al., 1998). A number of strategies have been used to develop NSAIDs that spare the GI tract. One approach is to produce NSAIDs that only inhibit the inducible isoform of cyclooxygenase (COX)-2, thereby exerting anti-inflammatory activity but sparing GI prostaglandin (PG) production (Arai et al., 1993; Chan et al., 1995). This approach has been demonstrated to lessen the incidence of damage in the GI tract (Arai et al., 1993; Wallace et al., 2000; Tanaka et al., 2001; 2002a), although some COX-2 selective inhibitors reportedly increased the risk of cardiac vascular diseases (Mukherjee et al., 2001).

Following the withdrawal of selective COX-2 inhibitors from the market, demand has intensified for drugs targeted to antagonize inflammatory pain receptors that do not injure the GI tract as do nonselective COX inhibitors. The discovery of downstream targets such as prostaglandin (PG) E receptors has opened new possibilities in this regard. Selective EP antagonists have increasingly been developed for this indication (Zeihofer and Brune, 2006). CJ-42794 [(S)-4-(1-(5-chloro-2-(4-fluorophenyoxy) benzamido)ethyl) benzoic acid], developed by Pfizer Inc., is a selective antagonist of EP receptor subtype 4 (EP4). These receptors are thought to be involved in the inflammatory as well as nociceptive actions of PGE2 (Lin et al., 2006; Woodhams et al., 2006). Woodhams et al. (2006) reported the localization and modulation of EP1 and EP4 receptors in a rat chronic constriction injury model of neuropathic pain. Lin et al. (2006) showed the involvement of EP4 receptors in hypersensitivity to inflammatory pain and
suggested the EP4 receptor to be a potential target for the pharmacological treatment of inflammatory pain. Recent study has indeed demonstrated that CJ-42794 is as effective as conventional NSAIDs in terms of its antinociceptive action (personal communication from Dr. Sakakibara).

PGE₂ exerts its many effects by binding to four different EP receptor subtypes, EP1-EP4 (Narumiya and FitzGerald, 2001). We previously investigated the relationship between EP receptor subtypes and the protective effect of PGE₂ in the GI tract and found that EP1 receptors are mainly involved in the protective action in the stomach and esophagus (Araki et al., 2000; Suzuki et al., 2001; Takeuchi et al., 2002; Yamato et al., 2005), while EP4 receptors are associated with the protective action in the duodenum as well as the small and large intestines (Takeuchi et al., 1999; Kunikata et al., 2002; Kabashima et al., 2002; Aoi et al., 2004, Aihara et al., 2007). It is possible, therefore, that CJ-42794 has a deleterious influence on the GI mucosa, similar to NSAIDs or COX-2 inhibitors under certain conditions such as in adjuvant-induced arthritic rats (Kato et al., 2002; Ohno et al., 2004).

In the present study, we examined in rats 1) whether CJ-42794, a selective EP4 antagonist, has any ulcerogenic influence on the GI mucosa in the absence or presence of adjuvant-induced arthritis, 2) its effect on the gastric ulcerogenic responses to stimuli such as stress and aspirin, and 3) its influence on the spontaneous healing of chronic gastric ulcers, and 4) compared its effects with those of various COX inhibitors such as indomethacin (the non-selective COX inhibitor), SC-560 (the COX-1 selective inhibitor), and rofecoxib (the COX-2 selective inhibitor).
Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River, GS, Yokohama, Japan; 200–230 g) or Dark Agouti rats (DA: SLC, Shizuoka, Japan; 140–160 g) were fed standard rat chow and tap water ad libitum. DA rats were used only in the experiment on adjuvant arthritis. Except for the studies on intestinal ulceration and gastric ulcer healing, the animals were kept in individual cages with raised mesh bottoms and deprived of food but allowed free access to tap water for 18 hr prior to the experiments. Studies were performed under unanesthetized conditions, unless otherwise specified. All experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Determination of duodenal HCO₃⁻ secretion

It is known that PGE₂, via the activation of EP₄ receptors, stimulates the secretion of mucus in both the stomach and small intestine as well as the secretion of HCO₃⁻ in the duodenum (Takahashi et al., 1999; Kunikata et al., 2001; Aoi et al., 2004; Aihara et al., 2007). Among these actions, the duodenal HCO₃⁻ response to PGE₂ is a well-reproducible event and can be observed optically on the computed recording system in acute experiments. Thus, we determined the effective doses of CJ-42794 under in vivo conditions by examining the inhibitory effect of this agent on the HCO₃⁻ secretion stimulated by PGE₂ or AE1-329, the EP₄ agonist (Amano et al., 2003; Aoi et al., 2004). HCO₃⁻ was measured in a duodenal loop under urethane anesthesia (1.25 g/kg, intraperitoneally), according to a previously published method (Takeuchi et al., 1986). In brief, the abdomen was incised, and a duodenal loop (1.7 cm) was made between the pyloric ring and the area just above the outlet of the common bile duct to exclude the
influences of bile and pancreatic juice. Then, the loop was superfused with saline that was gassed with 100% O2 and kept in a reservoir. The secretion of HCO3− was measured at pH 7.0 using a pH-stat method (Hiranuma Comtite-8, Mito, Japan) and adding 5 mM HCl to the reservoir. After basal HCO3− secretion had well stabilized, the selective EP4 agonist AE1-329 (3 µg/kg) or PGE2 (0.3 mg/kg) was administered intravenously (i.v.) as a single injection, and the secretion of HCO3− was measured for 1 hr thereafter. CJ-42794 (0.3, 1 and 3 mg/kg) was administered intraduodenally (i.d.) distal to the loop, 1 hr before the injection of AE1-329 or PGE2.

**Evaluation of GI ulcerogenic responses in normal rats**

Effects of CJ-42794 and various COX inhibitors on the gastric ulcerogenic responses were examined in the following (A~D) studies; A: the ulcerogenic effect the agent alone has on the gastric mucosa; B: the ulcerogenic effect the agent alone has on the small intestinal mucosa; C: the effect of the agent on the gastric ulcerogenic response to cold-restraint stress; D: the effect of the agent on aspirin-induced gastric ulceration. In these studies, the doses of various COX inhibitors such as indomethacin (a nonselective COX inhibitor), SC-560 (a COX-1 selective inhibitor) and rofecoxib (a COX-2 selective inhibitor) were chosen according to our previous papers (Tanaka et al., 2001; 2002a; 2002b). Since it is known that rofecoxib damages the GI mucosa of normal rats only when administered together with SC-560 (Wallace et al., 2000; Tanaka et al., 2001; 2002a), the ulcerogenic effects of CJ-42794 or rofecoxib co-administered with SC-560 were also examined in the gastrointestinal mucosa in studies A and B.

**Study A:** Animals fasted for 18 hr were administered CJ-42794 (30 and 50 mg/kg), SC-560 (30 mg/kg), rofecoxib (30 mg/kg), or indomethacin (30 mg/kg) orally (p.o.), and killed 4 hr later (Tanaka et al., 2001). In some cases, CJ-42794 (50 mg/kg) or rofecoxib (30 mg/kg) was co-administered with SC-560 (30 mg/kg). The stomachs were
removed, inflated by injecting 10 ml of 2% formalin for 10 min to fix the tissue walls, and opened along the greater curvature. The area (mm²) of macroscopically visible lesions was measured under a dissecting microscope with a square grid (x10; Olympus, Tokyo, Japan), summed per stomach, and used as a lesion score. The person measuring the lesions did not know the treatments given to the animals. These procedures were applied to Studies B–D.

**Study B:** Animals fed normally were administered CJ-42794 (30 and 50 mg/kg), SC-560 (30 mg/kg), rofecoxib (30 mg/kg), or indomethacin (10 mg/kg) p.o., and killed 24 hr later. In some cases, CJ-42794 (50 mg/kg) or rofecoxib (30 mg/kg) was co-administered with SC-560 (30 mg/kg). In each case, to delineate the damage, 1 ml of Evans blue (w/w) was injected i.v. 30 min before sacrifice (Tanaka et al., 2002a). The small intestines were excised, treated with 2% formalin and opened along the antimesenteric attachment, and examined for hemorrhagic lesions.

**Study C:** Animals fasted for 18 hr were kept in a Bollman cage and placed in a cold room for 6 hr; where the ambient temperature being 10°C (Tanaka et al., 2007). The animals were killed under deep ether anesthesia, and the stomachs were removed, treated with 2% formalin, and examined for hemorrhagic lesions. CJ-42794 (30 mg), SC-560 (10 mg/kg), rofecoxib (10 mg/kg) or indomethacin (5 mg/kg) was administered p.o. 30 min before the onset of stress.

**Study D:** Animals fasted for 18 hr were given aspirin (50 mg/kg) orally and killed 4 hr later (Fiorucci et al., 2002). The stomachs were removed, treated with 2% formalin, and examined for hemorrhagic lesions. CJ-42794 (30 mg/kg) or rofecoxib (10 mg/kg) was administered p.o. 30 min before the administration of aspirin.

**Evaluation of GI ulcerogenic responses in arthritic rats**

Arthritis was induced by injection of 50 µl of Freund's complete adjuvant
(FCA; 10 mg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra suspended in paraffin oil) into the plantar region of the right hindfoot (Kato et al., 1999). Normal rats were housed in the same manner for the same period of time, so that aged and batch-matched normal and arthritic rats were used in all of the experiments. Since the paw edema in the left (uninjected) hindfoot was observed from 10 days and reached a maximum 14 days after the injection of FCA, we used the animals 14 days after the injection in the subsequent experiments. In addition, because it was previously found that the gastrointestinal ulcerogenicity of indomethacin was increased in arthritic rats and the ulcerogenic doses became much less than those in normal rats (Kato et al., 1999; 2006), we used a low dose (3 mg/kg) of indomethacin in these experiments.

**Gastric ulcerogenic response:** The animals with or without arthritis were deprived of food but allowed free access to tap water for 18 hr before the experiments. Indomethacin (3 mg/kg), rofecoxib (30 mg/kg), SC-560 (10 mg/kg), or CJ-42794 (30 and 50 mg/kg) was administered orally, and the animals were killed under deep ether anesthesia 4 hr later. The stomachs were excised, treated with 2% formalin, opened along the greater curvature, and examined for lesions.

**Intestinal ulcerogenic response:** The animals with or without arthritis were administered indomethacin (3 mg/kg), rofecoxib (30 mg/kg), SC-560 (10 mg/kg), or CJ-42794 (30 and 50 mg/kg) orally, and killed 24 hr later. In each case, to delineate the damage, 1 ml of Evans blue (w/w) was injected i.v. 30 min before sacrifice. The small intestines were excised and treated with 2% formalin, opened along the anti-mesenteric attachment, and examined for macroscopically visible lesions.

**Induction of chronic gastric ulcers**

Chronic gastric ulcers were induced in rats by thermal-cauterization, according to a method described previously (Ukawa et al., 1998). Under ether anesthesia, the
stomach was exposed through a midline incision, the electric probe (Fuchigami, Kyoto, Japan; diameter: 8 mm\(^2\)) was attached to the mid corpus mucosa, and a gastric ulcer was induced by heating the tip at 70\(^\circ\)C for 20 sec. Then, the abdomen was closed, and the animals were routinely maintained with food and tap water. The animals were killed on day 17 after ulceration, and the stomach was removed and opened along the greater curvature. The area (mm\(^2\)) of ulceration was determined under a dissecting microscope. Since deep, well-defined ulcers were consistently observed 3 days following thermal-cauterization, the 3rd day after the operation was defined as the initial day of ulceration. Indomethacin (2 mg/kg), SC-560 (10 mg/kg), rofecoxib (10 mg/kg), or CJ-42794 (3, 10 and 45 mg/kg) was administered p.o. once daily for 14 days, starting 3 days after the operation. The person measuring the size of the ulcers was blinded as to which treatment had been administered to any animal. Control animals received the vehicle of each drug alone.

**Determination of Mucosal PGE\(_2\) Content**

Levels of PGE\(_2\) in the gastric mucosa were measured on day 10 after ulceration by thermocauterization (70\(^\circ\)C, 20 sec) in rats, with or without the p.o. administration of CJ-42794 (10 mg/kg), indomethacin (2 mg/kg), or rofecoxib (10 mg/kg) once daily for the last 7 days. Under deep ether anesthesia, the stomach was removed, and the corpus mucosa was isolated, weighed, and put in a tube containing 100% methanol plus 0.1 mM indomethacin (Futaki et al., 1994). The tissues were then minced with scissors, homogenized with a Polytron homogenizer (IKA, Tokyo, Japan), and centrifuged at 12,000 g for 10 min at 4\(^\circ\)C. After the supernatant of each sample had been evaporated with N\(_2\) gas, the residue was resolved in assay buffer and used for the determination of PGE\(_2\). The concentration of PGE\(_2\) was measured using a PGE\(_2\) enzymeimmunoassay kit (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK).
Histological Observations and Evaluation of Angiogenesis

At the autopsy on day 10 after ulcer induction, 12 µm frozen sections were prepared. For evaluation of angiogenesis, sections were incubated with an antibody for von Willebrand factor (factor VIII-related endothelial antigen; DAKO, Glostrop, Denmark) after the deactivation of endogenous peroxidase with 0.3% H$_2$O$_2$ and the blockade of nonspecific binding sites was performed (Szabo et al., 1994; Yue et al., 2007). Microvasculature was visualized by the avidin-biotin-peroxidase complex method using a Vectastain ABC-peroxidase kit (Vector, Burlingame, CA). The degree of microvasculature in the ulcer base granulation tissue was determined in three randomly chosen 1 mm$^2$ fields. The density of microvasculature was expressed as the number of vessels per mm$^2$ of ulcer base.

Western Blot Analysis for VEGF

Gastric ulcers were produced by thermocauterization (70°C, 20 sec), and the animals were killed 10 days later. Tissues from the ulcerated mucosa were minced with scissors, and collected and weighed. Samples were homogenized with protease inhibitor cocktail tablets (Complete; Roche, Penzberg, Germany) and centrifuged at 20,000 g for 30 min at 4°C, and the supernatant was collected as the protein samples. The protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). To analyze the expression of VEGF, the protein samples (20 µg) were electrophoresed on sodium dodecyl sulfate (SDS-15%) polyacrylamide slab gels, as described by Laemmli (1970), and electrically transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). Sequential immuno-blotting was performed using a monoclonal anti-VEGF (Santa Cruz, CA, USA) as a primary antibody. The membrane was then reacted with horseradish peroxidase-conjugated goat anti rabbit-IgG antibody (Santa Cruz, CA, USA) for 1 hr at room temperature. Western
blots were visualized with an enhanced chemiluminescence system (Western Blot Chemiluminescence Reagent Plus; NEN, Boston, USA). Indomethacin (2 mg/kg), SC-560 (5 mg/kg), rofecoxib (5 mg/kg), or CJ-42794 (10 mg/kg) was given p.o. once daily for 7 days, starting 3 days after ulceration.

**Preparation of Drugs**

The drugs used were CJ-42794 [(S)-4-(1-(5-chloro-2-(4-fluorophenyoxy)benzamido) ethyl)benzoic acid](Pfizer Inc. Japan, Aichi, Japan), indomethacin, aspirin (Sigma Chemicals, St. Louis, MO), rofecoxib (synthesized in our laboratory), SC-560 (Cayman Chemical, Ann Arbor, MI), AE1-329 [[3-[[[(1R,2S,3R)-3-hydroxy-2-[(1E,3S)-3-hydroxy-4-[3-(methoxymethyl)phenyl]-1-butenyl]-5-oxocyclopentyl]thio]propyl]thio]-acetic acid (Ono Pharmaceutical Co. Ltd, Osaka, Japan) and urethane (Tokyo Kasei, Tokyo, Japan). Urethane was dissolved in saline, while AE1-329 was first dissolved in absolute ethanol and then diluted with saline to a desired concentration. Other agents, including CJ-42794, were suspended in a hydroxypropylcellulose solution (HPC: Wako, Osaka, Japan). Each agent was prepared immediately before use and administered p.o. or i.p. in a volume of 0.5 ml/100 g body weight, or i.v. in a volume of 0.1 ml/100 g body weight. Control animals received vehicle alone.

**Statistics**

Data are presented as the mean±SE of 4~9 rats per group. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by a two-tailed Dunnett's multiple comparison test or, when appropriate, by Student-\(t\) tests, and values of \(P<0.05\) were regarded as significant.
Results

Effect of CJ-42794 on Duodenal $\text{HCO}_3^-$ Secretion Stimulated by the EP4 agonist AE1-329

It is known that PGE$_2$ stimulates duodenal HCO$_3^-$ secretion through activation of EP4 receptors (Aoi et al., 2004). To confirm the involvement of EP4 receptors in the stimulatory action of PGE$_2$ and the effectiveness of CJ-42794, a selective EP4 antagonist, under in-vivo conditions, we examined the effect of CJ-42794 on the HCO$_3^-$ response induced by AE1-329, the selective EP4 agonist.

The intravenous administration of AE1-329 (3 µg/kg) increased duodenal HCO$_3^-$ secretion over control levels, with a maximal value of 2.0±0.2 µEq/10 min; the $\Delta$ HCO$_3^-$ output was 2.9±0.6 µEq/hr, which is significantly greater than that (0.1±0.4 µEq/hr) observed in the animals given saline alone and is almost equivalent to that (3.2±0.6 µEq/hr) induced by PGE$_2$ (1 mg/kg, i.v.). As evident in Figure 1, CJ-42794 (0.3, 1 and 3 mg/kg) given i.d. dose-dependently antagonized the HCO$_3^-$ stimulatory action of AE1-329 in the duodenum, and the effect even at 1 mg/kg was significant, the inhibition being 68.9%. The ED$_{50}$ of CJ-42794 was calculated to be 0.5 mg/kg. Likewise, CJ-42794 dose-dependently attenuated the PGE$_2$-stimulated HCO$_3^-$ secretion, although the effect was less potent as compared to that against AE1-329 (data not shown). These results confirmed that CJ-42794 is an effective EP4 antagonist under in vivo conditions, exerting a significant effect at a dose of 1 mg/kg or greater.

Ulcerogenic Effect of CJ-42794 on GI Mucosa in Normal Rats

Consistent with previously published observations (Suzuki et al., 2001; Tanaka et al., 2001), indomethacin (30 mg/kg, p.o.), a nonselective COX inhibitor, produced hemorrhagic lesions in the stomach, the lesion score being 22.8±2.3 mm$^2$ (Figure 2).
Likewise, a selective COX-1 inhibitor, SC-560 (30 mg/kg, p.o.), also itself caused a few lesions in the stomach, whereas rofecoxib (30 mg/kg, p.o.), a selective COX-2 inhibitor, did not. However, when rofecoxib was given together with SC-560, this treatment provoked severe lesions in the stomach, the lesion score being 19.74.1 mm². On the other hand, CJ-42794 (30 and 50 mg/kg) given p.o. did not cause any damage in the gastric mucosa of normal rats. CJ-42794 slightly damaged the gastric mucosa when co-administered with SC-560, yet the lesion score at 50 mg/kg was 5.8±2.2 mm², a highly significant difference from the damage induced by rofecoxib plus SC-560.

We also confirmed that indomethacin (10 mg/kg, p.o.) produced multiple hemorrhagic lesions in the small intestine within 24 hr, and the lesion score was 266.3±34.1 mm² (Figure 3). Neither SC-560 nor rofecoxib caused any damage in the small intestine, yet combined treatment with these two agents produced hemorrhagic lesions, the severity of which was similar to that of indomethacin-induced lesions. As observed in the stomach, CJ-42794 (30 and 50 mg/kg) by itself had no effect on the small intestinal mucosa, but caused slight damage when co-administered with SC-560, the lesion score being 76.1±20.0 mm².

Effect of CJ-42794 on Gastric Ulcerogenic Response Induced by Cold-Restraint Stress

It has been reported that the gastric ulcerogenic response to stress was markedly aggravated under PG-deficient conditions induced by NSAID treatment (Konturek et al., 1990; Ukawa et al., 1998; Tanaka et al., 2007). We compared the effects of CJ-42794 and various COX inhibitors on stress-induced gastric lesions and examined whether CJ-42794 has a deleterious influence on the gastric ulcerogenic response to stress, similar to NSAID.

Cold-restraint stress resulted in multiple hemorrhagic lesions in the stomach,
the lesion score being $14.2 \pm 1.6 \text{ mm}^2$ \textbf{(Figure 4)}. The severity of these lesions was significantly aggravated by prior p.o. administration of indomethacin (5 mg/kg), and the lesion score was $44.7 \pm 5.2 \text{ mm}^2$, which is about 2.3 times greater than that observed in control animals. Likewise, SC-560 (10 mg/kg) significantly increased the gastric ulcerogenic response to cold-restraint stress, the lesion score being $36.1 \pm 4.9 \text{ mm}^2$, whereas neither rofecoxib (10 mg/kg) nor CJ-42794 (30 mg/kg) had any effect.

\textbf{Effect of CJ-42794 on Gastric Ulcerogenic Response Induced by Aspirin}

It has been reported that the acetylated COX-2 induced to form by aspirin results in the formation of 15-epi-lipoxin A4 (AL4), which in turn protects the stomach against the ulcerogenic action of aspirin (Fiorucci et al., 2002). The same authors also showed that COX-2 selective inhibitors such as rofecoxib suppress AL4 production and aggravate the aspirin-induced gastric ulceration (Souza et al., 2003). Thus, we examined whether CJ-42794 has a deleterious effect on the aspirin-generated gastric lesions, similar to rofecoxib.

When aspirin (50 mg/kg) was given orally through esophageal intubation, the stomach suffered some damage with hemorrhaging, the lesion score being $5.2 \pm 0.3 \text{ mm}^2$ \textbf{(Figure 5)}. The severity of these lesions was markedly worsened by prior administration of rofecoxib (10 mg/kg, p.o.), the lesion score ($21.1 \pm 4.6 \text{ mm}^2$) being about 4 times greater than control values. Pretreatment of the animals with CJ-42794 (30 mg/kg), however, did not significantly affect the severity of the gastric lesions caused by aspirin, and the lesion score was equivalent to control values.

\textbf{Ulcerogenic Effect of CJ-42794 on GI Mucosa in Rats with Adjuvant Arthritis}

No damage was observed macroscopically in the GI mucosa of DA rats given the vehicle alone, with or without adjuvant arthritis. Neither indomethacin (3 mg/kg),
rofecoxib (30 mg/kg), nor CJ-42794 (30 and 50 mg/kg), given orally, caused any visible damage in the gastric mucosa of normal rats (Figure 6). SC-560 (30 mg/kg) induced some damage in the normal rat stomachs (7.9±0.2 mm²), yet no significant difference was observed among the groups. In adjuvant arthritic rats, however, both indomethacin and SC-560 produced severe lesions in the stomach within 4 hr, the lesion score being 54.1±14.2 mm² and 81.0±12.3 mm², respectively. Rofecoxib also damaged the gastric mucosa in arthritic rats, though the lesions (20.1±4.8 mm²) were much less severe than those induced by indomethacin or SC-560. However, CJ-42794 even at 50 mg/kg did not cause much damage in the arthritic rat stomach; the lesion score was 6.1±3.1 mm², which was not significantly different from that (2.4±1.2 mm²) in normal rats.

Oral administration of neither indomethacin (3 mg/kg), SC-560 (30 mg/kg), nor rofecoxib (30 mg/kg) had an ulcerogenic effect in the small intestine of normal rats (Figure 7). Likewise, CJ-42794 (30 and 50 mg/kg) given p.o. in normal rats did not damage the mucosa of the small intestine. In arthritic rats, however, indomethacin produced severe hemorrhagic lesions in the small intestine, the lesion score being 51.6±12.1 mm². SC-560 also generated hemorrhagic lesions in the arthritic rat small intestine, similar to indomethacin, the lesion score being 36.1±8.2 mm². By contrast, rofecoxib did not damage the small intestine of arthritic rats. Similarly, CJ-42794 at 30 mg/kg had little injurious effect on the small intestine of arthritic rats as compared to normal rats, yet at 50 mg/kg did slightly damage the mucosa, the lesion score being 10.1±1.9 mm², which was significantly greater than that observed in normal rats.

**Effect of CJ-42794 on Spontaneous Healing of Gastric Ulcers**

Three days after the thermo-cauterization, well-defined ulcers developed in the mucosa, the ulcer score being 17.1±1.8 mm². These ulcers healed gradually within 14 days, and the ulcer score on day 17 was 1.6±0.2 mm² (Figure 8). The healing of gastric
ulcers was markedly impaired when the animals were given indomethacin (2 mg/kg, p.o.) once daily for 14 days starting from 3 days after the ulcers were induced; the ulcer score on day 17 was 8.2±1.6 mm², which was significantly greater than the values for the control group. Rofecoxib (10 mg/kg) also significantly delayed the healing of gastric ulcers, the ulcer score being 7.2±1.9 mm². Likewise, the healing was dose-dependently delayed by the daily administration of CJ-42794 (3, 10 and 45 mg/kg), and a significant effect was observed at both 10 and 45 mg/kg. The ulcer score in rats treated with CJ-42794 at 10 mg/kg was 6.3±1.2 mm², almost equivalent to that observed in the animals treated with rofecoxib at 10 mg/kg. SC-560 (10 mg/kg) had no effect on the healing of ulcers (data not shown).

**Effect of CJ-42794 on PGE₂ Content, VEGF Expression and Angiogenesis in the Ulcerated Gastric Mucosa**

Several studies have demonstrated the COX-2/PGE₂/VEGF pathway to be involved in the healing of gastric ulcers (Miura et al., 2004). We examined the effects of CJ-42794, indomethacin, and rofecoxib on PGE₂ content and VEGF expression as well as the angiogenic response in the ulcerated mucosa of the stomach.

Levels of PGE₂ in normal rat gastric mucosa were 5.0±0.3 pg/mg tissue. PGE₂ content was markedly increased in the ulcerated mucosa (on day 10), reaching a value of 28.7±2.4 pg/mg tissue, about 8 times that in normal rats (Figure 9). The increase was significantly suppressed when animals were treated with indomethacin (2 mg/kg, p.o.) given once daily for 7 days, the inhibition being 90.6%. Likewise, the repeated administration of rofecoxib (10 mg/kg, p.o.) for 7 days also significantly decreased the amount of PGE₂ in the ulcerated mucosa. However, CJ-42794 (10 mg/kg, p.o.) did not have a significant effect on the increased production of PGE₂ in the ulcerated mucosa.

Conventional Western blotting revealed VEGF protein to be constitutively
expressed in both the normal mucosa and the ulcerated mucosa on day 10 after ulceration, although the expression was clearly up-regulated in the latter (Figures 10A and 10B). However, the expression of VEGF in the ulcerated mucosa was significantly down-regulated when the animals were treated with indomethacin (2 mg/kg) and rofecoxib (5 mg/kg) once daily for 7 days. Likewise, CJ-42794 (10 mg/kg, p.o.) also significantly suppressed the increase of VEGF expression, similar to indomethacin or rofecoxib. SC-560 (10 mg/kg) had no effect on the VEGF expression (data not shown). On day 10 after ulceration, the ulcer base was spontaneously reconstructed by the growth of granulation tissue and newly formed microvasculature, as represented by Factor VIII-positive cells (Figure 11A). As shown in Figure 11B, one-week treatment with indomethacin (2 mg/kg) and rofecoxib (10 mg/kg) apparently prevented the growth of granulation in the ulcer base; the degree of re-vascularization was 8.1±0.6 microvessels/mm² and 7.8±0.5 microvessels/mm², respectively, both of which were significantly less than that (20.0±0.3 microvessels/mm²) in control mice. Likewise, CJ42794 (10 mg/kg) also significantly decreased the angiogenic response, the degree of re-vascularization being 6.5±0.4 microvessels/mm².
Discussion

PGE₂ is both an inflammatory mediator released at the site of tissue inflammation and a neuromodulator that alters neuronal excitability and synaptic processing. Recent study demonstrated that the EP4 antagonist AH23848 attenuated inflammation-induced thermal and mechanical behavioral hypersensitivity in vivo and also reduced the PGE₂-mediated sensitization of capsaicin-evoked currents in dorsal root ganglion neurons in vitro (Lin et al., 2006). It is thus assumed that EP4 antagonists may be used for the pharmacological treatment of inflammatory pain, similar to NSAIDs or selective COX-2 inhibitors. The latter drugs, even selective COX-2 inhibitors, reportedly cause adverse reactions in the GI tract, especially, in the stomach (Ukawa et al., 1998; Wallace et al., 2000; Tanaka et al., 2001; 2002a Kato et al., 2002; Ohno et al., 2004). An EP4 antagonist free of adverse reactions in these tissues would be the ideal therapeutic agent for treatment of inflammatory pain. In the present study, we therefore tested the novel selective EP4 antagonist, CJ-42794, in a variety of standard models of GI injury and repair, in order to determine its potential for mucosal injury.

First, CJ-42794 even at 50 mg/kg did not by itself have an injurious effect in either the stomach or small intestine. Since the ED₅₀ of this agent given i.d. was found to be about 0.5 mg/kg under in vivo conditions, judging from the inhibition of the HCO₃⁻ response to the EP4 agonist, it is assumed that CJ-42794 does not cause deleterious effects in the GI mucosa at a therapeutic dose. We also confirmed that this agent dose-dependently antagonized the HCO₃⁻ stimulatory action of PGE₂, although the effect was less potent as compared to that observed against AE1-329. This is understandable, because the HCO₃⁻ response to PGE₂ is mediated by the activation of both EP3 and EP4. Consistent with our previous observations (Tanaka et al., 2001; 2002a), indomethacin at the doses used produced hemorrhagic lesions in both the stomach and small intestine,
whereas neither SC-560 (selective COX-1 inhibitor) nor rofecoxib (selective COX-2 inhibitor) caused any damage in these organs. Rofecoxib, however, provoked damage in these tissues when co-administered with SC-560, confirming that inhibition of both COX-1 and COX-2 is required for NSAID-induced GI damage (Wallace et al., 2000; Tanaka et al., 2001; 2002a). We have previously reported that SC-560 suppressed production of PGs through inhibition of COX-1 but up-regulated COX-2 expression in the GI mucosa and that PGE$_2$ derived from COX-2 plays a compensatory role in the mucosal defense of these tissues (Tanaka et al., 2001; 2002a). Although CJ-42794 did not have an adverse effect in the stomach when co-administered with SC-560, at 50 mg/kg it produced some damage in the small intestine in the presence of SC-560. Since PGE$_2$ protects the intestinal mucosa through the activation of EP4 receptors (Kunikata et al., 2002), it is understandable that CJ-42794, though at a high dose, damaged the intestinal mucosa when co-administered with SC-560.

NSAIDs not only are themselves ulcerogenic in the GI mucosa but also potentiate the gastric ulcerogenic response to various stimuli including stress. Konturek et al (1990) reported that gastric lesions produced by water-immersion stress were markedly worsened by indomethacin at a low dose that did not cause any damage in the stomach. We also showed that the gastric ulcerogenic response to cold stress was increased by pretreatment with indomethacin and SC-560 (Ukawa et al., 1998; Tanaka et al., 2007). Since these agents decreased mucosal PGE$_2$ production, it is assumed that the aggravating effect on stress ulcers is related to a deficiency of endogenous PGs. The selective COX-2 inhibitor rofecoxib had no effect on basal PG levels in the stomach and did not affect the ulcerogenic response to cold stress, consistent with the observations of Tanaka et al. (2007). As expected, CJ-42794 did not affect the ulcerogenic response to cold stress in the stomach, confirming our previous finding that endogenous PGE$_2$ contributes to the gastric mucosal defense mediated by the activation of EP1 but not

Unlike other NSAIDs, COX-2’s acetylation by aspirin switches eicosanoid biosynthesis from PGE\textsubscript{2} to AL4, which exerts protective effects in the stomach. Co-administration of aspirin and a selective COX-2 inhibitor, such as celecoxib or rofecoxib, resulted in substantially more severe gastric injury than that produced with either agent alone (Fiorucci et al., 2002; Souza et al., 2003). In the present study, we observed that the gastric ulcerogenic response to aspirin was significantly worsened by co-administration of rofecoxib but not CJ-42794. These results confirmed the importance of COX-2’s inhibition in this phenomenon related to the suppression of AL4’s production and suggested that EP4 receptors have nothing to do with the aggravation of aspirin-induced gastric ulceration.

We have previously demonstrated that NSAID-induced gastric lesions were markedly aggravated in rats with adjuvant-induced arthritis (Kato et al., 1999). We further showed that arthritic conditions up-regulated COX-2 expression in the stomach, where selective COX-2 inhibitors by themselves produced lesions, although they caused no damage in normal rat stomachs (Kato et al., 2002). The present study confirmed that rofecoxib produced damage in the stomach of arthritic rats. Interestingly, we observed that SC-560, a selective COX-1 inhibitor, also damaged the arthritic rat stomach, whereas CJ-42794 did not. Adjuvant arthritis is often used for animal models of rheumatoid arthritis, and these arthritic animals are known to suffer from chronic systemic inflammation and severe pain. Since the present and previous studies showed that SC-560, but not CJ-42794, worsened stress-induced gastric lesions, similar to indomethacin (Tanaka et al., 2007), it is possible that SC-560 produced hemorrhagic lesions in the stomach by potentiating the ulcerogenic response to arthritis-related stress.

On the other hand, both SC-560 and indomethacin, at doses that do not damage the normal rat intestine, produced lesions in the small intestine of arthritic rats, whereas
rofecoxib did not. We have recently found that indomethacin-induced intestinal lesions were markedly worsened in adjuvant-induced arthritic rats and suggested the increased intestinal ulcerogenic response to be related to the up-regulation of Toll-like receptor 4 (TLR4) in these animals (Kato et al., 2006). Enterobacteria that have invaded the mucosa exert a pathogenic influence via TLR4, and this response may be exaggerated in arthritic rats. We also previously reported that SC-560, similar to indomethacin, caused a marked increase in intestinal motility as well as a decrease in the secretion of mucus, somehow leading to derangement of the intestinal barrier to pathogen and resulting in bacterial invasion (Tanaka et al., 2002b; Takeuchi et al., 2002). The pathogenic bacterial insult due to the increased TLR4 expression may overcome the compensatory action of PGs derived from COX-2 up-regulated by SC-560 (Tanaka et al., 2002a). It is understandable that rofecoxib did not damage the small intestine in arthritic rats, because this agent by itself had no effect on intestinal motility (Tanaka et al., 2002b). By contrast, CJ-42794 at 50 mg/kg produced slight damage in the small intestine of arthritic rats, although this agent at 30 mg/kg had no effect. Kunikata et al. (2001) showed that the presence of EP4 receptors is essential for the protective action of PGE$_2$ against NSAID-induced intestinal lesions (Kunikata et al., 2001). Even in normal rats, CJ-42794 slightly damaged the small intestine when co-administered with SC-560, although this agent alone had no injurious effect.

We also found in the present study that the healing of gastric ulcers in rats was markedly delayed by the daily administration of CJ42794, in addition to indomethacin or rofecoxib, although this drug, unlike the latter two agents, did not affect the increase in PGE$_2$ production in the gastric mucosa after ulceration. These results are consistent with our recent observation that endogenous PGE$_2$ contributes to the healing of indomethacin-induced intestinal ulcers via the activation of EP4 receptors (Hatazawa et al., 2006) and suggest the involvement of EP4 receptors in the healing-promoting
action of PGE₂. It is assumed that the PGE₂ produced by COX-2 accelerates the healing of gastric ulcers via the activation of EP4 receptors. The healing mechanism in wounded tissues involves multiple steps, such as the formation of granulation tissue, and these processes are regulated by growth factors, such as VEGF, produced locally by regenerating cells (Tarnawski, 2005). Angiogenesis, an essential component of the wound healing process, is induced by VEGF, which is known as a fundamental regulator of angiogenesis (Szabo et al., 1998). As expected, we found in the present study that both rofecoxib and CJ-42794 down-regulated the expression of VEGF protein in the gastric mucosa after ulceration, similar to indomethacin. These results suggest that endogenous PGE₂ derived from COX-2 stimulates both VEGF expression and angiogenesis in the ulcerated mucosa through the activation of EP4 receptors.

Taking all the present findings together, we confirmed that the untoward effects of indomethacin, a conventional NSAID (nonselective COX inhibitor), included ulcerogenic properties in the GI mucosa of normal and arthritic rats, aggravation of the gastric ulcerogenic response to stress, and the impairment of gastric ulcer healing. It was also found that rofecoxib, a selective COX-2 inhibitor, was less ulcerogenic in the GI tract, even under stressful conditions, yet provoked apparent lesions in the arthritic rat stomach, and also impaired the healing process. The most important finding of the present study is that CJ-42794, a selective EP4 antagonist, did not cause any damage in either the normal rat GI mucosa or the arthritic rat stomach, and did not worsen the gastric ulcerogenic response to stress or aspirin in normal rats, although this agent slightly damaged the small intestine of arthritic rats and impaired the healing of gastric ulcers. Thus, since this EP4 antagonist shows apparently less adverse effects in the GI mucosa, as compared with various COX inhibitors, it would be an ideal therapeutic agent for the treatment of inflammatory pain.
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Footnotes

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Legends for Figures

Figure 1. Effect of CJ-42794 on duodenal HCO\textsubscript{3}\textsuperscript{-} secretion stimulated by the EP4 agonist AE1-329 in anesthetized rats. HCO\textsubscript{3}\textsuperscript{-} secretion was measured in the proximal duodenum under urethane anesthesia. AE1-329 (3 µg/kg) was given i.v. as a single injection. CJ-42794 (0.3-3 mg/kg) was administered i.d. 30 min before the injection of AE1-329. Data show the net HCO\textsubscript{3}\textsuperscript{-} output for 1 hr after the administration of AE1-329 and are presented as the mean±SE for 4~6 rats per group. Significant difference at P<0.05; * from control; # from saline.

Figure 2. Effects of indomethacin, SC-560, rofecoxib, and CJ-42794, either alone or in combination, on the rat gastric mucosa. The animals were administered indomethacin (30 mg/kg), SC-560 (30 mg/kg), rofecoxib (30 mg/kg), or CJ-42794 (30 and 50 mg/kg) orally, and killed 4 hr later. In some cases, rofecoxib and CJ-42794 were co-administered with SC-560. Data are presented as the mean±SE for 5~8 animals. * Significant difference from control, at P<0.05.

Figure 3. Effects of indomethacin, SC-560, rofecoxib, and CJ-42794, either alone or in combination, on the small intestinal mucosa in rats. The animals were administered indomethacin (10 mg/kg), SC-560 (30 mg/kg), rofecoxib (30 mg/kg), or CJ-42794 (30 and 50 mg/kg) orally, and killed 24 hr later. In some cases, rofecoxib and CJ-42794 were co-administered with SC-560. Data are presented as the mean±SE for 6~8 animals. * Significant difference from control, at P<0.05.

Figure 4. Effects of indomethacin, SC-560, rofecoxib, and CJ-42794 on the gastric ulcerogenic response induced by cold-restraint stress in rats. The animals mildly
restrained in Bollman cages were placed in a cold room at an ambient temperature of 10°C for 4 hr. Indomethacin (5 mg/kg), SC-560 (10 mg/kg), rofecoxib (10 mg/kg) or CJ-42794 (10 mg/kg) was administered orally 1 hr before the onset of stress. Data are presented as the mean±SE for 5~8 animals. * Significant difference from control, at P<0.05.

**Figure 5.** Effects of rofecoxib and CJ-42794 on the gastric ulcerogenic response induced by aspirin in rats. The animals were administered aspirin (50 mg/kg) orally and killed 4 hr later. Rofecoxib (30 mg/kg) or CJ-42794 (50 mg/kg) was administered 1 hr before the administration of aspirin. Data are presented as the mean±SE for 8 animals. * Significant difference from vehicle, at P<0.05.

**Figure 6.** Gastric ulcerogenic effect of indomethacin, rofecoxib, SC-560, and CJ-42794 in rats without or with adjuvant-induced arthritis. Arthritis was induced by injection of Freund’s complete adjuvant (FCA) into the plantar region of the right hindfoot, and the experiments were performed 14 days after the injection. Indomethacin (3 mg/kg), rofecoxib (30 mg/kg), SC-560 (30 mg/kg), or CJ-42794 (30 and 50 mg/kg) was administered orally, and the animals were killed 4 hr later. Data are presented as the mean±SE for 4~8 animals. * Significant difference from the corresponding group in normal rats, at P<0.05.

**Figure 7.** Intestinal ulcerogenic effect of indomethacin, rofecoxib, SC-560, and CJ-42794 in rats without or with adjuvant-induced arthritis. Arthritis was induced by injection of Freund’s complete adjuvant (FCA) into the plantar region of the right hindfoot, and the experiments were performed 14 days after the injection. Indomethacin (3 mg/kg), rofecoxib (30 mg/kg), SC-560 (30 mg/kg) or CJ-42794 (30 and 50 mg/kg) was
administered orally, and the animals were killed 24 hr later. Data are presented as the mean±SE for 4–8 animals. * Significant difference from the corresponding group in normal rats, at P<0.05.

**Figure 8.** Effects of CJ-42794 and various COX inhibitors on the healing of gastric ulcers in rats. Gastric ulcers were produced by thermocauterization (70°C, 20 sec), and the animals were killed 17 days later. Indomethacin (2 mg/kg), rofecoxib (10 mg/kg) or CJ-42794 (3, 10 and 45 mg/kg) was administered p.o. twice daily for 14 days, starting 3 days after the acid application. Data are presented as the mean±SE for 6–9 animals. *Significant difference from control, at P<0.05.

**Figure 9.** Effects of CJ-42794 and various COX inhibitors on PGE₂ content in the ulcerated gastric mucosa in rats. Gastric ulcers were produced by thermo-cauterization (70°C, 20 sec), and the animals were killed 10 days later. CJ-42794 (10 mg/kg), indomethacin (2 mg/kg), or rofecoxib (10 mg/kg) was given p.o. once daily for 7 days starting 3 days after the ulceration. Mucosal PGE₂ content was measured by EIA. Data are presented as the mean±SE for 7–8 rats. Significant difference at P<0.05; * from normal; # from control.

**Figure 10.** Effects of CJ-42794 and various COX inhibitors on VEGF expression in the ulcerated gastric mucosa in rats. Gastric ulcers were produced by thermo-cauterization (70°C, 20 sec), and the animals were killed 10 days later. CJ-42794 (10 mg/kg), indomethacin (2 mg/kg), or rofecoxib (10 mg/kg) was given p.o. once daily for 7 days starting 3 days after the ulceration. Mucosal expression of VEGF was determined by Western blotting. **Figure B** showed the densitometric quantification determined by Quantity One software and the results are expressed as dimeric/total ratio. Data are
presented as the mean±SE from 3–4 rats. Significant difference at P<0.05: * from normal; # from control.

**Figure 11.** Effects of CJ-42794 and various COX inhibitors on the angiogenic response in the rat ulcerated gastric mucosa. Gastric ulcers were produced in rats by thermocauterization (70°C, 20 sec), and the animals were killed on day 10 after ulceration. Indomethacin (2 mg/kg), rofecoxib (10 mg/kg) or CJ42794 (10 mg/kg) was given p.o. once daily for 7 days starting 3 days after ulceration. Frozen sections were prepared, and immunostaining with anti-Factor VIII antibody was performed. Factor VIII-positive cells represent newly formed microvasculature. **Figure A** shows the histological observation of the ulcerated mucosa (x40). **Figure B** shows the number of vessels per mm² of ulcer base and represent the mean±SE from 6 rats. * Significant difference from control (untreated mucosa of untreated rats), at P<0.05.
Figure 1

Net HCO₃⁻ Output (µEq/hr)

Control

Saline

0.3 1 3 mg/kg

CJ-42794

AE1-329 (3 µg/kg)

N=4-6

*# P<0.05

ED₅₀: 0.5 mg/kg

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Figure 2
Figure 3
Figure 4
Figure 5

Gastric Lesions (mm$^2$)

- Control
- Vehicle
- Aspirin (50 mg/kg)
- Rofecoxib
- CI-427/4

$N=8$

* $P<0.05$
Figure 7
Figure 8
Figure 9
Figure 10
Figure 11