The anti-inflammatory agent etodolac is used worldwide and it has a good gastrointestinal safety profile. Etodolac consists of two enantiomers, \( S \)- and \( R \)-etodolac. Here, we investigated the beneficial activities of racemic etodolac and its enantiomers. First, we compared \( S \)- and \( R \)-etodolac in terms of their inhibition of cyclooxygenase (COX) activity in vitro and their suppression of paw swelling in adjuvant-induced arthritic rats. The COX-2 inhibitory and anti-inflammatory effects of etodolac were found to be due to the \( S \)-enantiomer. We previously reported that etodolac attenuates allodynia in a mouse model of neuropathic pain by a COX-2-independent mechanism [N. Inoue et al., J. Pharmacol. Sci., 109, 600—605 (2009)]. In the present study, we showed that the anti-allodynic effects of etodolac in mice were also due to the \( S \)-enantiomer. In addition, we investigated the ulcerogenic activity of racemic etodolac and its enantiomers. At high doses, racemic etodolac showed a lower gastric lesion index in rats than the equivalent dose of \( S \)-etodolac. In contrast, \( R \)-etodolac showed no ulcerogenic activity and even showed protection against HCl/ethanol-induced gastric damage in rats. In conclusion, \( S \)-etodolac exhibited anti-inflammatory effects mediated by COX-2 inhibition and anti-allodynic effects that were independent of COX-2 inhibition, while \( R \)-etodolac showed gastroprotective effects that may contribute to the low gastrointestinal toxicity of racemic etodolac. Our results show that each enantiomer plays a different role in the efficacy and gastrointestinal safety of etodolac.

Key words etodolac; enantiomer; adjuvant arthritis; neuropathic pain; ulcerogenic activity

The therapeutic effects of non-steroidal anti-inflammatory drugs (NSAIDs) include anti-pyretic, analgesic and anti-inflammatory effects, while their adverse effects are primarily gastrointestinal (GI) and renal toxicity. The major therapeutic and adverse effects are mediated by the inhibition of cyclooxygenase (COX), which catalyzes the rate-limiting step in the formation of prostanooids from arachidonic acid.

There are two membrane-bound COX isoenzymes, the constitutively expressed COX-1 and the highly inducible COX-2. COX inhibitors include conventional NSAIDs and COX-2-selective inhibitors. Conventional NSAIDs, at therapeutic doses, are non-selective COX inhibitors and inhibit both isoenzymes. The anti-inflammatory benefits of NSAIDs are primarily due to COX-2 inhibition, while inhibition of COX-1 often elicits GI toxicity. Etodolac is widely known as a COX-2-selective inhibitor. It has good clinical anti-inflammatory efficacy and a good safety profile for the GI tract.

Many NSAIDs are chiral and are marketed as the racemate (which contains equal amounts of each enantiomer). The anti-inflammatory activity of NSAIDs and their inhibition of prostaglandin synthetase is largely stereospecific in favor of the \( S \)-enantiomer. Etodolac too is a racemate consisting of \( S \)- and \( R \)-enantiomers, and \( S \)-etodolac inhibits prostaglandin synthesis in sheep vesicular glands and decreases hind-paw volume in established adjuvant arthritic rats.

Neuropathic pain is a common symptom caused by injury to peripheral or central nerves, and it can result from pathological changes induced by metabolic disease, viral infection, traumatic injury or chemotherapeutically induced nerve damage. Clinically, neuropathic pain is characterized by mechanical and thermal allosthenia, hyperalgesia and spontaneous ongoing pain that are often refractory to treatment. Patients with neuropathic pain do not respond to NSAIDs, and resistance or insensitivity to opiates is common. We recently reported that etodolac attenuates allodynia in a mouse model of neuropathic pain. The mechanism of the anti-allodynic effects of etodolac has not yet been established, but COX inhibition is unlikely to be involved because the COX inhibitors indomethacin and celecoxib do not attenuate the allodynia. There is still a question which enantiomer of etodolac produces the anti-allodynic effect in mice. And there is also a question whether the ulcerogenic activity of etodolac is attributable to the \( S \)-enantiomer alone.

In the present study, we evaluated the inhibitory effects of the enantiomers of etodolac on COX-1 and COX-2. We also investigated the beneficial actions of racemic etodolac and its enantiomers, especially their anti-inflammatory effects, anti-allodynic effects and ulcerogenic activity, in mouse and rat models. \( S \)-Etodolac, but not \( R \)-etodolac, inhibited COX-2 and showed anti-inflammatory and anti-allodynic effects, while \( R \)-etodolac showed a gastroprotective effect that may explain the low GI toxicity of racemic etodolac.

**MATERIALS AND METHODS**

**Materials** Racemic etodolac and the \( S \)- and \( R \)-enantiomers were synthesized in our laboratories, and diclofenac and meloxicam were purchased from Merck (Darmstadt, Germany). For in vivo studies, each compound was suspended 0.5% methylcellulose solution and administered orally.

**Animals** Male Scl:ddY mice (five weeks of age) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Male Crl:CD (Sprague-Dawley; SD) rats (six weeks of age) and male LEW/Crl:CD (Lewis) rats (seven weeks of age) were purchased from Charles River Japan (Yokohama, Kanagawa, Japan). The animals were used after quarantine and acclimation for one week. They were housed four to six per room.
cage under a 12-h light–dark cycle (lights on, 8:00—20:00) at 20—26 °C, a relative humidity of 35—75%, and a ventilation frequency of at least 15 times/h. They were allowed free access to pellet chow (F-2; Funabashi Farm, Funabashi, Chiba, Japan) and tap water. The study was conducted in compliance with the Internal Regulations on Animal Experiments at Nippon Shinyaku Co., Ltd., which are based on the Law for the Humane Treatment and Management of Animals (Law No. 105, 1 October 1973, as amended on 1 June 2006).

**COX-1 and COX-2 Assay** Inhibition of COX-1 and COX-2 was measured with the Colorimetric COX (ovine) Inhibitor Screening Assay Kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) according to the manufacturer’s instructions. The assay is based on the measurement of peroxidase activity by monitoring the appearance of oxidized N,N,N′,N″-tetramethyl-p-phenylenediamine (TMPD) at 595 nm with a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.). IC_{50} values were calculated by nonlinear regression with SAS software, version 8.2 (SAS Institute, Cary, NC, U.S.A.) as the concentration required to produce 50% inhibition of TMPD oxidation.

**Adjuvant-Induced Arthritis (AIA)** Adjuvant arthritis was induced by injecting 50 μl of a 1.2% suspension of dried heat-killed Mycobacterium butyricum (Difco Laboratories, Detroit, MI, U.S.A.) in paraffin oil intradermally into the plantar region of the right hind paw of male Lewis rats on day 0. Vehicle or compounds were administered orally once a day from day 0 to day 20. The volume of the right hind paw (ipsilateral paw; injected with adjuvant) was measured on day 0 and day 3, and that of the left hind paw (contralateral paw; not injected with adjuvant) on day 0 and day 21, with a plethysmometer (TK-105; Unicomp, Yachiyo, Chiba, Japan). The severity of the adjuvant arthritis was assessed by the increase in paw volume after the injection of adjuvant.

**Partial Sciatic Nerve Ligation (PSNL)** Partial ligation of the sciatic nerve was performed on ddY mice under pentobarbital anesthesia (50 mg/kg intraperitoneally (i.p.)) as described by Seltzer et al. Briefly, the sciatic nerve of the right hind limb was exposed and one-third to one-half of the center of the ventral surface of the paw until the filament was bent slightly. If the mouse withdrew or lifted the paw upon application of the hair, then a hair one size smaller was tried. Conversely, if no response was observed, a hair one size larger was tried. In this way, the minimum hair size (in grams) required to induce a response in at least three of ten trials was determined and recorded as the PWT.

**In Vitro Profiling of S-Etodolac** An in vitro profiling test was performed by Ricerca Bioscience (Concord, OH, U.S.A.). S-Etodolac (10 μm) was tested for binding to a variety of receptors (glutamate [N-methyl-D-aspartic acid (NMDA), kainate, and AMPA], purinergic [P2X and P2Y], bradykinin [B1 and B2], tachykinin [NK1 and NK2], adenosine [A1, A2A, and A3], adrenergic [α1A, α1B, α1D, α2A, α2B, α2C, β1, β2, and β3], muscarinic [M1, M2, M3, M4, and M5], dopamine [D1, D2L, D3, and D4], cannabinoid [CB1], chemokine [CCR2B, CCR4, CCR5, and CXCR2], neuropeptide Y [Y1 and Y2], opiate [μ, κ, and δ], serotonin [1A, 1B, 2B, 2C, 3, 4, and 6], and histamine [H1 and H2]), channels (sodium [site 2], chloride [γ-aminobutyric acid (GABA)], calcium [benzodiazepine, dihydropyridine, and phenylalkylamine binding site], and potassium [K_{ST}], and transporters (adenosine, dopamine, norepinephrine, serotonin, and GABA), and its inhibition of enzymes (COX-1, COX-2, 15-lipoxygenase, nitric oxide synthases [inducible and neuronal], neutrophil elastase 2, matrix metalloproteinases [MMP-1 and MMP-9], tyrosine kinases [EGF receptor, Lck, and Fyn], serine/threonine kinases [p38α, ERK1, ERK2, and PKCα], phosphodiesterases [PDE-3, PDE-4, and PDE-5], and aldose reductase).

**Gastric Mucosal Lesions** For observation of gastric mucosal lesions induced by test compounds, normal SD rats were deprived of food, but not water, for 18 h before compound administration. The rats were orally administered compound and euthanized 7 h later under deep ether anesthesia. The gastroprotective effect of R-etodolac was assessed in a model of gastric mucosal injury induced by HCl/ethanol. First, rats were pretreated by oral administration of R-etodolac or 0.5% methylcellulose vehicle. After 30 min, 1 ml of 150 mM HCl in 60% ethanol was orally administered. One hour after HCl/ethanol treatment, the rats were killed and their stomachs were removed, inflated by injection of 8 ml of 2% formalin, immersed in 2% formalin for 10 min to fix the gastric wall, and opened along the greater curvature. The length of each lesion in the glandular mucosa was measured under a dissecting microscope (SZH; Olympus, Tokyo, Japan) with a square grid (×10 magnification), and the lengths of the lesions were summed for each stomach.

**Statistical Analysis** The statistical significance of differences between normal and control groups was analyzed by the student’s t-test for paw swelling in AIA rats and by the Wilcoxon’s rank-sum test for PSNL-induced decrease in the PWT. Control and compound-treated groups were analyzed by Dunnett’s multiple-comparison test for paw swelling in AIA rats and the HCL/ethanol-induced gastric lesion index and the Shirley–Williams test for PSNL-induced decrease in the PWT. Differences in the gastric lesion index between racemic etodolac and the equivalent dose of S-etodolac in normal rats were analyzed by the Wilcoxon’s rank-sum test. Values of p<0.05 were regarded as statistically significant. Statistical analyses were carried out with SAS software, version 8.2.

**RESULTS**

**Inhibition of COX-1 and COX-2** Racemic and S-etodolac both inhibited COX-2 activity in a concentration-dependent manner with IC_{50} values of 21.6 and 11.6 nm, respectively (Fig. 1A), whereas the inhibitory effects of both
racemic and S-etodolac on COX-1 were weak (Fig. 1B). R-Etodolac did not inhibit either COX-1 or COX-2. Diclofenac potently inhibited both isoenzymes with IC$_{50}$ values of 0.114 and 0.399 nM, respectively. The IC$_{50}$ value of meloxicam for COX-2 was 195 nM, an order of magnitude higher than that of racemic and S-etodolac.

**Anti-inflammatory Effects in AIA Rats** In control rats, the volume of the ipsilateral paw increased within a few days (this is the acute, or primary, swelling), while the volume of the contralateral paw increased over a period of a few weeks (this is the immune-mediated, or secondary, swelling). By day 3, the volumes of the ipsilateral paw in normal and control rats were 1.76±0.03 and 3.30±0.04 ml, respectively, corresponding to the primary swelling (Fig. 2). On day 21, the volumes of the contralateral paw in normal and control rats were 1.95±0.03 and 3.09±0.12 ml, respectively, corresponding to the secondary swelling. S-Etodolac significantly inhibited both primary and secondary swelling at 1 and 10 mg/kg. In contrast, R-etodolac did not inhibit either type of swelling at 10 or even 100 mg/kg.

**Effects of Racemic Etodolac, Meloxicam and Diclofenac on Mechanical Allodynia in PSNL Mice** PSNL treatment greatly reduced the PWT of the hind paw (Fig. 3). Etodolac (10 mg/kg) had brought about significant recovery of the PWT by day 14 after PSNL (after one week’s administration of etodolac) and day 21 after PSNL (after two weeks’ administration of etodolac), whereas neither meloxicam (10 mg/kg) nor diclofenac (3 mg/kg) had any significant effect throughout the assessment period.

**Effect of S- and R-Etodolac on Mechanical Allodynia in PSNL Mice** In PSNL groups, the mechanical PWT of the ipsilateral hind paw was greatly reduced (Fig. 4). Compounds were administered orally for two weeks starting 7 d after PSNL. By day 14, S-etodolac (5 mg/kg) had increased the PWT significantly before and 1 h after the dose. By day 21, S-etodolac had increased the PWT significantly at all measurement points. In addition, the efficacy of S-
etodolac gradually increased throughout the administration period, and had restored pre-ligation PWT values by day 21. In contrast, R-etodolac (5 mg/kg) had no effect on the PWT throughout the assessment period (Fig. 4).

**In Vitro Profiling of S-Etodolac**  S-Etodolac (10 μM) was tested in vitro on the receptors, channels, transporters, and enzymes listed in Materials and Methods. S-Etodolac inhibited COX-2 by 86%, but all other tests were negative.

**Gastric Mucosal Ulcerogenic Activity of Racemic Etodolac and Its Enantiomers in Normal Rats**  Gastric mucosal lesions were induced in normal rats that had been given racemic, S-, or R-etodolac 7 h before being euthanized. The gastric lesion index calculated when racemic etodolac was administered was lower than when the equivalent dose of S-etodolac was administered, with a significant difference being observed when racemic etodolac was administered at a dose of 30 mg/kg (Fig. 5). R-Etodolac showed no ulcerogenic activity at 50 mg/kg (data not shown).

**Anti-ulcerogenic Activity of R-Etodolac in a Rat Model of Gastric Mucosal Injury Induced by HCl/Ethanol**  Oral administration of HCl/ethanol to rats induced multiple lesions in the glandular mucosa of the stomach. These gastric lesion index were suppressed in a dose-dependent manner by prior administration of R-etodolac (Fig. 6), with a significant effect being observed at 50 mg/kg.

**DISCUSSION**

In this study, we investigated the in vitro and in vivo effects of racemic etodolac and its S- and R-enantiomers. First, we showed that the COX-2 inhibitory activity, anti-inflammatory effect and anti-allodynia effect of etodolac are attributable to the S-enantiomer. In many NSAIDs, inhibition of prostaglandin E<sub>2</sub> production is due to the S-enantiomer. The S-enantiomer of etodolac suppresses prostaglandin synthesis in sheep vesicular glands and paw swelling in adjuvant arthritis rats. Therefore, the COX-inhibitory effects and anti-inflammatory effects of etodolac might also be expected to be due to the S-enantiomer. However, the dominant isoenzyme in vesicular glands was later recognized to be COX-1, and COX-1 and COX-2 inhibition were not separately evaluated in the earlier study. Therefore, in the present study, we separately measured the inhibition of COX-1 and COX-2 by each etodolac enantiomer. In vitro, S-etodolac potently inhibited COX-2 but only weakly inhibited COX-1. Meloxicam also inhibited only COX-2, but not as strongly as S-etodolac did. In contrast, R-etodolac did not inhibit either isoenzyme. Etodolac is a COX-2-selective drug, and in the present study we have shown that its anti-inflammatory effect in adjuvant-induced arthritis rats is entirely due to the S-enantiomer. Similar results have been reported in an established arthritis model demonstrating therapeutic effects of the S-enantiomer. In the present study, we administered etodolac from the date of adjuvant injection, demonstrating prophylactic effects of S- and racemic etodolac. S-Etodolac showed highly selective COX-2-inhibitory and anti-inflammatory effects, while R-etodolac showed neither effect.

A number of animal models have been reported to mimic human peripheral neuropathic conditions. Most neuropathic pain models have been produced in the rat or mouse, and, among these, peripheral nerve injury models have been the most extensively studied. In peripheral nerve injury models, partial injury to the sciatic nerve is commonly used to induce neuropathic pain behavior. Such models include chronic constriction injury (CCI) of the sciatic nerve, partial sciatic nerve ligation (PSNL), and spinal nerve ligation (SNL). In these models, the cutaneous sensory threshold of the ipsilateral hind paw is measured. The presence of neuro-
pathic pain is mainly assessed by hyperalgesia to thermal and mechanical stimuli and allodynia to cold and tactile stimuli.\textsuperscript{22-23} Although upregulation of COX-2 expression in the peripheral nervous system after injury has been reported in neuropathic pain models,\textsuperscript{24} neither rofecoxib (a selective COX-2 inhibitor) nor ibuprofen (a non-selective COX inhibitor) ameliorated established mechanical allodynia by chronic oral dosing in CCI rats.\textsuperscript{15} We have previously reported that etodolac, but not indomethacin or celecoxib, attenuates allodynia in PSNL mice.\textsuperscript{13} In the present study, neither diclofenac nor meloxicam showed anti-allodynic effects in AIA and nor- necotic activity of racemic etodolac and other NSAIDs when we previously compared the gastric mucosal ulcerogenic activity of racemic etodolac and other NSAIDs. All results were negative except for inhibition of COX-2, providing evidence that S-etodolac is COX-2-selective. Staaf \textit{et al.}\textsuperscript{25} recently reported differential regulation of transient receptor potential (TRP) channels in the dorsal root ganglion (DRG) in SNL rats. TRP channels are a family of non-selective cation-permeable channels that are important for sensory signaling in the peripheral nervous system.\textsuperscript{26} TRPML3 (mucolipin-3) is induced on nerve injury, whereas the levels of other TRP channels are decreased.\textsuperscript{25} In the SNL model, several proteins involved in the mediation of pain, including neuronal nitric oxide synthase, the α2δ subunit of the Ca\textsuperscript{2+} channel, and cyclic AMP-dependent transcription factor ATF-3, are upregulated in the DRG.\textsuperscript{27} Our finding in the present study that the anti-allodynic effects of etodolac gradually increased throughout the administration period is consistent with the idea that etodolac regulates the expression of genes involved in neuropathic pain. We intend to study the effects of etodolac on the expression of receptors, channels and other proteins involved in the mediation of neuropathic pain.

The low GI toxicity of etodolac can be explained by its high COX-2 selectivity, because inhibition of COX-1 is thought to be the main cause of GI ulcers and bleeding. When we previously compared the gastric mucosal ulcerogenic activity of racemic etodolac and other NSAIDs (meloxicam, diclofenac and indomethacin) in AIA and normal rat models,\textsuperscript{3} racemic etodolac showed the lowest ulcerogenic activity in both models. Here, we compared the gastric mucosal ulcerogenic activity of racemic etodolac and its enantiomers in normal rats (Fig. 5). At high doses, the gastric lesion index for racemic etodolac was lower than that for the equivalent dose of S-enantiomer. From this result, we hypothesized that the R-enantiomer has a gastroprotective effect that contributes to the low GI toxicity of racemic etodolac. This hypothesis is supported by our finding that R-etodolac provided gastric protection against HCl/ethanol-induced damage in rats (Fig. 6), although the extent of the protection was significant only at 50 mg/kg. In a future study, we hope to investigate the effect of R-etodolac on NSAID-induced gastric ulcer formation by monitoring gastric mucosal prostaglandin E\textsubscript{2} concentrations. Although there are several reports on the biological activity of R-etodolac, especially its cytotoxicity in multiple myeloma cells,\textsuperscript{28-29} the gastroprotective effects that we have observed have not been reported previously. Instillation of HCl/ethanol into the stomach of rats induces severe epithelial desquamation, deep mucosal necrosis and submucosal edema associated with leucocyte accumulation.\textsuperscript{30} The mucosal integrity of the stomach is maintained by multiple factors, including both paracrine and neuronal systems. Further work is required to elucidate the mechanism of the gastroprotective effects of R-etodolac as well as the anti-allodynic mechanism of S-etodolac.

In conclusion, S-etodolac has anti-inflammatory effects mediated by COX-2 inhibition, and anti-allodynic effects due to an unknown mechanism not mediated by COX-2 inhibition. R-etodolac has gastroprotective effects and may contribute to the low GI toxicity of racemic etodolac. This is a point in favor of racemic etodolac as a therapeutic agent for chronic inflammatory diseases and neuropathic pain.

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