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Pharmacological profile of FK881(ASP6537), a novel potent and selective cyclooxygenase-1 inhibitor

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are now understood to fall into one of two agent classes in clinical use. Traditional NSAIDs inhibit both cyclooxygenases-1 and 2 (COX-1, 2), which act as key enzymes catalyzing the same reaction in the production of prostaglandins (PGs), while the second class of NSAIDs selectively inhibit COX-2. Inhibition of the inducible COX-2 isoform is believed to be responsible for the therapeutic effects of NSAIDs, such as anti-inflammatory, analgesic, and antipyretic effects, while COX-1 inhibition results in side-effects on the gastrointestinal (GI) system. In the present study, however, we changed this notion that inhibiting only COX-1 causes adverse effects. We discovered FK881, a specific COX-1 inhibitor which exhibits a 650-fold ratio for human whole blood COX-1/COX-2 and rats *in vivo*. In rats, FK881 dose dependently inhibited carrageenan-induced paw edema (ED30: 22 mg/kg; diclofenac ED30: 3.6 mg/kg, rofecoxib ED30: 26 mg/kg) and paw swelling associated with adjuvant arthritis (ED50: 17 mg/kg; diclofenac ED50: 1.4 mg/kg, rofecoxib ED50: 1.8 mg/kg). Further, FK881 dose dependently inhibited acetic acid-induced writhing in mice (ED50: 19 mg/kg; diclofenac ED50: 14 mg/kg, rofecoxib ED50: >100 mg/kg) and adjuvant arthritis hyperalgesia in rats (ED50: 1.8 mg/kg; diclofenac ED50: 1.0 mg/kg, rofecoxib ED50: 0.8 mg/kg). However, unlike traditional NSAIDs, GI tolerability was improved, although the antipyretic effect of FK881 was weak (NOEL: >320 mg/kg; diclofenac NOEL: <1 mg/kg, rofecoxib NOEL: 100 mg/kg). These results suggest that FK881 may be useful in treating symptoms of rheumatoid arthritis and osteoarthritis.

1. Introduction

Conventional nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, ibuprofen, and diclofenac inhibit cyclooxygenase (COX), the key enzyme in prostaglandin (PG) biosynthesis, and exert strong anti-inflammatory, analgesic, and antipyretic effects. However, a number of adverse events are also associated with these compounds, the most common of which occur in a dose-dependent fashion in the upper GI tract and include discomfort, ulcers, and bleeding. A US survey estimated that 10–20% of patients prescribed traditional NSAIDs may experience dyspepsia during treatment, and that 50,000–100,000 hospitalizations each year were related to NSAID use. Approximately 16,000 NSAID-related deaths secondary to GI complications were estimated at the beginning of this century [1].

In the early 1990s, a second isoform of COX was identified and named COX-2, with the previously known COX denoted as COX-1. COX-1 mRNA and protein are expressed constitutively in most tissues and cells, particularly in the normal gastric mucosa and platelets, and help produce PGs. In contrast, COX-2 mRNA and proteins are predominantly expressed in inflamed tissues, rapidly producing proinflammatory PGs after proinflammatory stimulation [2–4]. Since the discovery of COX-2, COX-1-derived prostaglandin E2 (PGE2) have been found to be involved in constitutive defense mechanisms operating under physiological conditions [5], and inhibition of COX-1 is believed to be the root of adverse events associated with traditional NSAIDs, which were found to inhibit both isozymes. Selective COX-2 inhibitors were therefore developed to minimize side effects. This new class of NSAIDs, which includes rofecoxib and celecoxib, known as coxibs, reduces the risk of GI toxicity in both animal models [3,6] as well as humans [1,7–9].

However, a new hypothesis regarding COXs is gaining acceptance in recent years, which citing that while PG reduction in the stomach does not induce GI damage when COX-1 alone is inhibited, simultaneous inhibition of both COX isotypes will result

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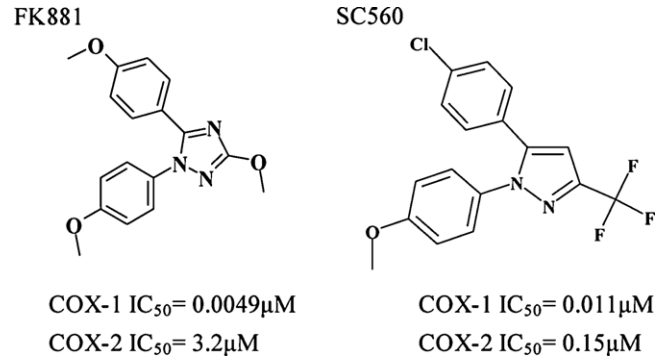

Fig. 1. Selective COX-1 inhibitors.

in the formation of gastric ulcers. Although COX-1 is mainly expressed in the GI tract both in animals and humans [4] and almost all PGE2 synthesis was reduced in the GI tissues of COX-1 deficient mice, these animals exhibited no GI injury [10,11]. In addition, the slightly selective COX-1 inhibitor SC560 was found to not induce GI damage in rats when administered alone. However, severe gastric lesions developed when rats received concurrent treatment with SC560 and a COX-2 inhibitor [12]. Similar findings were reported by other authors [13–15].

In our research over the past decade, we have found that our selective COX-1 inhibitors exert a potent analgesic effect without inducing gastrointestinal toxicity. In particular, our most selective COX-1 inhibitor, FK881 [3-methoxy-1,5-bis(4-methoxyphenyl)-1H-1,2,4-triazole], exhibited 650-fold selectivity to COX-1 over COX-2 in human whole blood assay (hWBA), while SC-560 showed only slight selectivity in the same assay. NSAIDs may be grouped into three main classes: (a) carboxylic acids, (b) phenazones (pyrazolones, oxicams) and (c) non-acidic compounds (biaryl heterocycles), the third of which includes FK881 [52] and other COX-1 selective inhibitors such as SC-560 (Fig. 1). Here, we have constructed the pharmacological profile of FK881 and compared its effects to a COX-1, 2 dual inhibitor and a COX-2-only inhibitor.

2. Materials and methods

2.1. Drugs and reagents

FK881 and rofecoxib were synthesized at Astellas Pharmaceutical Company (Tokyo, Japan), and diclofenac sodium salt, indomethacin, and SC-560 were purchased from Sigma–Aldrich Japan (Tokyo, Japan). All drugs were dissolved in dimethylsulfoxide for *in vitro* assay. For murine *in vivo* assay, drugs were suspended or dissolved in 0.1% methylcellulose (MC) solution, and in 0.5% MC solution for rats.

Enzyme immunoassay kit for thronboxane 2 (TxB2) and PGE2 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Acetic acid was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Lambda-carrageenan and A23187 were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Dried brewers' yeast (*Saccharomyces* genera) was purchased from Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). *Mycobacterium tuberculosis H37 RA* strain was purchased from Difco Laboratories (Detroit, MI, USA).

2.2. Cells and animals

Fresh human blood was collected from male volunteers in a manner approved by the Astellas Research Ethics Committee. The subjects had not taken any drugs for at least seven days prior and had not consumed any alcohol for at least one day prior to blood collection. Male ddY mice aged five weeks and male Sprague-Dawley (SD) rats aged six weeks were obtained from Japan SLC Inc. (Shizuoka, Japan). Female Lewis rats (7–9 weeks old) were obtained from Charles River Inc. (Yokohama Japan). All animals were acclimated for at least three to seven days before experiments and were maintained in the institutional animal facilities approved by the American Association for Accreditation of Laboratory Animal Care.

2.3. Recombinant human COX isozymes assay

Recombinant human (rh) COX-1 and COX-2 were expressed in Chinese hamster ovary cells. The COX enzymes, derived from the supernatant of homogenized cells, were suspended in reaction buffer. After addition of arachidonic acid (10 μM), enzyme samples were incubated for 5 min (COX-1) or 10 min (COX-2) at room temperature. Reactions were terminated by adding indomethacin (final concentration: 1 mM), and PGE2 productions were measured using an enzyme immunoassay kit.

2.4. Human whole blood assay

For COX-1, fresh blood was incubated with test drugs for 1 h at 37 °C to achieve clotting. Reactions were terminated by adding indomethacin (final concentration: 2.5 mM) and blood was centrifuged to obtain serum. For COX-2, fresh blood containing anticoagulants was incubated with test drugs and lipopolysaccharide (LPS; 100 μg/mL) for 24 h at 37 °C, after which the samples were centrifuged to obtain plasma. Serum or plasma was mixed with methanol to precipitate protein and then centrifuged to obtain the supernatant. The supernatant was assayed for TXB2 for COX-1 and PGE2 for COX-2 using an enzyme immunoassay kit.

2.5. In vivo COX inhibition

In vivo COX-1/COX-2 inhibition of test compounds (FK881 and rofecoxib) was evaluated using a modified version of Smith's methods in normal rats [33]. To investigate COX-1 inhibition *in vivo*, male SD rats were sacrificed 1 h after oral administration of drugs. The stomachs were then removed, washed, and homogenized in methanol containing 10 μM indomethacin. Supernatants were freeze-dried and dissolved in assay buffer from a PGE2 EIA kit, and concentration of PGE2 was subsequently determined using the same kit.

To investigate COX-2 inhibition, male SD rats were orally dosed with aspirin (320 mg/kg) to achieve irreversible inhibition of constitutively expressed COXs, and air was subcutaneously injected into the back to produce a cavity. Test compounds were orally administered 23 h after aspirin dosing, and lambda-carrageenan saline suspension (2% [w/v]) was injected into the air cavity 24 h after aspirin dosing. Six hours after saline suspension administration, the exudates in the air pouch were collected, and PGE2 concentration was determined using an EIA kit.

2.6. Acetic acid-induced writhing in mice

Test drugs were administered to the ddY mice orally 1 h before acetic acid injection. Writhing was induced by an intraperitoneal injection of 0.6% acetic acid (20 mL/kg). Three minutes after injection, the number of writhing reactions was counted for subsequent period of 10 min.

2.7. Hyperalgesia in adjuvant arthritis rats

Arthritis was induced in Lewis rats by injecting a suspension of 0.5 mg of *M. tuberculosis* in liquid paraffin into the right hind foot

pads [16]. Eighteen days after injection, we measured analgesic activity in the left hind paws 2 h after administration of test drugs, via the method used by Sakuma et al. [17], which was modified from that used by Randall-Selitto [16]. The analgesic coefficients were calculated as the pain threshold ratio against control groups, and the ED50 value was defined as the dose that raised the analgesic coefficient to 1.5.

2.8. Carrageenan-induced paw edema in rats

SD rats were administered test drugs orally 1 h before carrageenan injection. Paw edema was induced by intraplantar injection of 0.1 mL of 1% carrageenan-saline solution into the right hind paws. The right hind paw volumes were measured with a volumeter 3 h after carrageenan injection.

2.9. Adjuvant arthritis in rats (therapeutic treatment)

Arthritis was induced in Lewis rats by injecting 0.5 mg of *M. tuberculosis* into the right hind foot pads. Test drugs were then administered orally once daily from Days 15 to 24 (for FK881, three times daily). The left hind paws volumes were measured with a volumeter, and paw swelling values were defined as the increased paw volume of each animal.

2.10. Measurement of ulcerogenic effect

After fasting for 24 h, oral administrations were performed for each drug. On the other hand, SD rats in control group were dosed 0.5% MC solution. Five hours later, the stomachs were removed and immersed in 2% formalin to fix the gastric tissue wall and opened from the pyloric region along the great curvature. Presence or absence of visible mucosal ulceration was then noted and scored using the following criteria: 0, no alteration; 1, large ecchymosis or some small ulcers; 2, five or more small ulcers or one ulcer 3 mm or larger in diameter; 3, a large number of ulcers.

2.11. Yeast-induced hyperthermia

Rectal temperature was measured using a thermoelectric thermometer. Yeast saline suspension (1:5 [w/v]) was injected subcutaneously into the backs of SD rats in the hyperthermic groups, while rats in normothermic groups received saline alone. Rectal temperature was measured again 15 h after injection. Both the hyperthermic and normothermic groups included control and drug-treated groups. Drugs were administered orally 17 h after saline or yeast injection. Animals in the normothermic drug-treated groups were administered the maximum dose of each drug to examine whether or not normal temperatures were affected by

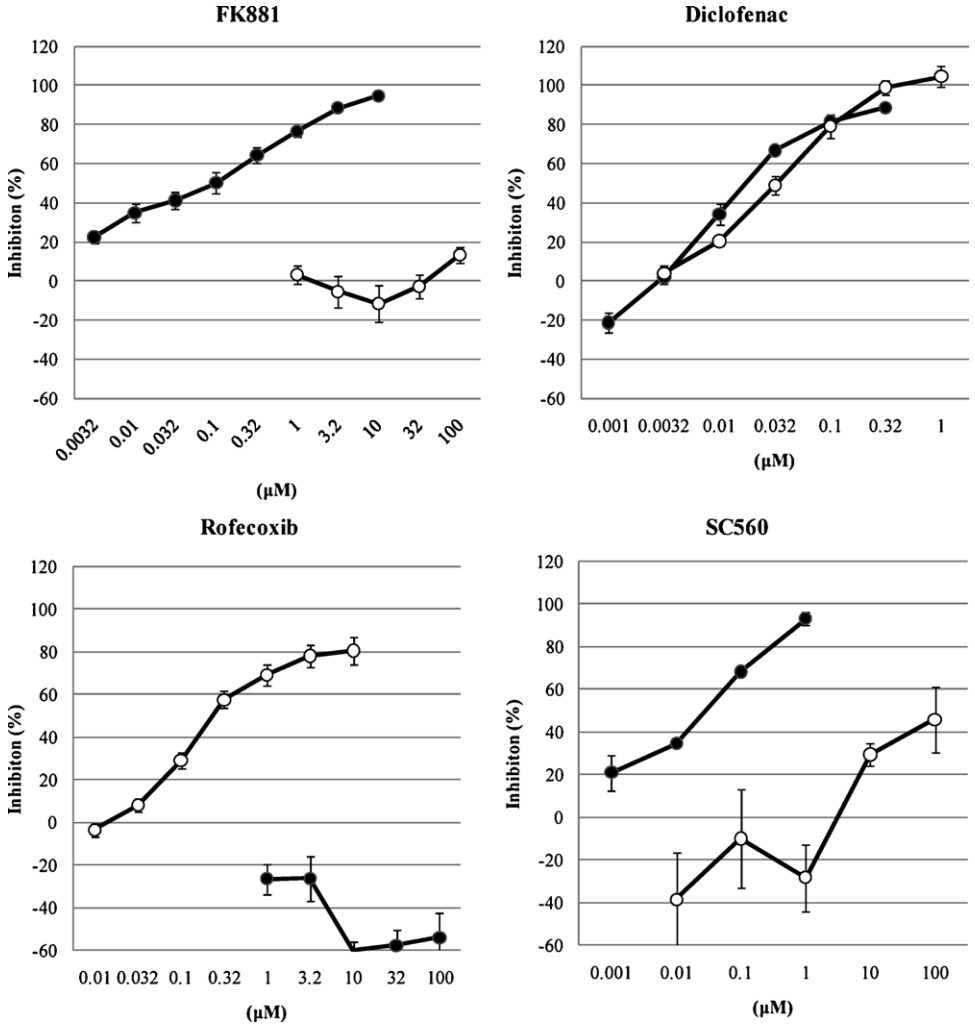

Fig. 2. Inhibition of rhCOX-1 or rhCOX-2 activity by FK881 and reference drugs. The COX enzymes were preincubated with test drugs for 10 min prior to the addition of arachidonic acid (final concentration: 10 μM) for 5 (COX-1) or 10 min (COX-2) at room temperature. Reactions were terminated by adding indomethacin (final concentration: 1 mM), and PGE2 production was measured using an enzyme immunoassay kit. Closed circles indicate inhibitory activities for rhCOX-1 by test drugs, and open circles those for rhCOX-2. Data are expressed as mean values ± S.E.M. for $n = 3$.

test drugs. Temperature was measured at 1-h intervals for 5 h after administration.

2.12. Data analysis and statistics of hyperthermia induced by yeast

2.12.1. Normothermic groups

In normothermic groups, "A" values were calculated as the ratio between temperature values at each hour and that at 15 h, with values of "A" expressed as a percent of that at 15 h. Average values of "A" for each rat were calculated as the mean value of "A" at each hour.

2.12.2. Hyperthermic groups

For rats in the hyperthermic groups, "B" values for each hour were calculated as follows:

$$B(\%) = \frac{(\text{Temperature difference between each hour and Hour 0})}{(\text{Temperature difference between Hours 15 and 0})} \times 100$$

Average values of "B" for each rat were calculated as the mean value of "B" at each hour and expressed as "average of hyperthermia."

3. Results

3.1. Effects of FK881 on activities of recombinant human COXs

We examined the effects of FK881 on the activities of rhCOX isozymes. The IC50 value of FK881 for inhibiting rhCOX-1 activity was 0.064 μM, while that for inhibiting rhCOX-2 activity exceeded 100 μM. These results showed that FK881 had COX-1 selective inhibition activity (1562-fold or more) (Fig. 2). FK881 inhibited COX-1 activity as potent as an earlier compound (diclofenac IC50 value for rhCOX-1 = 0.024 μM, rhCOX-2 = 0.032 μM) and exhibited wider COX-1 selectivity than SC560 (IC50 value for rhCOX-1 = 0.22 μM, rhCOX-2 = >100 μM; selectivity was 454-fold or more). In contrast, the selective COX-2 inhibitor rofecoxib exhibited highly selective COX-2 inhibition activity in this assay (IC50 value for rhCOX-1 = >100 μM, COX-2 = 0.31 μM) (Fig. 2).

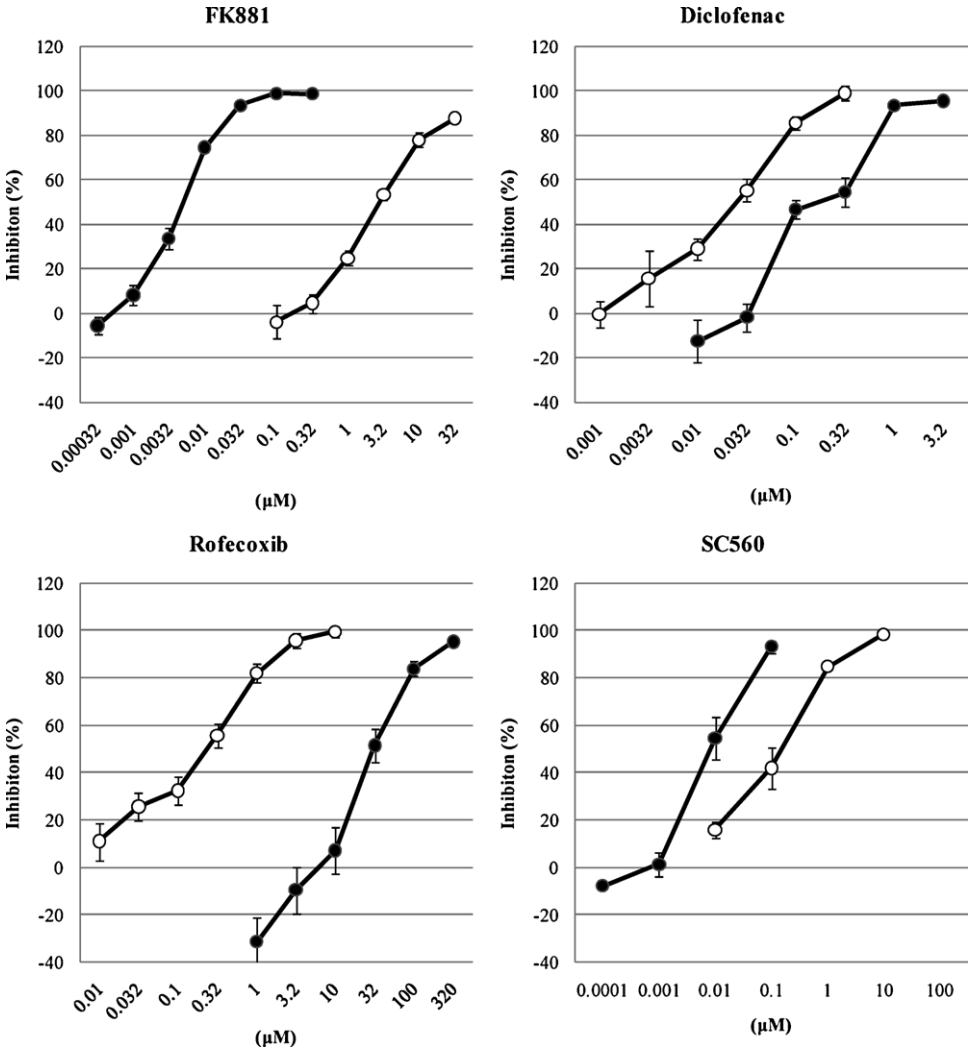

Fig. 3. Inhibition of WB COX-1 or COX-2 activity by FK881 and reference drugs. Human WBA for COX-1: The blood was incubated with test drugs for 1 h at 37 °C to allow clotting. After incubation, the blood was centrifuged to obtain serum and assayed for TXB2 using an enzyme immunoassay kit. For COX-2: The blood was incubated with test drugs for 15 min at 37 °C, followed by incubation with LPS (final concentration: 100 μg/mL) for 24 h at 37 °C. After incubation, the blood was centrifuged to obtain plasma and assayed for PGE2 using an enzyme immunoassay kit. Closed circles indicate inhibitory activities for WB COX-1 by test drugs, and open circles those for WB COX-2. Data are expressed as mean values ± S.E.M. for $n = 6$.

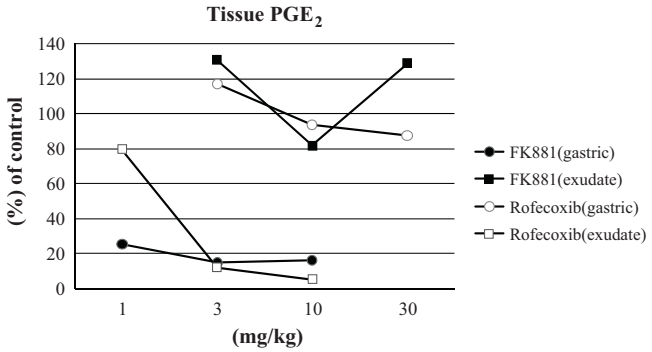

Fig. 4. *In vivo* selectivity of COXs inhibitior. To evaluate COX-1 inhibition, male SD rats were fasted for 24 h and sacrificed 1 h after oral administration of FK881 (●) or rofecoxib (○). The washed stomachs were homogenized in methanol (10 mL with 10 μM indomethacin), and supernatants were freeze-dried. For COX-2, male SD rats were orally dosed with aspirin (320 mg/kg), after which air (10 mL) was subcutaneously injected into the back. FK881 (■) or rofecoxib (□) was orally administered 23 h after aspirin dosing, and lambda-carrageenan saline suspension (2% (w/v)) was injected into the air pouch 24 h after aspirin. Six hours after saline suspension administration, the exudates in the air pouch were collected, and PGE2 concentration in the supernatants was measured. Each value is expressed as the percent of control value. $n = 4–5$.

3.2. Effects of FK881 on activities of whole blood human COXs

We examined the effects of FK881 on the activity of whole blood human COX isozymes. TXB2 levels following human whole blood coagulation and PGE2 levels in LPS-challenged human whole blood were measured as biochemical indices for COX-1 and COX-2 activity respectively. The IC50 value of FK881 for inhibiting COX-1 activity was 0.0049 μM, while that for inhibiting COX-2 activity was 3.2 μM. These results showed that FK881 had COX-1 selective inhibition activity in human whole blood (650-fold) (Fig. 3). The COX-1-inhibiting potency of FK881 was far superior to that of diclofenac (IC50 value for COX-1 = 0.20 μM, COX-2 = 0.024 μM), and FK881 had far greater selectivity than SC560 (IC50 value for COX-1 = 0.011 μM, COX-2 = 0.15 μM; 14-fold selectivity) in hWBA. The IC50 values of rofecoxib were 38 and 0.25 μM for COX-1 and COX-2 respectively (Fig. 3).

3.3. Result of in vivo COX inhibition

To investigate COX-1/COX-2 inhibition *in vivo*, PGE2 concentration in the stomach (COX-1 activity) and in inflammatory exudates (COX-2 activity) was determined as in previous studies [3,33]. In this assay, rofecoxib potently reduced PGE2 levels in exudates without notable effect on gastric levels at 10 mg/kg, while FK881 reduced gastric levels of PGE2 significantly at 10 mg/kg (83.6% inhibition, Fig. 4) with no suppression of exudate PGE2 up to 10 mg/kg. PGE2 secretion from rat PBMCs incubated for 10 min with A23187 was almost completely inhibited even 24 h after 320 mg/kg aspirin administration (data not shown). We therefore confirmed that both COXs were irreversibly inactivated at the time of carrageenan injection, indicating that FK881 is a highly selective and orally active COX-1 inhibitor.

3.4. Effect of FK881 on acetic acid-induced writhing in mice

The acetic acid-induced writhing reaction model in mice is a model of chemical-induced acute pain useful in evaluating analgesic activity. The ED50 value of FK881 for inhibiting the writhing reaction was 19 mg/kg, comparable to the effects of diclofenac (ED50: 14 mg/kg) and SC560 (67% inhibition at 32 mg/kg). In contrast, rofecoxib did not inhibit this reaction, even at a dosage of 100 mg/kg (Fig. 5a and b); however, COX-2 inhibitors are widely understood not to inhibit this reaction, so this finding is not surprising.

3.5. Effect of FK881 on hyperalgesia in rat adjuvant arthritis

Given that the rat model of adjuvant arthritis has long been used as an animal model for rheumatoid arthritis (RA), hyperalgesia in adjuvant arthritis in these animals, a source of chronic inflammatory pain, is believed to reflect that in human RA patients. The ED50 value of FK881 for inhibiting hyperalgesia in an adjuvant arthritis rat model was 1.8 mg/kg, while values for diclofenac and rofecoxib were 1.0 mg/kg and 0.8 mg/kg, respectively (Fig. 6a and b). SC560 did not show 50% inhibition at 3.2 mg/kg (pain threshold ratio = 1.45 at 3.2 mg/kg). The analgesic effect of FK881 was comparable to that of diclofenac and rofecoxib in a rat model of chronic inflammatory pain.

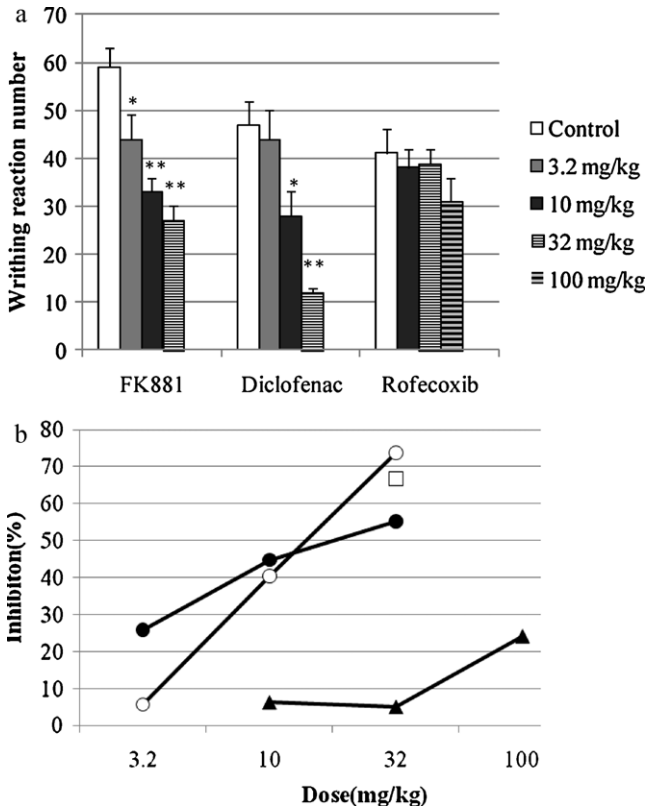

Fig. 5. (a) Analgesic effect of test drugs on acetic acid-induced writhing in mice. Drugs were administered orally 1 h before acetic acid injection. Writhing was induced by an intraperitoneal injection of 0.6% acetic acid (20 mL/kg). Three minutes after injection, the number of writhing reactions was counted for 10 min. Statistical significance was analyzed using Dunnett's multiple comparison test. Significant difference from the control was set at $^{*}P < 0.05$, $^{**}P < 0.01$. Data are expressed as mean values ± S.E.M. for $n = 10$.
(b) Inhibition of acetic acid-induced writhing in mice. The number of writhing reactions was used to calculate inhibition. FK881 (●), diclofenac (○), rofecoxib (▲) and SC560 (□).

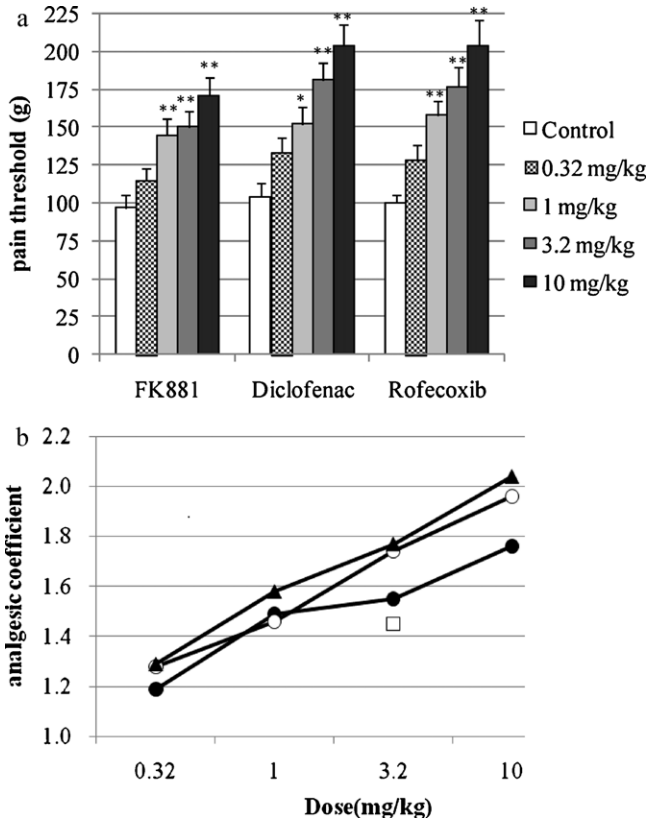

Fig. 6. (a) Analgesic effect of drugs on adjuvant-induced arthritis in rats. Arthritis was induced in rats by injecting 0.05 mL of a 10 mg/mL suspension of *M. tuberculosis* in liquid paraffin into the right hind foot pad. Analgesic activity was then measured via the modified method of Randall-Selitto. Eighteen days after inoculation, test compounds were orally administrated 2 h before measurement. The pain threshold of the left hind paw was assessed using an algesimeter, and the analgesic coefficient was calculated as the pain threshold ratio against control groups. Statistical significance was analyzed using Dunnett's multiple comparison test with respect to the control group: $^{*}P < 0.05$, $^{**}P < 0.01$. Data are expressed as mean values ± S.E.M. for $n = 10$.
(b) Analgesic coefficients for adjuvant-induced arthritis in rats. Analgesic coefficients of FK881 (●), diclofenac (○), rofecoxib (▲), and SC560 (□) were calculated for the pain threshold.

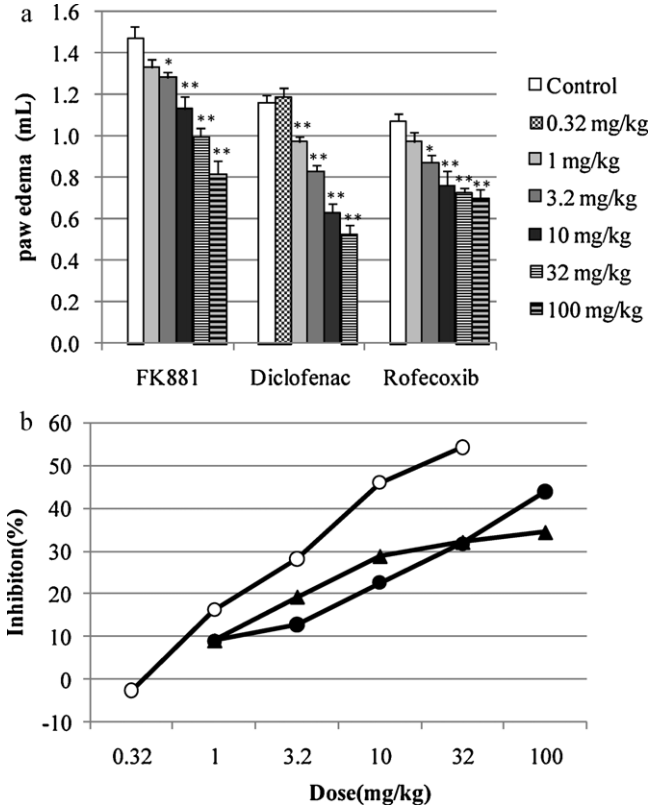

Fig. 7. (a) Anti-inflammatory effect of test drugs on carrageenan-induced paw edema in rats. Drugs were administered orally 1 h before carrageenan injection. Paw edema was induced by intraplantar injection of 0.1 mL of 1% carrageenan-saline solution into the right hind paw. Edema was measured 3 h after injection, and statistical significance was analyzed using Dunnett's multiple comparison test. Significant difference from the control was set at $^{*}P < 0.05$, $^{**}P < 0.01$. Data are expressed as the mean values ± S.E.M. for $n = 10$.
(b) Inhibition of carrageenan-induced paw edema in rats. Paw edema measurements were used to calculate inhibition. FK881 (●), diclofenac (○), rofecoxib (▲).

3.6. Effect of FK881 on carrageenan-induced paw inflammation in rats

The rat model of carrageenan-induced paw inflammation, a model of acute inflammation, has proven useful in predicting the anti-inflammatory effects of NSAIDs in clinical settings [18]. The ED30 value of FK881 for inhibiting paw edema in the present study was 22 mg/kg, a value comparable to the inhibitory effect of rofecoxib on acute inflammation (ED30: 26 mg/kg). However, diclofenac inhibited edema more strongly at 0.32–32 mg/kg than FK881 (ED30: 3.6 mg/kg) and was approximately 6 times as potent (Fig. 7a and b).

3.7. Effect of FK881 on paw inflammation in rat adjuvant arthritis (therapeutic treatment)

To determine whether or not FK881 inhibits paw swelling in our rat model of adjuvant arthritis, FK881 was administered from 15 to 24 days after arthritis induction during the therapeutic period. The ED50 value of FK881 for inhibiting paw swelling was 17 mg/kg, while values for diclofenac and rofecoxib were 1.1 and 1.4 mg/kg, respectively (Fig. 8a and b). This weak efficacy may be due to an effective concentration of FK881 not being maintained in rat blood over the lengthy dosing period.

3.8. Effect of FK881 on yeast-induced hyperthermia in rats

The ED50 value of FK881 for inhibiting a febril response was 19 mg/kg, which was less potent than that of diclofenac and rofecoxib (respective ED50 values: 0.23 and 0.65 mg/kg). None of the tested drugs had any effect on the temperature of normal rats (data not shown) (Fig. 9a and b).

3.9. Effect of FK881 on gastrointestinal toxicity in rats

To determine potential to induce gastrointestinal toxicity, drugs were administrated to fasted rats at 100 mg/kg. While neither FK881 nor rofecoxib induced gastrointestinal toxicity at this concentration, diclofenac caused serious gastric ulcers even at 1 mg/kg. After SC560 administration at 100 mg/kg, 30% of rats developed gastric ulcers (Table 1).

4. Discussion

In the present study, we determined whether or not FK881 and reference drugs selectively inhibited COX-1 or COX-2 activity by conducting recombinant human enzyme assay and hWBA to assess selectivity of both isozymes (Figs. 2 and 3). After confirming the selectivity of FK881 *in vivo* (Fig. 4), we investigated its ameliorating effect on acute pain by observing acetic acid-induced writhing reactions in mice (Fig. 5) and on chronic pain using the modified Randall-Selitto method in adjuvant arthritis rats (Fig. 6) to evaluate

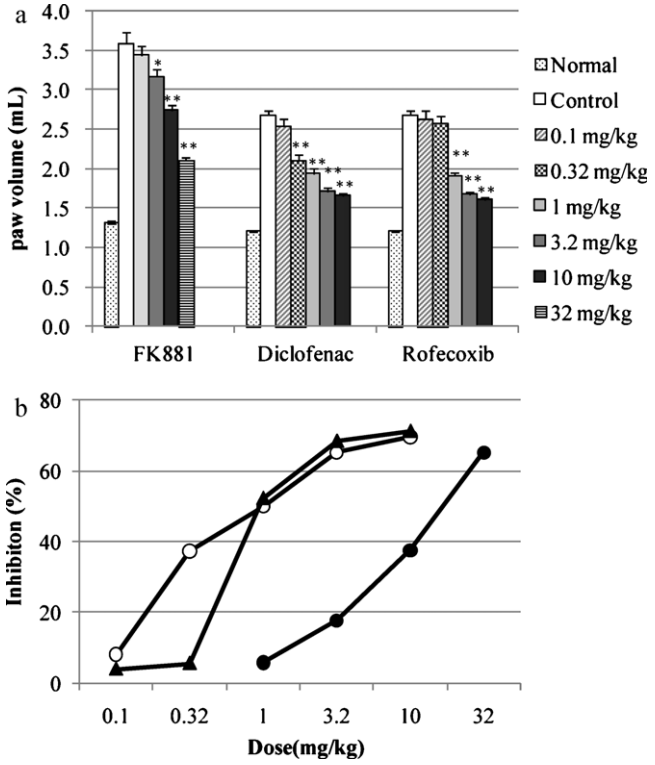

Fig. 8. (a) Anti-inflammatory effects of drugs on paw volume in rats with adjuvant-induced arthritis. Arthritis was induced as described above. The normal group received no injections. Drugs were administered orally from Days 15 to 24 while normal and control group animals received 0.5% MC solution. Final paw volume was measured between Days 24 and 25. Statistical significance was analyzed using Dunnett's multiple comparison test. Significant difference from the control was set at $^{*}P < 0.05$, $^{**}P < 0.01$. Data are expressed as the mean values ± S.E.M. for $n = 10$.
(b) Inhibition of paw swelling in rats with adjuvant-induced arthritis. Inhibition curves are shown for FK881 (●), diclofenac (○) and rofecoxib (▲) in adjuvant arthritis in rats. Paw swelling vales were used to calculate inhibition.

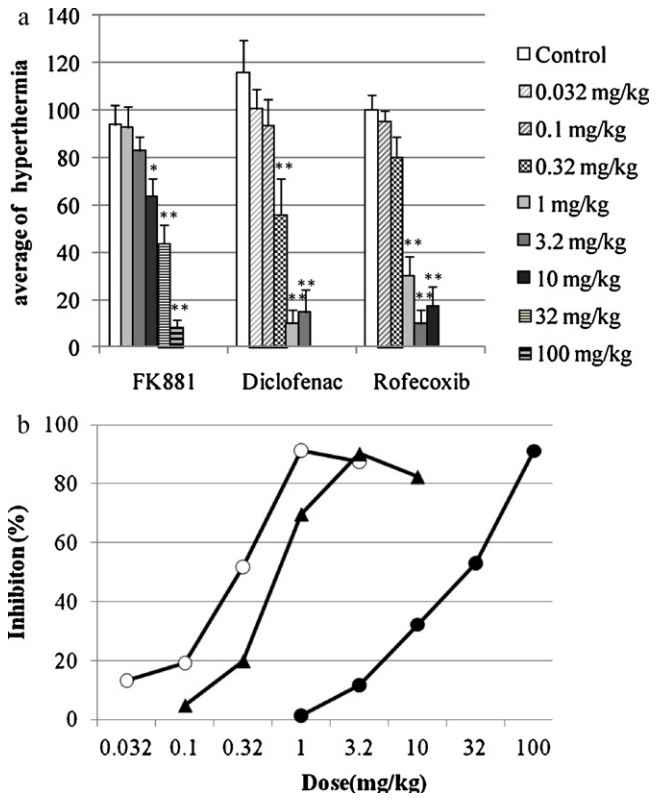

Fig. 9. (a) Antipyretic effects of drugs on yeast-induced hyperthermia in rats. Drugs were administered orally 17 h after yeast injection, and rectal temperatures were measured between 18 and 22 h after yeast administration. The inhibition (%) values represent means of inhibition at five time points. Significant differences between drug groups and the control group were analyzed using Dunnett's multiple comparison test ($^{*}P < 0.05$, $^{**}P < 0.01$). Data are expressed as mean values ± S.E.M. for $n = 10$.
(b) Inhibition of yeast-induced fever in rat. Inhibition curves of FK881 (●), diclofenac (○), and rofecoxib (▲) in yeast-induced fever in rats.

the *in vivo* effects of FK881. FK881 showed dose-dependent analgesic activity in both models, and potencies were comparable to those of diclofenac. Although rofecoxib was potently effective in treating chronic inflammatory pain, the compound did not inhibit writhing, even at 100 mg/kg.

Although differences between the human enzyme assay and hWBA, such as in reaction time and enzyme volume, may cause differing IC50 values and selectivity to be obtained for tested drugs, these assays are widely used to compare conventional NSIADs and COX-2 inhibitors [19,20]. In particular, the hWBA is generally accepted as the best method of predicting COX-1/2 selectivity under *in vivo* physiological conditions [21,22], because hWBA reflects certain factors such as protein binding, cell–cell interaction, stability, and cell-permeability of drugs in human blood. For example, rofecoxib has been shown to have COX-2 selective inhibition activity both in hWBA [23] and in clinical settings [21,24], findings which corroborate our own *in vivo* findings of selectivity for rofecoxib at doses ≤30 mg/kg and 6 h after p.o. administration (Fig. 4). Our hWBA and *in vivo* findings for FK881 therefore suggest that the compound possess selectivity for COX-1 in a clinical setting as well as in our study, and further, is far more selective than SC560.

Table 1
Ulcerogenic effect of drugs in rats.

	FK881	Diclofenac	Rofecoxib
UD50 (mg/kg) (95% CI)	–	11 (5.5–19)	–
NOEL	>320	<1	>100

CI, confidence interval; UD50, The doses producing ulcers in 50% of the treated rats; NOEL, no-observed effect level.
The UD50 value and 95% CI for diclofenac were calculated by probit analysis ($n = 10$).

Stimulation of spinal nerve pathways by pain-producing substances, including PGs released from damaged tissues, is needed to induce the writhing reaction [25]. Ochi reported that levels of 6-keto prostaglandin F1α, a stable degradation product of PGI2, were significantly increased in the peritoneal cavity of mice after acetic acid injection. Indomethacin dose-dependently inhibited the writhing responses and reduced the production of 6-keto prostaglandin F1α, while a COX-2 inhibitor failed to affect the reaction and prostaglandin production [26]. Further, writhing responses induced by acetic acid are generally regarded as dependent on PGI2 derived from COX-1, as the number of writhing responses has been reported to be decreased in COX-1-knockout mice [27] and IP-receptor-knockout mice [28] but not COX-2-knockout mice [27]. A number of other reports also have demonstrated the significant roles of COX-1 in nociception [29,30].

Moreover, the time-course in the present study may be insufficient to induce COX-2 and writhing. For example, although COX-2 mRNA and protein levels in the spinal cord increased from 3 to 6 h after injection of acetic acid into the hind paw [31], the number of writhing reactions was observed for only the first 20 min after inflammatory agent administration in the present study. In a previous study, when COX-2 production was induced by pre-treatment with LPS 8 h before the injection of acetic acid, the number of writhing reactions significantly increased, likely due to

the additive amount of PGs induced by COX-2 [32]. In this LPS-pretreatment model, selective COX-2 inhibitor suppressed only the increase in number of writhes. Taken together, these findings indicate that acute pain depends on PGs from COX-1.

Adjuvant inoculation in rats induces upregulation of COX-2 and microsomal PGE synthase followed by high levels of PGE2 production [33,34]. However, in a previous study, we demonstrated that PGI2 in the spinal cords of adjuvant arthritis rats, one of the main factors for pain of this model (in submission), was derived from COX-1. Adjuvant arthritis rats show hyperalgesia on mechanical stimulation [17,25] and are recognized as a model of RA, as the pain induced in this model resembles that experienced by human RA patients [17]. In fact, the analgesic activities of NSAIDs in adjuvant arthritis have been found to be correlated with clinical doses for pain control [35], suggesting that selective COX-1 inhibitors may be useful in treating inflammatory pain in a clinical setting.

COX-2 inhibitors used in clinical settings, such as rofecoxib (Vioxx), have been reported to have weaker analgesic effects than conventional NSAIDs [16]. For example, the clinical dose of rofecoxib used to treat chronic pain is only 12.5–25 mg, while that for treating postoperative pain is 50 mg. Given that acute pain in clinical settings, such as postoperative pain, may be induced more often by COX-1 than COX-2, FK881 may be useful in achieving pain relief in patients experiencing postoperative pain and RA, given its equivalent efficacy to that of conventional NSAIDs in relieving both acute and chronic pain.

To determine whether or not FK881 exerts anti-inflammatory effects in an acute inflammatory model, we examined its effect on carrageenan-induced paw edema in rats. This model proved useful for reflecting anti-inflammatory effects of NSAIDs in clinical settings [18]. Although PG levels in the paws after carrageenan injection were found to be elevated [33], given that several inflammatory mediators such as histamine, bradykinin, and kinin were also noted in addition to PGs [34] and that NSAIDs were not found to exert potent inhibition in this model in our initial experiments, we determined ED30 values in order to characterize compounds' actions. We found that all tested drugs dose-dependently inhibited carrageenan-induced paw edema, and the effect of FK881 was comparable to that of rofecoxib but inferior to that of diclofenac. Further, simultaneous administration of the COX-1 and COX-2 inhibitors resulted in equivalent amelioration of COX-1, 2 dual inhibitor in this animal model (data not shown), indicating that both isozymes of COX were involved in carrageenan-induced inflammation to a similar extent. However, we did note that the ED50 value of FK881 for adjuvant arthritis paw swelling in rats was higher than those of rofecoxib and diclofenac, possibly due to the short elimination half-life of FK881 in rats (data not shown). Taken together, these findings suggest that COX-1 inhibitors exert an anti-inflammatory effect comparable to that of COX-2 inhibitors in a clinical setting.

PGs synthesized in the brain and spinal cord evoke various biological phenomena, including hyperthermia in the central nervous system [36]. Indeed, several reports have indicated that PGE2 is a neural mediator in the fever response. For example, fever was induced by direct injection of PGE2 into the third ventricle of the brain [37], and mice lacking the PGE receptor subtype EP3 showed impaired febrile response [38]. In the brain, COX-2 mRNA and protein were expressed constitutively [4,39] and were induced in the brain by systemic pyrogenic stimuli such as LPS, IL-1β or TNFα [39–43]. These observations suggest that fever induction is primarily mediated by the COX-2 isoform, a theory strongly supported by additional evidence that COX-2-deficient mice are unable to develop a febrile response to exogenous or endogenous pyrogens [39] and that selective COX-2 inhibitors such as NS-398, JTE-522, and rofecoxib exert an antipyretic effect in the rat fever model [23,44,45] and humans [46]. Zymosan and mannans, well-known reagents in the cell walls of yeast, activate toll-like-receptors (TLRs) 2/6 and 4, respectively [47], to induce pro-inflammatory cytokines such as IL-1β or TNFα in blood [48,49]. Given that plasma levels of IL-1β and TNFα have been found to remain elevated even 18 h after zymosan administration [48], we determined that COX-2 must have been induced in the brain in the present experiment. Our present findings therefore indicate that FK881 has poor antipyretic activity, contrary to rofecoxib or diclofenac, in our rat model of yeast-induced febrile response, due to the compound's high selectivity for COX-1.

In the present study, we found that FK881 caused no ulceration in gastric mucosa, even when administered at an extremely high dose (320 mg/kg), while our findings for rofecoxib and diclofenac ulcer induction concurred markedly well with previous reports [23,50]. Given these findings, FK881 is expected to have favorable GI tolerability in clinical use. Despite the fact that the pathological relationship between COX isozymes and NSAID-induced GI injury remains unknown, we posited several possible reasons to explain the need for both isoforms in order to maintain a healthy stomach environment. For instance, each isoform helps to maintain mucosal integrity, and vicarious up-regulation of COX-2 after inhibiting COX-1 may induce PGs and subsequently prevent GI injury [15]. In addition, non-selective NSAIDs have been shown to exert GI toxicity through a systemic mechanism via reduction of blood flow around the stomach by inhibiting COX-1 and attracting neutrophils, which can cause mucosal injury by COX-2 inhibition [51,52]. Further studies are needed to clarify the mechanism of GI toxicity.Here, we clearly demonstrated that FK881 has analgesic activity equivalent to that of classical NSAIDs in preclinical animal models while having a substantially different GI side-effect profile. Highly selective COX-1 inhibitors can therefore be denoted as a new class of therapeutic agent for treating acute and chronic inflammatory conditions, which will offer patients experiencing such pain another choice in relief.

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