Profile of JTE-522 as a Human Cyclooxygenase-2 Inhibitor

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ABSTRACT—Inhibitory activity and the mechanism of action of JTE-522 (4-(4-cyclohexyl-2-methyl-oxazol-5-yl)-2-fluorobenzensulfonamide), a novel selective cyclooxygenase (COX)-2 inhibitor, on human COX-1 and COX-2 were investigated and compared with those of reference compounds. In an enzyme assay, JTE-522 inhibited yeast-expressed human recombinant COX-2 with an IC₅₀ value of 0.085 μM. In contrast, JTE-522 did not inhibit human COX-1 prepared from human platelets at concentrations up to 100 μM. In a cell-based assay, JTE-522 diminished lipopolysaccharide-induced prostaglandin E₂ production in human peripheral blood mononuclear cells (COX-2) (IC₅₀ value = 15.1 nM). On the other hand, JTE-522 was less potent at inhibiting calcium ionophore-induced thromboxane B₂ production in washed human platelets (COX-1) (IC₅₀ value = 6210 nM). JTE-522 showed highly selective inhibition of human COX-2, and its activity was more selective than that of other COX-2 inhibitors (NS-398 and SC-58635). Human recombinant COX-2 activity was attenuated by JTE-522 in a dose-dependent and time-dependent manner. In contrast, the inhibitory activity of JTE-522 on human COX-1 was not affected by preincubation time. COX-2 inhibition by JTE-522 could not be recovered by gel filtration. These results indicate that JTE-522 is a highly selective, time-dependent and irreversible inhibitor of human COX-2.

Keywords: Cyclooxygenase, Cyclooxygenase-1, Cyclooxygenase-2, JTE-522, Non-steroidal anti-inflammatory drug (NSAID)

Cyclooxygenase (COX) converts arachidonic acid to prostaglandin (PG) H₂, which serves as the common precursor for the synthesis of various PGs and thromboxane (TX). It has been shown that there are two major isoforms of COX (I). The constitutive enzyme COX-1 is believed to be involved in the maintenance of essential physiological functions such as platelet aggregation and cytoprotection in the stomach. A second isozyme, COX-2, has been recently identified, and it has been found to show significant induction under inflammatory conditions in vitro (2–5) and in vivo (6–10). COX is an important target of non-steroidal anti-inflammatory drugs (NSAIDs) (11). The anti-inflammatory properties of NSAIDs are achieved by the inhibition of COX-2, whereas the common adverse effects of NSAIDs such as gastrointestinal damage and renal dysfunctions appear to be associated with the inhibition of COX-1 (12). These findings have suggested that selective inhibitors of COX-2 may have an anti-inflammatory effect without the adverse effects of NSAIDs.

NSAIDs can be divided into two groups according to their mechanism of COX inhibition (13, 14). One group inhibits COX in a time-independent manner with the formation of reversible-inhibitor complexes, and includes flufenamic acid and ibuprofen. The other group shows time-dependent COX inhibition involving a two-step mechanism, and includes indomethacin and diclofenac. In general, the latter group of NSAIDs is more potent since the time-dependent step involves the formation of an essentially irreversibly inhibited non-covalent enzyme-inhibitor complex.

We have recently shown that JTE-522, 4-(4-cyclohexyl-2-methylazol-5-yl)-2-fluorobenzensulfonamide, possesses selective inhibitory activity on ovine COX-2 with potent anti-inflammatory activities and very little gastrointestinal ulcerogenicity in rats (15). In the present study, we evaluated the inhibitory activity of JTE-522 on human COX-1 and COX-2, and compared it with those of reference compounds. Furthermore, the mode of inhibitory action of JTE-522 on human COXs was investigated.
MATERIALS AND METHODS

Materials
JTE-522, NS-398 and SC-58635 (celecoxib) were synthesized at the Central Pharmaceutical Research Institute of Japan Tobacco, Inc. (Osaka). The other drugs used in this study were as follows: 12-O-tetradecanoyl-phorbol-13-acetate, indomethacin and calcium ionophore A23187 (Sigma Chemicals, St. Louis, MO, USA); mefenamic acid and L-tryptophan (Wako Pure Chemical Industries Ltd., Osaka); hematin (Chroma-Gesellschaft, Köngen, Germany); [14C]arachidonic acid (New England Nuclear, Boston, MA, USA); and lipopolysaccharide (LPS) (Difco, Detroit, MI, USA).

Preparation of human COX-1
Platelet microsome fraction was used as human COX-1. Platelets were prepared according to a modification of the method of Hammarström and Falcardeau (16). Briefly, blood was collected in heparin (final concentration of 5 units/ml) from volunteers who had not taken medication for at least 1 week. Blood samples were centrifuged at 350×g for 10 min at 4°C, and the supernatants were centrifuged at 900×g for 15 min at 4°C. After washing twice with 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 10 mM EDTA, the pellets were suspended in 10 mM Tris-HCl (pH 7.4) containing 5 mM EDTA and disrupted by sonication (5 sec×6, at setting 10, on ice) with a Handy Sonic model UR-20P (Tomy Seiko Co., Ltd., Tokyo). The samples were then centrifuged at 100,000×g for 15 min at 4°C. The final pellets were suspended in 10 mM Tris-HCl (pH 7.4) containing 5 mM EDTA at a concentration of 10 mg protein/ml, and stored at −80°C. Protein concentration was determined by bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA).

Preparation of human COX-2
Human recombinant COX-2 was expressed in yeast. cDNA coding for human COX-2 was obtained from human histiocytic lymphoma cell line U937. The U937 cells were differentiated with 12-O-tetradecanoyl-phorbol-13-acetate and stimulated with LPS as reported by Nanyama et al. (17). Total RNA was extracted using the acid guanidinium thiocyanate procedure (18). cDNAs were then prepared using the Superscript prem下amplification system (Gibco BRL Life Technologies, Inc., Rockville, MD, USA). We acquired the full coding region of human COX-2 cDNA by the polymerase chain reaction with the 5′ primer 5′-GCT CTA GAC TGC GAT CTT CCG C3′ and 3′ primer 5′-ATT GGA TCC TAC AGT TCA GTC GAA C3′. The obtained cDNA was ligated with the shuttle vector pHIL-D2. The recombinant vector was used for transformation of Pichia pastoris using the Pichia expression kit (Invitrogen, NV Leek, Netherlands) (19). The obtained transformant was grown in minimal glycerol medium and stimulated with methanol for one day to induce COX-2 protein by the same method used to express porcine 12-lipoxygenase (19). The cells were harvested by centrifugation and washed once with ice-cold breaking buffer (50 mM sodium phosphate pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, 50 μM pepstatin, 50 μM chymostatin, 1 mM EDTA and 5% glycerol). The pellet was resuspended in the same volume of the breaking buffer and the cells were disrupted by sonication (5 sec sonication×120, at setting 7, on ice; Branson Sonifier 250; Danbury, CT, USA). The sample was centrifuged at 7,000×g for 10 min at 4°C. Protein concentration of the supernatant was determined by BCA protein assay reagent and adjusted at 10 μg protein/ml. The supernatant was stored at −80°C and used as the enzyme source.

Enzyme assay
Human COX-1 and COX-2 activities were determined by a modification of the method of Takahashi et al. (20). Briefly, human COX-1 (0.3 mg protein/assay) or COX-2 (1 mg protein/assay) was suspended in 0.2 ml of 100 mM Tris-HCl buffer (pH 8.0) containing hematin (2 μM) and tryptophan (5 mM) as cofactors. The reaction mixture was preincubated with each test drug for 5 min at 24°C. [14C]Arachidonic acid (100,000 dpm, 30 μM) was then added to the mixture and incubated for 2 min (COX-1) or 5 min (COX-2) at 24°C. The reaction was stopped by the addition of 400 μl of a stop solution composed of diethyl ether/methanol/1 M citric acid (30:4:1, v/v/v). After centrifugation of the mixture at 1,700×g for 5 min at 4°C, 50 μl of the upper phase was applied to a thin-layer chromatography (TLC) plate. TLC was performed at 4°C with a solvent system consisting of diethyl ether/methanol/acetic acid (90:2:0.1, v/v/v). Enzyme activity was calculated from the percent conversion of arachidonic acid to PGE2 and its decomposition products, using a BAS2000 system (Fuji Photo Film Co., Ltd., Tokyo).

Reversibility of human COX-2 activity
The reversibility of COX-2 inhibition by JTE-522 was examined by the gel filtration method. After preincubation of enzyme with inhibitors (JTE-522 and mefenamic acid) or vehicle for 5 min at 24°C, 2.5 ml of the assay mixture was applied to the PD 10 column (Pharmacia, Uppsala, Sweden) equilibrated with 100 mM Tris-HCl buffer (pH 8.0) containing hematin (2 μM) and tryptophan (5 mM) and was eluted with 3.5 ml of the equilibrating buffer. COX-2 activities of the pre-filtrate fractions and the eluted fractions were estimated by the
method described above, and recovery of enzyme activity was evaluated.

**COX-1 activity in human washed platelets**

COX-1 activity in human washed platelets was determined by A23187-induced TXB₂ production (21). Briefly, blood was collected in a bag containing anti-coagulant (final concentrations: 22.3 mM sodium citrate, 3.4 mM citric acid, 28.2 mM glucose, 3.5 mM sodium dihydrogenphosphate dihydrate; Terumo, Tokyo) from volunteers who had not taken medication for at least 1 week. Platelet-rich plasma was obtained from blood samples by centrifugation at 350 × g for 10 min at room temperature and then diluted with 0.14 M NaCl solution containing 12 mM Tris-HCl and 1.2 mM EDTA (pH 7.4). The mixture was centrifuged at 900 × g for 15 min at room temperature. Platelets were washed and suspended in Ca²⁺-free Hanks' buffer containing 0.2% BSA and 20 mM Hepes (platelet buffer). Platelets (final 2.0 × 10⁵ cells/ml) were preincubated in the presence or absence of the inhibitor for 10 min and were stimulated with 1.26 mM CaCl₂ and 10 μM A23187 for 15 min at 37°C. After stopping the reaction by addition of an equal volume of 15.4 mM EDTA, the supernatant was obtained by centrifugation. TXB₂ levels were measured by an enzyme-immunoassay (EIA) system (Amersham Life Science, Buckinghamshire, England).

**COX-2 activity in human peripheral blood mononuclear cells (PBMC)**

COX-2 activity in human PBMC was determined by LPS-induced PGE₂ production (22). Briefly, PBMC were separated from heparinized whole blood by Ficoll-Paque (Pharmacia). After centrifugation at 350 × g for 30 min at room temperature, PBMC were removed, washed once with Ca²⁺-free Hanks' buffer and twice with RPMI 1640 culture medium containing 10% fetal calf serum (FCS), and resuspended in RPMI 1640 containing 5% FCS. PBMC (final 5.0 × 10⁵ cells/ml) were preincubated with or without inhibitor for 1 hr and stimulated with 1 μg/ml LPS for 24 hr at 37°C in a 5% CO₂-humidified atmosphere. The supernatant was obtained and assayed for PGE₂ levels by an EIA system (Amersham Life Science).

**Data analyses**

Results were expressed as the mean ± S.E.M. of three or four experiments. IC₅₀ values were determined by non-linear regression.

**RESULTS**

**Inhibition of human COX-1 and COX-2**

The activities of human COX-1 and COX-2 were determined after 5 min preincubation with JTE-522, indomethacin, NS-398 and SC-58635. COX-1 and COX-2 converted arachidonic acid to PGH₂ and its decomposition products by 16–26% (n=3–4) and 7–23% (n=3–4), respectively. JTE-522 inhibited COX-2 activity in a concentration-dependent manner (Fig. 1a). The activity of JTE-522 was greater than those of the other compounds, with IC₅₀ values for JTE-522, indomethacin, NS-398 and SC-58635 of 0.085, 2.39, 0.167 and 0.257 μM, respectively. In contrast, JTE-522 did not inhibit COX-1 at concentrations up to 100 μM (Fig. 1b). IC₅₀ values for indomethacin, NS-398 and SC-58635 on COX-1 activity were 0.15, 75.1 and 18.4 μM, respectively. JTE-522 had over 1100 times higher selectivity for human COX-2 than for human COX-1. NS-398 and SC-58635 showed selectivity for COX-2, with the ratio of IC₅₀ values (COX-1/COX-2) of 450 and 72, respectively, whereas indomethacin showed lower selectivity (0.063) for COX-2.

**Time-dependency of inhibition of human COX-2**

The kinetic mode of the COX-2 inhibitory action by JTE-522 and indomethacin was investigated with human recombinant COX-2. At concentrations of 1 and 10 μM, JTE-522 showed about 20% and 60% inhibition of human COX-2 without any preincubation time, respectively (Fig. 2a). With preincubation, inhibitory activity of JTE-522 was potentiated in a time-dependent manner. Inhibition of COX-2 by JTE-522 was dependent on the length of preincubation of enzyme and inhibitor before the addition of arachidonic acid. COX-2 activity was also attenuated by indomethacin (10 μM) in a time-dependent manner.

**Time-dependency of inhibition of human COX-1**

The time-dependent inhibition of JTE-522 and indomethacin for human COX-1 was investigated by increasing the preincubation time (0–10 min) for the enzyme in the presence of the test compounds. JTE-522 at a concentration of 10 μM did not inhibit human COX-1, irrespective of preincubation time (Fig. 2b). JTE-522 at a concentration of 100 μM inhibited human COX-1 activity by about 15% without preincubation, and the inhibitory activity of JTE-522 was not affected by preincubation time. Indomethacin at concentrations of 1 and 10 μM inhibited human COX-1 in a time-dependent and dose-dependent manner.
Reversibility of human COX-2 activity

Before and after gel filtration, COX-2 converted arachidonic acid to PGH₂ and its decomposition products by 15.1 ± 3.1% (n = 3) and 13.8 ± 2.1% (n = 3), respectively, in the control group. Before gel filtration, JTE-522 (1 μM) and flufenamic acid (50 μM) inhibited human COX-2 activity almost completely, by 86.4% and 100%, respectively, with 5 min preincubation (Fig. 3). For COX-2 activity of the vehicle control group, no significant difference was observed between before and after gel filtration. Gel-filtered human COX-2 was inhibited to a similar extent (79.2%) by JTE-522 as human COX-2 before gel filtration, but it was not inhibited by flufenamic acid.

Inhibition of human cyclooxygenase in cell-based assay

The production of PGE₂ by LPS-stimulated human PBMC was evaluated as a cell-based assay for COX-2.

![Graphs](image)

**Fig. 1.** Effects of JTE-522 and reference compounds in human COX-2 (expressed in yeast) (a) and COX-1 (platelet microsome fraction) (b) enzyme assay. JTE-522 (●), indomethacin (○), NS-398 (□) or SC-58635 (△) were preincubated with the enzyme for 5 min before addition of arachidonic acid (final concentration of 30 μM). Enzyme activity was calculated from the percent conversion of arachidonic acid to PGH₂ and its decomposition products, using a BAS2000 system. Values represent the mean ± S.E.M. of 3 or 4 experiments.

**Fig. 2.** Time-dependent inactivation in human COX-2 (expressed in yeast) (a) and COX-1 (platelet microsome fraction) (b) enzyme assay by JTE-522 and indomethacin. JTE-522 (1 μM ●, 10 μM ▲ or 100 μM ■) or indomethacin (1 μM ○ or 10 μM △) were preincubated for the indicated times with the enzyme before addition of arachidonic acid (final concentration of 30 μM). Enzyme activity was calculated from the percent conversion of arachidonic acid to PGH₂ and its decomposition products, using a BAS2000 system. Values represent the mean ± S.E.M. of 3 or 4 experiments.
Unstimulated human PBMC contained small amounts of PGE₂ (0.35 ± 0.09 ng/ml, n = 4). LPS stimulation for 24 hr produced a substantial increase of PGE₂ (2.47 ± 1.46 ng/ml, n = 4). The inhibitory effects of test compounds on cell-based COX-2 activity was concentration-dependent (Fig. 4a). IC₅₀ values for JTE-522, indomethacin, NS-398 and SC-58635 were 15.1, 1.78, 2.54 and 4.35 nM, respectively. On the other hand, intact cell-based human COX-1 activity was determined with A23187-induced TXB₂ production by human platelets. A23187 stimulation of human platelets resulted in the generation of large amounts of TXB₂ (144 ± 5 ng/ml, n = 4). Test compounds inhibited TXB₂ formation in a concentration-dependent manner (Fig. 4b). IC₅₀ values for JTE-522, indomethacin, NS-398 and SC-58635 were 6210, 28.4, 562 and 813 nM, respectively. JTE-522 showed the highest selectivity for the inhibition of cell-based COX-2 relative to cell-based COX-1. The ratio of IC₅₀ values (COX-1/COX-2) for JTE-522, indomethacin, NS-398 and SC-58635 were 411, 16, 221 and 187, respectively.

DISCUSSION

In the present study, we investigated the inhibitory effects of JTE-522 on human COX-1 and COX-2. In our human COX-2 enzyme assay, NS-398, a well-known COX-2 inhibitor, diminished COX-2 activity with an IC₅₀ value of 0.167 µM, which is in agreement with the literature (23, 24). Under the present experimental conditions, JTE-522 strongly inhibited human COX-2 activity with an IC₅₀ value of 0.085 µM. JTE-522 was the most potent inhibitor of human COX-2 of the compounds tested. On the other hand, JTE-522 at concentrations up

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**Fig. 3.** Recovery of JTE-522 or flufenamic acid inhibited COX-2 activity after gel filtration. After preincubation of the enzyme in the presence or absence of the inhibitor for 5 min at 24°C, the assay mixture was applied to the PD 10 column and then eluted with the equilibrating buffer. COX-2 activities of the pre-filtrate fractions (open column) and eluted ones (hatched column) were estimated and the recovery of enzyme activity was evaluated. Values represent the mean ± S.E.M. of 3 experiments.

**Fig. 4.** Effects of JTE-522 and reference compounds in human cell-based cyclooxygenase assay. a: Cell-based COX-2 activity was determined by LPS-induced PGE₂ production in human PBMC. PBMC were preincubated with compounds for 1 hr at 37°C and stimulated with 1 µg/ml LPS for 24 hr at 37°C. PGE₂ levels were measured by an EIA system. b: Cell-based COX-1 activity was determined by A23187-induced TXB₂ production in human washed platelets. Platelets were preincubated with compounds for 10 min at room temperature and stimulated with 1.26 mM CaCl₂ and 10 µM A23187 for 15 min at 37°C. TXB₂ levels were measured by an EIA system. Compounds: JTE-522 (●), indomethacin (○), NS-398 (□) and SC-58635 (△). Values represent the mean ± S.E.M. of 4 experiments.
to 100 μM did not exert an inhibitory effect against human COX-1 activity, while indomethacin strongly inhibited human COX-1 activity, and NS-398 and SC-58635 also attenuated COX-1 activity at 10 μM order of concentration. Therefore, JTE-522 had the highest selectivity for human COX-2 as compared with COX-1 under the conditions used. Similar results were obtained in ovine COX enzyme assays (15). We have reconfirmed that JTE-522 is a selective COX-2 inhibitor.

It has been reported that the time-dependent inhibition of COX by NSAIDs such as indomethacin occurs via a two-step process in which initial rapid formation of a reversible complex (EI) is followed by slow isomerization to an essentially irreversibly bound form (EI*-). (13, 14). The degree of selectivity of NS-398 towards COX-2 results from the difference in the nature of time-dependent COX-2 inhibition and time-independent COX-1 inhibition (25). Besides, mutation of two residues in the active site of COX-1 into those of COX-2 resulted in inhibition of the COX-1 mutant by COX-2 selective inhibitors (NS-398, SC-58125 and DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5H)-furanone)) (26). It has been suggested that residues in the active site of COX-2 are essential determinants in differentiating between the interaction of nonselective and selective COX-2 inhibitors and their mechanism of action. To clarify the COX inhibitory mechanism by JTE-522, we investigated the time-dependency and reversibility of JTE-522 for COX. In the present study, JTE-522 showed time-dependent and irreversible inhibition of human COX-2, suggesting that JTE-522, like indomethacin, inhibits human COX-2 by a two-step mechanism with formation of an irreversibly inhibited complex (EI*-). In contrast, inhibition of human COX-1 by JTE-522 was not time-dependent after preincubation for 10 min, while indomethacin inhibited both human COX-1 and COX-2 in a time-dependent manner as described in other reports (13, 14). Therefore, the time-dependency of JTE-522 for human COX-2 might be, at least in part, an apparent basis for COX-2 selectivity, and the structure of JTE-522 may interact with the residues for the active site of COX-2.

It has been reported that significant differences exist in the subcellular locations of COX-1 and COX-2 (27). The former functions predominantly in the endoplasmic reticulum, while the latter may be involved in events in both the endoplasmic reticulum and nuclear envelope. The differences in site-specific functions may reflect the different sensitivities of COX-1 and COX-2 to NSAIDs. Therefore, a cell-based assay is needed for evaluating the pharmacological features of NSAIDs for COX activities. In the present study, cell-based COX activities were evaluated with the production of TXB2 by A23187-stimulated human platelets for COX-1 and the generation of PGE2 by LPS-stimulated human PBMC for COX-2. NS-398 and SC-58635 diminished cell-based COX-2 activity with IC50 values of 2.54 and 4.35 nM, respectively. Although JTE-522 was less potent in inhibiting cell-based COX-2 than the other inhibitors tested, the order of selectivity for COX-2 relative to COX-1 was JTE-522 > NS-398 > SC-58635 > indomethacin. JTE-522 also showed the highest selectivity for human COX-2 as compared with COX-1 in the cell-based assay used.

JTE-522 did not cause inhibition of prostaglandin synthesis in COX-1 enzyme assays. However, inhibition of TXB2 by JTE-522 could be observed with platelets stimulated with A23187. The reason was not clear, but similar results were obtained with other COX inhibitors (indomethacin, NS-398 and SC-58635). The difference in potency of COX inhibitors between the enzyme assay and the cell-based assay might be explained by a higher effective concentration of arachidonic acid. In assays where inhibitors were preincubated with enzyme prior to the addition of arachidonic acid, lowering the substrate concentration will result in an increase in potency for competitive inhibitors. In fact, assays with low arachidonic acid concentration (0.1 μM) permit the detection of COX-1 inhibition with selective COX-2 inhibitors (28). In our enzyme assay, arachidonic acid was added to the assay system at a concentration of 30 μM, while in the cell-based assay, the concentration of arachidonic acid derived from platelets might be much less than 30 μM.

The difference between the mechanism of inhibition of COX-1 and COX-2 observed for JTE-522 has been noted previously for other selective COX-2 inhibitors, and this makes it difficult to determine accurately the selectivity ratio of the various COX inhibitors. However, the facts that JTE-522 can inhibit 100% of LPS-induced PGE2 synthesis in PBMC assays and shows efficacy in various in vivo models of inflammations indicate that the kinetic characteristics of COX-2 inhibition by this compound allow effective inhibition of the activity of the inducible enzyme. In fact, we have previously demonstrated that JTE-522 possesses potent anti-inflammatory, antinociceptive, antipyretic and anti-arthritic activities, and very little gastrointestinal ulcerogenicity (15, 29). Oral JTE-522 was efficiently absorbed and available systemically in rats. Bioavailability in rats was about 80%. In a one-month oral toxicity study in rats, histopathology in the gastrointestinal tract was not observed at high doses of JTE-522. These results support the possibility that JTE-522 may be clinically useful for the treatment of inflammatory diseases without the adverse effects related to COX-1 inhibition.

In conclusion, the present results indicate that JTE-522 has a strong, highly selective inhibitory activity on human COX-2, as compared with indomethacin or other COX-2
inhibitors, and that the inhibition is time-dependent and irreversible as seen for other COX-2 selective inhibitors.

REFERENCES


