Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of pain and inflammation. NSAIDs act by inhibiting the catalytic activity of cyclooxygenase (COX), which results in a blockage of the formation of prostaglandins (PGs). Two isoforms of COX have been identified. COX-1, a constitutively expressed form, displays in gut and kidney that produce PGs which are required for normal physiological functions. COX-2, an inducible isoenzyme, is encoded by a different gene from COX-1 and only exists in high concentrations under the inflammatory condition or following mitogenic stimulation. Inhibition of COX-2 accounts for the anti-inflammatory effects of NSAIDs, whereas interruption of COX-1 leads to gastrointestinal toxicity. Therefore, it is proposed that a selective COX-2 inhibitor would have a superior safety profile to traditional NSAIDs.

Since the discovery of COX-2, a large number of selective COX-2 inhibitors have been described of which vicinal diaryl heterocycles represent the most important group. These selective COX-2 inhibitors achieve therapeutic efficacy in acute and chronic inflammation management while avoiding the serious side effects. Celecoxib, in the 1,5-diarylpyrazole class of compound, was the first launched selective COX-2 inhibitor, and has excellent selectivity and potent anti-inflammatory activity; however, its aqueous solubility is relatively low, which decreases its oral bioavailability. One approach to address this problem is to convert the compound into a prodrug that is readily soluble in water. Researchers attempted to create a water-soluble form by N-acetylation of the sulfonamide group of celecoxib followed by preparation of its sodium salt, but the result was unsatisfactory. To improve the oral absorption of celecoxib, a prodrug strategy was used in this study. In our experiments, we designed and synthesized a novel 1,5-diarylpyrazole derivative, 4-{5-[3-(2-amino-acetamide)-4-methylphenyl]-3-(trifluoromethyl)-1H-pyrazol-5-yl}phenyl acetamide hydrochloride (CC06), which was intended to act as a prodrug and would exert potent anti-inflammatory activity after being converted to its parent compound in vivo. In vitro cell-based biological assay, CC06 showed decreased inhibitory effects on cyclooxygenase (COX)-1 and COX-2 compared with its parent compounds, but it exhibited potent anti-inflammatory activity in vivo. The anti-inflammatory effect was evaluated in a carrageenan-induced rat paw edema model and CC06 (15, 30, 60 mg/kg, intragastrically) reduced rat paw edema in a dose-dependent manner. CC06 is also a selective inhibitor of COX-2 since it can reduce prostaglandin E2 (PGE2) production in the inflamed pouch dose-dependently without affecting PGE2 production in stomach in rat air pouch model. Furthermore, preliminary pharmacokinetics experiments were conducted using high performance liquid chromatography/mass spectrometry (HPLC/MS) to detect whether CC06 can convert to its parent compound or not. Our results supported the hypothesis that CC06 was actually converted to its parent compound. These suggested that CC06 served as an anti-inflammatory prodrug and actually converted to its parent compound to exert its anti-inflammatory effect. This finding will be of great benefit in carrying out structural modifications of prodrug-like selective COX-2 inhibitors.

Key words cyclooxygenase-2; inflammation; carrageenan-induced paw edema; air pouch model; prodrug
MATERIALS AND METHODS

Animals Male Sprague Dawley (SD) rats, weighing 180—200 g, were supplied by Shanghai Experimental Animal Center, Chinese Academy of Sciences. All animals were housed in Plexiglas cages and kept on a 12/12 h light–dark cycle in temperature and humidity controlled rooms. Food was withheld 12 h before the experiments, with free access to water. Unless otherwise indicated in the text, standard laboratory food and water were provided ad libitum. Experiments were performed between 9:00—17:00 h. All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Cell Culture Insect cell line Spodoptera frugiperda (SF9), obtained from the Institutes of Biochemistry and Cell Biology, Shanghai Institutes for Biological Science, Chinese Academy of Sciences, was cultured in monolayer at 28 °C in Grace’s supplemented medium (Gibco BRL) with 10% heat-inactivated fetal calf serum (Gibco BRL).

In Vitro Cell-Based Assay of COX Inhibition Cell-based assay was performed as previously described. Briefly, 24 h after infecting SF9 cells with hCOX-1 or hCOX-2 recombinant baculovirus, the cells were collected and washed in Hank’s solution buffered with 15 mM N-(2-hydroxyethyl)pipperazine-N’’-2-ethanesulfonic acid (HEPES) (HHBS, pH 7.4) adjusted to 1×10⁶/ml, placed in 24-well plates and incubated for 15-min with drugs or vehicles at 37 °C. Then cells were challenged with 10 µM arachidonic acid (Sigma) in ethanol and incubated for 10 min. The cells were pelleted for 10 min at 300×g and the levels of prostaglandin E₂ (PGE₂) in the supernatant were determined by a PGE₂-specific radioimmunoassay (RIA). The concentration of PGE₂ was then determined by interpolation from a standard curve and inhibition calculated by comparison of the PGE₂ production by drug-treated cells with that of dimethylsulphoxide (DMSO)-treated cells.

Carrageenan-Induced Paw Edema The inflammatory response was induced by sub-plantar injection of 100 µl of 1% (w/v) sterile carrageenan in saline into the right hind paw as described previously. The volume of the injected paw was measured with a plethysmometer (Shandong Academy of Medical Sciences, China) before and 3 h after the injection. The inflammation index was calculated as the difference between the final volume of the carrageenan injected paw (V₂) and the initial volume of the same paw before injecting it (V₁), i.e., inflammation index (Ii)=V₂−V₁. The edema inhibition (%) was calculated as percentage of the difference of Ii according to the following formula: % inhibition=([pre-drug Ii]−[post-drug Ii])×100/[pre-drug Ii]×100. CC06 (15, 30, 60 mg/kg) were given intragastrically (i.g.) 12 h before carrageenan challenged. Celecoxib (30 mg/kg, i.g.) only reduced rats paw edema by 30%, thus we choose 50 mg/kg (i.g.) in this study. Celecoxib and indomethacin (10 mg/kg, i.g.) were administered 0.5 h before carrageenan injected. Rats of the control group received the same volume of saline according to their weights.

Air Pouch Model of Inflammation Air pouch was produced by subcutaneous injection of 20 ml sterile air into the intrascapular area of the back as previously described. One day after initial air injection, carrageenan (0.5%, 5 ml) was injected directly into the pouch using a 5-ml syringe and a 20-G, 1-in needle to induce inflammation. After 3 h of carrageenan treatment, 10 ml of 5.4 mM ethylenediaminetetra-acetic acid (EDTA) disodium was injected into the air pouch of each rat using a 10-ml syringe and 18-G, 1.5-in needle. Mix the contents of the pouch by gently massaging the area. Then animals were euthanized by CO₂ asphyxiation and the pouch fluid was collected for PGE₂ determination by RIA. Stomachs of these animals were excised, opened, and cleaned, and the mucosal lining was dissected, weighed and frozen in liquid nitrogen. Stomach tissue was processed by homogenization with 0.9% saline and absolute alcohol (1:4). After centrifuged, the supernatants were stored at −30 °C for PGE₂ determination by RIA. The dose and administration protocols of drugs were the same as those of the paw edema test.

Preliminary Pharmacokinetic Study of the Prodrug CC06 As described previously, HPLC/MS was used to confirm the conversion of prodrug-like derivative CC06 in vivo in rats. A single dose (60 mg/kg) of CC06, which showed potent inhibitory effects on rat paw edema, was orally administered to rats (n=6). Blood samples (50 µl) were taken before or 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 60 and 72 h after intragastric administration, and immediately centrifuged at 10000 g at 4 °C for 15 min. Plasma concentration–time curves were evaluated by InnaPhase Kinetic (InnaPhase Corporation, Philadelphia, PA, U.S.A.).

Drugs Carrageenan (Sigma, St. Louis, MO, U.S.A.) was suspended in saline. CC06, 2-amino-N-(2-methyl-5-(1-(4-sulfamoylphenyl)-3-(trifluoromethyl)-1H-pyrrozol-5-yl) phenylacetamide hydrochloride, CC05, 4-[5-(3-amino-4-methylphenyl)-3-(trifluoromethyl)-1H-pyrrozol-1-yl]benzenesulfonylamide and celecoxib were kindly provided by Professor Jing-Kang Shen (Shanghai Institute of Materia Medica, Chinese Academy of Sciences). CC06, celecoxib and indomethacin (Sigma) were dissolved in 0.5% carboxymethylcellulose immediately before experiments and administered intragastrically. PGE₂-specific RIA kits was purchased from Beijing East Asia Institute of Immunology.

Statistical Analysis Data were expressed as mean±S.E.M., and subjected to one-way analysis of variance (ANOVA) and Dunnett’s test. p<0.05 was considered to be statistically significant.

RESULTS

Effect of CC06 on COX-1 and COX-2 Enzymatic Activity in Vitro Cell-based biological assay was performed as described previously. CC06 and reference compounds were evaluated in intact infected SF9 cells expressing comparable amounts of recombinant hCOX-1 or hCOX-2. Parent compound CC05 and celecoxib exhibited potent and selective inhibition of COX-2 activity in vitro, whereas the inhibitory activities of the hydrochloride salts CC06 were greatly decreased compared with its parent compound. IC₅₀ values were illustrated in Table 1.

Effect of CC06 on Carrageenan-Induced Paw Edema In vivo, CC06 (15, 30, 60 mg/kg, i.g.) can inhibit the inflammatory edema dose-dependently. The reductions of paw edema were 13.94%, 25.76% and 71.15%, respectively. Indomethacin (10 mg/kg, i.g.) reduced the paw edema by...
and celecoxib (50 mg/kg, i.g.) only reduced by 47.56% (Fig. 2).

Selectivity of CC06 on COX-1 and/or COX-2 in Air Pouch Model

Like the paw edema induced by carrageenan, injection of carrageenan into an established subcutaneous air pouch in rats induced marked PGE2 production. PGE2, produced from air pouch was inhibited by CC06 in a dose-dependent manner, and CC06 at a dose of 60 mg/kg produced a significant reduction. However, PGE2, produced from the stomach was not inhibited by CC06 at any dose. The inhibition manner was similar to that of celecoxib, different from indomethacin, which could inhibit the production of PGE2 driven either from the air pouch or the stomach (Fig. 3).

Preliminary Pharmacokinetic Study

A preliminary pharmacokinetic study of compound CC06 in rats (n=6) was undertaken to prove that it was converted in vivo to compound CC05 (Fig. 1). CC06 converted to its parent compound CC05 several hours after intragastric administration and we detected CC05 in rat plasma 4 h after CC06 administration. Plasma drug concentration of CC05 released from compound CC06 was calculated by non-compartmental model analysis based on the drug concentration–time curve of 0—72 h sample dotting. Peak plasma concentration (Cmax), the corresponding time (Tmax), the area under the plasma concentration–time curve (AUC0—∞) sample dotting and T1/2 were 7.1±0.9 mg/l, 12.2±1.7 h, 155.1±11.7 mg·l·h, and 5.9±1.0 h, respectively.

DISCUSSION

In the present study, an amino acid-binding 1,5-diarylpyrazole derivatives was designed and synthesized as prodrug. As expected, the aqueous solubility of it was greatly improved...
compared with that of celecoxib. For example, the solubility of CC06 in water at 18°C was 19—22 g/l, whereas that of celecoxib was <0.05 g/l. Good aqueous solubility should enhance the dissolution rate of a drug and thus improve its oral bioavailability. We also presented evidence that the diarylpyrazole derivative CC06, inhibits COX-2 enzymatic activity in vivo, however, it showed extremely weak inhibition on COX activity in vitro cell-based assay. Our results demonstrated that 1,5-diarylpyrazole derivatives CC06 possessed potential and selective inhibition of COX-2 activity in two in vivo assays carrageenan-induced inflammatory model (Figs. 2, 3). As shown in Fig. 2, celecoxib (50 mg/kg, i.g.) can only reduced the paw edema by 47.56%. It was probably related to the degree of inhibition of COX-2. Anti-inflammatory effects (edema reduction and analgesia) of celecoxib were nevertheless attributed to inhibition of COX-2, however, there was a window of COX-2 inhibition. Edeima inhibition was not expressed at a particular level of COX-2 inhibition and that level did appear hypoalgesia. Francischi et al. reported that celecoxib (30 mg/kg) can only reduce paw edema by 30%.

In our study, CC06 was administrated intragastrically 12 h before carrageenan challenging since the results of HPLC/MS showed that plasma drug concentration reached peak at that point. And CC06 could exert its maximal anti-inflammatory effect and convert to its parent compound CC05 at that point. The preliminary pharmacokinetic studies suggested that the T_{max} of COX05 (as derived from CC06) was found at 12 h after oral administration (Fig. 4B). However, the T_{max} of celecoxib was at 3 h after administration. In order to compare the maximal potencies of these drugs, we chose different administration protocols. In our experiments, celecoxib and indomethacin were given 0.5 h before carrageenan challenging, and CC06 was given 12 h before carrageenan administration, we observed that they all possessed marked anti-inflammatory effects on edema. As Francischi et al. reported, rat paw edema volume induced by carrageenan reached a peak 3 h after intraplantar injection. Therefore, we detected whether CC06, celecoxib and indomethacin possessed potent anti-inflammatory effect at that time-point. The anti-inflammatory effect of CC05 has been examined and CC05 exerted its maximal anti-inflammation when administrated 12 h before carrageenan challenging.

The selective inhibition of CC06 on COX-2 in vivo was assessed in carrageenan-induced air pouch model. This model was chosen because the inflammatory response includes the production of large amounts of PGE2, derived from expression of COX-2. In order to detect whether CC06 exerted its anti-inflammatory effect by selective inhibition on COX-2, we chose the same time-point (3 h after carrageenan challenging) as rat paw edema model. Although as Masferrer et al. reported, the expression of COX-2 and the production of PGE2 is apparent at 6 h, there are definitely COX-2 expression and PGE2 production 3 h after carrageenan injection. Whittle et al. first reported that NSAIDs can selectively inhibit PGs biosynthesis in different tissues in vivo. Infection of a carrageenan solution into the pouch produces an inflammatory reaction that is characterized by an infiltration of cells, an increase in exudates, and a marked production of biochemical mediators such as prostaglandins, leukotrienes, and cytokines. Futaki et al. reported the selective inhibition of NS-398 on PGs production in inflamed tissue, such as carrageenan induced air pouch, but not the normal gastric mucosa in rats. Thus, the carrageenan induced air pouch model has been extensively used to analyze the selectively potential of inhibition on COX-1/2 in vivo. This model is also an acute or sub-acute model to mimic clinical arthritis or other chronic inflammatory disorders since the pouch consists mainly of macrophages and fibroblasts and bears a remarkable resemblance to a synovial cavity. CC06 could inhibit PGE2 production in the air pouch rather than PGE2 in the stomach (Fig. 3). The results suggested that CC06 had selective action on COX-2 in vivo. We also observed that CC06 did not produce any gastric or intestinal lesions after a single dose of 1.25 g/kg administration intragastrically in a 2-week study in mice (data not shown). These findings suggest that CC06 might be a promising candidate agent in the management of chronic rheumatoid arthritis and osteoarthritis with less usage of concomitant gastroprotective drugs.

We hypothesized that hydrochloride salt should bring about beneficial anti-inflammatory effects after delivering its parents compound in vivo. Therefore, anti-inflammatory evaluation in vivo was performed in carrageenan-induced rat model. Compound CC06 showed potent inhibition of edema after intragastric administration. Preliminary pharmacokinetic studies in rats also proved that CC06 actually served as a prodrug, which has an anti-inflammatory effect once it is converted into its active form, CC05. We can detect CC05 in plasma drug concentration reached 12 h before carrageenan challenging since the results of HPLC/MS showed that plasma drug concentration reached peak at that point. And CC06 could exert its maximal anti-inflammation on the level of transcription and translation of COX.

In conclusion, our data presented here demonstrated that celecoxib analog was combined with natural amino acid to obtain a novel hydrochloride salts, CC06, served as a prodrug and exerted its anti-inflammatory effects in vivo. By biological evaluation and preliminary pharmacokinetic studies in vivo, CC06 exhibited potent anti-inflammatory activity and was confirmed to act as a prodrug. These findings will be of great benefit to the structural modifications of prodrug-like selective COX-2 inhibitors.

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REFERENCES


