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Research Article

Analgesic Effect of the Newly Developed S(+)-Flurbiprofen Plaster on Inflammatory Pain in a Rat Adjuvant-Induced Arthritis Model

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT This article describes the properties of a novel topical NSAID (Nonsteroidal anti-inflammatory drug) patch, SFPP (S(+)-flurbiprofen plaster), containing the potent cyclooxygenase (COX) inhibitor, S(+)-flurbiprofen (SFP). The present studies were conducted to confirm human COX inhibition and absorption of SFP and to evaluate the analgesic efficacy of SFPP in a rat adjuvant-induced arthritis (AIA) model. COX inhibition by SFP, ketoprofen and loxoprofen was evaluated using human recombinant COX proteins. Absorption of SFPP, ketoprofen and loxoprofen from patches through rat skin was assessed 24 h after application. The AIA model was induced by injecting *Mycobacterium tuberculosis* followed 20 days later by the evaluation of the prostaglandin PGE_2 content of the inflamed paw and the pain threshold. SFP exhibited more potent inhibitory activity against COX-1 (IC_{50} = 8.97 nM) and COX-2 (IC_{50} = 2.94 nM) than the other NSAIDs evaluated. Absorption of SFP was 92.9%, greater than that of ketoprofen and loxoprofen from their respective patches. Application of SFPP decreased PGE_2 content from 15 min to 6 h and reduced paw hyperalgesia compared with the control, ketoprofen and loxoprofen patches. SFPP showed analgesic efficacy, and was superior to the ketoprofen and loxoprofen patches, which could be through the potent COX inhibitory activity of SFP and greater skin absorption. The results suggested SFPP can be expected to exert analgesic effect clinically. Drug Dev Res 76 : 20–28, 2016.

Key words: S(+)-flurbiprofen; analgesic; nonsteroidal anti-inflammatory drugs

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Conflict of interest: M Sugimoto, Y Toda, M Hori, A Mitani, T Ichihara, S Sekine, T Hirose, H Endo, N Futaki, S Kaku, and N Otsuka are employees of Taisho Pharmaceutical Co., Ltd. H Matsumoto receives consulting fees from Taisho Pharmaceutical Co., Ltd.

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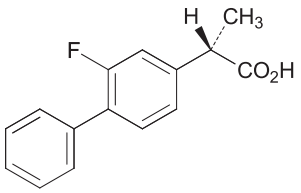

S(+)-flurbiprofen

Fig. 1. Chemical structure of S(+)-flurbiprofen The molecular weight of S(+)-flurbiprofen: (2S)-2-(2Fluorobiphenyl-4-yl) propionic acid is 244.26.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to relieve the chronic pain associated with musculoskeletal disorders such as rheumatoid arthritis (RA) and osteoarthritis (OA) that is a major factor in decreasing patient quality of life. NSAIDs exert their pharmacological and toxicological effects by inhibition of arachidonic acid binding to cyclooxygenase (COX), thereby inhibiting the production of PGs, including PGE_2, a major mediator of inflammatory pain [Narumiya et al., 1999]. However, since chronic oral administration of NSAIDs involves the risk of a wide range of adverse effects, including gastrointestinal, renal, hepatic, and cardiovascular disorders, percutaneous absorption of NSAIDs via the transdermal patch method has been used for pain relief with reduced risk of several adverse effects. Topical administration of NSAIDs has several advantages, including protection of the active compound from gastric enzymes, avoidance of hepatic first-pass metabolism, and the lower risk of gastrointestinal adverse effects [Mason et al., 2004]. Moreover, more constant plasma levels are achieved by administration via the transdermal route. Topical NSAID preparations are widely used to treat acute and chronic musculoskeletal disorders. Conversely, the efficacy of the patch method in humans remains unclear due to the possibility of a placebo effect [Zhang et al., 2008; Zeidler, 2011]. Thus, it might be expected that novel topical NSAID patches containing a potent NSAID that is more efficiently absorbed when applied topically will be more effective in relieving chronic pain without severe adverse effects.

The 2-aryl propionic acid, "profen" NSAIDs, are prescribed for the treatment of articular diseases. Chemically, the 2-aryl propionic acids are weak acids, and because of the presence of an asymmetric carbon atom on the propionic acid they occur in enantiomeric pairs (R- and S-profens). Flurbiprofen (FP) is representative of the chiral NSAIDs of the 2-arylpropionic acid class and is extensively used as anti-inflammatory and analgesic agents in the form of a racemic mixture. S(+)-flurbiprofen (SFP, Fig. 1) is a more potent COX inhibitor than the R(-)-enantiomer (RFP), demonstrating that COX inhibition by flurbiprofen is predominantly attributable to the S-enantiomer [Peskar et al., 1991; Carabaza et al., 1996]. SFP has more potent COX inhibitory activity in in vitro assays than other NSAIDs [Carabaza et al., 1996]. Both potent COX inhibitory activity and high skin permeability are critical for topical NSAIDs to achieve optimal efficacy. Although FP has relatively good skin penetration potency compared with other NSAIDs [Yano et al., 1986; Goi et al., 2010], the skin absorption rate of FP from commercially available patches is low (4%, relative bioavailability to oral dosing) [Taburet et al., 1995]. The skin permeation efficacy of FP patches may be expected to be further optimized and led to the development of a novel anti-inflammatory analgesic patch containing SFP (SFPP) where transdermal absorption is improved by the addition of certain agents (Yataba et al., 2015). SFPP could exert potent pain relief as a topical NSAID patch.

The purpose of this study was to compare the analgesic efficacy of the SFPP and clinically available NSAID patches in an animal model. We first assessed the inhibitory activity of SFP, ketoprofen and loxoprofen against human COX, and second assessed rat skin absorption of SFP, ketoprofen, and loxoprofen patches to confirm their in vitro pharmacological activity and pharmacokinetics. We then evaluated the effect of a single application of the SFPP on the PGE_2 level in paw exudate and pain response in a rat adjuvant-induced arthritis (AIA) model in comparison with effect of a ketoprofen and loxoprofen patch.

MATERIALS AND METHODS

Animals

Male Lewis (7 week old) and male Sprague Dawley (SD; 8 week old) rats (Charles River Japan, Kanagawa, Japan) were used for the pharmacology and skin absorption studies, respectively. Animals were housed under controlled temperature (23 ± 3°C), humidity (55 ± 20%), and lighting (lights on from 07:00 to 19:00) conditions and used after at least two days of acclimation. All animal experiments reported here were reviewed and approved by the Institutional Animal Care and Use Committee of Taisho Pharmaceutical Co., Ltd., and were in accordance with the Guidelines for Proper Conduct of Animal Experiments published by Science Council of Japan at 2006.

Drugs and reagents

SFP was obtained from Aesica Pharmaceuticals Ltd (Newcastle, UK). Ketoprofen was purchased from Sigma-Aldrich (St Louis, MO) and Loxoprofen-SRS (an

active metabolite of loxoprofen sodium) was synthesized by Taisho Pharmaceutical Co., Ltd (Tokyo, Japan). The following products were evaluated in this study: SFPP (40 mg/140 cm^2, Tokuhon Corporation, Tokyo, Japan), ketoprofen patch (40 mg/140 cm^2, Morus® tape L; Hisamitsu Pharmaceutical Co., Inc., Tosu, Japan), and loxoprofen patch (100 mg/140 cm^2, Loxonin® tape; Daiichi-Sankyo Co., Ltd., Tokyo, Japan). Morus® tape L and Loxonin® tape were purchased as commercially available products. Clinically, all the drugs used in this study were administered to the affected area in the form of a patch (10.0 × 14.0 cm). *Mycobacterium tuberculosis* H37 RA (DIFCO Laboratories, Detroit, MI, USA), liquid paraffin (Wako, Tokyo, Japan), PGE_2 enzyme-immunoassay (EIA) kit, and Human COX-2 recombinant protein (Cayman Chemical, Ann Arbor, MI, USA) were purchased as indicated in parentheses.

Human recombinant COX-1 and COX-2 assay

Recombinant COX-1 was engineered as previously described [Smith et al., 2000]. The coding region of human COX-1 was cloned into the pFastBac1 vector. Recombinant baculovirus was isolated from Sf9 insect cells using the Bac-to-Bac® Baculovirus Expression System (Invitrogen, Carlsbad, CA). The cells were harvested 3 days after infection with the recombinant baculovirus and stored at −80°C. Human recombinant COX-1 was purified as previously described [Gierse et al., 1995, 1999] with the following minor modifications. The cell pellet after infection with the recombinant baculovirus was suspended in buffer A (25 mM Tris-HCl, pH 8.0, 0.25 M sucrose) and then homogenized and pelleted by centrifugation at 105,000 g for 1 h. This microsomal pellet after centrifugation was suspended in buffer B (buffer A: 10% CHAPS = 9:1), and the suspension sonicated for 5 sec 3 times and agitated for 1h at 4°C. Samples of the solution obtained were centrifuged at 105,000 g for 30 min and the supernatants centrifuged at 105,000 g for 60 min. The supernatants from this second centrifugation were used for the human COX-1 enzyme assays. The purity of the recombinant human COX-1 was determined by SDS-PAGE and by Western blot analysis with specific primary antibody (Anti Cox-1 (C-20), Code.sc-1752, Santa Cruz Biotechnology, Santa Cruz, CA) and secondary antibody (Anti-Goat HRP, Code.V805A, Promega, Madison, WI).

Enzyme activity was assayed in glass tubes each containing 200 μL of a reaction mixture consisting of 100 mM Tris-HCl buffer (pH 8.0), 1μM hematin, 2 mM phenol, 12.6 μg/mL COX-1 or 10.3 units/mL COX-2, various concentrations of each NSAID (dissolved in dimethyl sulfoxide (DMSO)) or DMSO (control), and [1-^{14}C] arachidonic acid 4.7 μmol/L(COX-1) or 1.1 μmol/L(COX-2). The COX-1 or COX-2 was preincubated at 37°C for 15 min with the drug or DMSO. The reaction was started by adding arachidonic acid and allowed to proceed by incubating the mixture at 37°C for 2 min. The reaction was then stopped by adding 1 mL/tube of n-hexane: ethyl acetate (2:1). The aqueous phase was frozen, and the organic solvent phase was discarded to eliminate any remaining [1-^{14}C] arachidonic acid. The extraction procedure was repeated three times. As PGE_2 selectively remained in the aqueous phase, the radioactivity of the aqueous phase was measured in a liquid scintillation counter. Prostaglandin production was calculated based on the conversion of ^{14}C-arachidonic acid. The same experiments were repeated performed four times. The drugs were dissolved in DMSO (final DMSO concentration: 1%)

Absorption After Drug Patch Application to Rat Skin

The dorsal skin of rats was shaved one day before the experiment. All patches, measuring 1.4 × 2.5 cm each, were applied to the dorsal skin of healthy SD rats (n=5/group) and covered with three layers of elastic bandage tape to prevent the patch from falling off. After 24 h the patches were peeled off, and each patch was shaken in extraction solvent to extract the remaining drug. The SFPPs, ketoprofen patches, and loxoprofen patches were shaken in acetone for 15 h at room temperature. The concentration of each drug was determined by high-performance liquid chromatography (HPLC). Absorption was calculated by using the formula:

$$\text{Absorption } (\%) = 100-(A/B * 100)$$

in which A is the concentration of the drug extracted from the patch after cutaneous application, and B is the concentration of the same drug extracted from nonapplicated control patch.

Induction of AIA and Tissue Preparations and Measurement of PGE_2 Levels in Paw Exudate from AIA Rats

Arthritis was induced in Lewis rats by injecting adjuvant (0.8 mg of *M. tuberculosis* in 0.1 mL of liquid paraffin) into the left hind footpad (day 0). On day 20, different patches were applied to the right hind paw to assess their analgesic effect on chronic inflammatory pain (n = 5/group). In the clinic applied in the size of 10.0 × 14.0 cm to the affected area. NSAID doses in this study were standardized by applying patches measuring size of

1.0 cm × 0.88 cm, considering the difference in body weight between human and rat. At each time point after patch application, the rats were anesthetized with sodium pentobarbital and exsanguinated. Exudate was collected from the right hind paw as described [Noguchi et al., 2005]. Briefly, 0.1 mL of a 10 μM indomethacin solution was injected intravenously to prevent further eicosanoid production, and the paw was incised with a scalpel, suspended off the bottom of a polypropylene centrifuge tube with an Eppendorf pipette tip, and centrifuged (2000 g, 15 min, 4°C) to obtain the inflammatory exudate. The inflammatory exudate samples were then centrifuged again (700 g, 1 min, 4°C), and the supernatant stored at −80°C until PGE_2 was measured using a PGE_2 EIA kit and expressed as of percentage of the PGE_2 level of the pre-treatment group.

Evaluation of Paw Hyperalgesia in AIA Rats

The flexion test previously reported as a reproducible method of evaluating the analgesic effect of the patches in the AIA model was used [Kuzuna et al., 1975; Futaki et al., 2009]. The pain threshold was measured by counting the number of squeaking vocalizations induced by five consecutive gentle flexions of the ankle joint of the right paw. Prior to patch application, it was confirmed that all the rats tested expressed a vocalization response to the flexion test 19 days after injection of the adjuvant. In the test of drug efficacy, the vocalization response was evaluated by the constant angle and amplitude of the flexion being confirmed in the flexion test prior to the patch applications. At 19 days, the Lewis rats were divided into 8 equal sized groups in such a way that the mean pain thresholds in all of the groups on day 19 were approximately the same. Different patches were applied to the right hind paw of the AIA rats for 6 h and pain thresholds were measured at the indicated time points for 6 h on day 20. The patches applied to the rats contained 0.25 mg SFP, 0.25 mg ketoprofen, or 0.63 mg loxoprofen ($n = 8$/group). A group of rats to which no patches were applied was used as the control group. The purpose of this study was to determine whether SFPP exerts analgesic efficacy in vivo including the effects of the patch base and to predict the clinical analgesic efficacy of SFPP by comparing it with other patches. The area under the curve (AUC) of the analgesic effect data was calculated by the area method based on squeaking vocalizations counts over a 0.5–6 h period. To reduce experimenter bias, two experimenters independently conducted the flexion test, the mean value of the two data sets was evaluated as the flexion score of each rat.

TABLE 1. Inhibitory Effect of SFP, Ketoprofen, and Loxoprofen-SRS on Human Recombinant COX-1 or COX-2 activity

NSAID	COX-1 IC_{50}(nM) [95% CI]	COX-2 IC_{50}(nM)[95% CI]
S(+)-flurbiprofen	8.97 [3.82–21.1]	2.94 [1.41–6.12]
Ketoprofen	38.2 [6.87–213]	26.1 [14–48.7]
Loxoprofen-SRS	1470 [1030–2100]	25.9 [15.6–43.0]

Data are expressed as the mean (95 percent confidence interval) obtained from four individual determinations.
IC_{50} values were estimated by nonlinear least-squares method.

Data Analysis

For the measurement of COX activity dose-response curves for the percentage of the vehicle (DMSO) control were fitted with a four-parameter logistic function by a nonlinear least squares regression method. The IC_{50} values for COX inhibition activity were calculated as the concentration of inhibitor which inhibit halfway between maximum and minimum inhibition calculated by a nonlinear least-squares method. The results of the pharmacological study are expressed as the mean ± SE, and the results of the skin absorption study are expressed as the mean ± SD. The percentage for PGE_2 level was calculated using the difference between the patch-treated and the pre-treatment group. The differences between the control group and the patch experimental groups and the differences between the SFPP group and the other patch groups were tested by Bartlett test followed by a multiple comparison test, i.e., Dunnett or Steel test. A P value <0.05 was considered statistically significant.

RESULTS

Inhibitory Effect of SFP, Ketoprofen, and Loxoprofen-SRS on Human Recombinant COX-1 or COX-2 Activity

We first confirmed in vitro human COX-1 and COX-2 activity using recombinant COX-1 and COX-2 proteins by SFP and conventional NSAIDs, as summarized in the IC_{50} values in Table 1. SFP strongly inhibited COX-1 and COX-2 activity with IC_{50} values of 8.97 nM and 2.94 nM, respectively, whereas the IC_{50} values for the conventional NSAIDs were: ketoprofen, 38.2 nM and 26.1 nM, respectively and loxoprofen-SRS, 1470 nM and 25.9 nM, respectively. Thus SFP was 4-9 times more potent than ketoprofen and 9–164 more potent than loxoprofen-SRS.

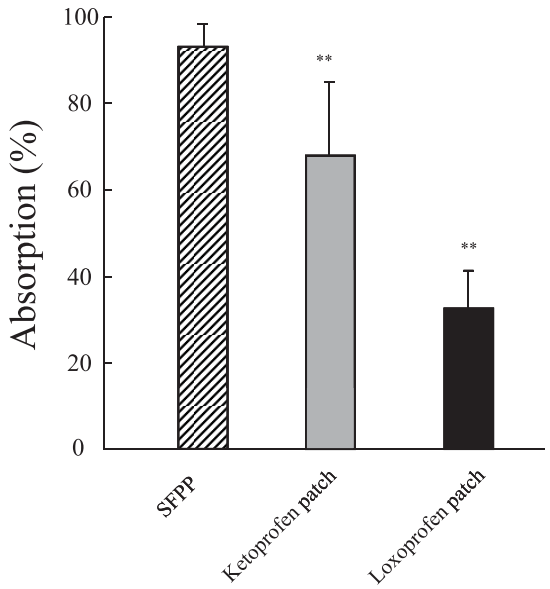

Fig. 2. Absorption after application of SFPP, ketoprofen patch and loxoprofen patch to rat skin.Skin absorption by rat dorsal skin was assessed 24 h after application of each patch. Each drug concentration in the patch after application was measured by HPLC. Data are expressed as the mean ± SD of the results obtained in 5 animals.**: $P < 0.01$, significant difference from SFPP (Dunnett test). The patch size was 1.4 cm × 2.5 cm.

Skin Absorption After a Single Application of SFPPs, Ketoprofen Patches, and Loxoprofen Patches to SD Rat Skin

The dorsal rat skin drug absorption rates 24 h after a single application of the SFPP, ketoprofen patch, and loxoprofen patch are shown in Figure 2. The rates were: SFPP 92.9%, ketoprofen patch 67.8%, loxoprofen patch 32.4%, respectively. The order of the absorption efficacy of each patch was SFPP > ketoprofen patch > loxoprofen patch, indicating that SFPP exhibited the highest absorbability difference, compared to ketoprofen and loxoprofen patch.

Effects of SFPP and Ketoprofen Patch, and Loxoprofen Patch Application on the PGE_2 Level of the Inflamed Paw Exudate of AIA Rats

The inhibitory effect of SFPP, ketoprofen patch, and loxoprofen patch on PGE_2 levels in the inflammatory exudate was examined, PGE_2 being the key factor of inflammation with inhibition of its formation being the mechanism of action of NSAIDs. As shown in Figure 3, SFPP rapidly decreased PGE_2 levels in the inflammatory exudate from the inflamed paw of the AIA rats from 15 min to 6 h after patch application. The SFPP inhibited sustainably PGE_2 production more than 50% of the pre-treatment group. The ketoprofen patch decreased PGE_2 levels at 1 h, 2 h, and 6 h with a slower onset to maximum effect while the loxoprofen patch decreased PGE_2 levels only at 6 h after application.

Effects of the SFPP, Ketoprofen and Loxoprofen Patch Application on Paw Hyperalgesia in AIA Rats

The analgesic effects of 6 h application of the SFPP, ketoprofen, and loxoprofen patches to the dorsal skin of AIA rats are shown in Figure 4. The SFPP exerted an analgesic effect from 30 min to 6 h after skin application as compared with the control group with more than 50% inhibition of the pain response being observed 1 h after patch application. The ketoprofen patch showed a modest analgesic effect compared with the control group and maintained this effect at the same level for the remainder of the 6 h observation period (Fig. 4A). The loxoprofen patch also showed a similar modest analgesic effect after application. The AUC of the time-course of paw hyperalgesia from 30 min to 6 h (Fig. 4B) showed that the SFPP had the most potent analgesic effect, a reduction of 67% in vocalization counts that was significantly different to the effects of the ketoprofen patch (31%) and the loxoprofen patch (25%).

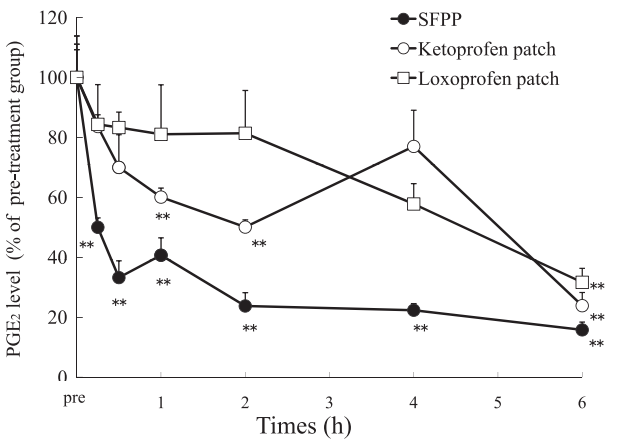

Fig. 3. Effect of the SFPP, ketoprofen patch, and loxoprofen patch application on the PGE_2 level of the inflamed paw exudate in a Lewis rat AIA model. On day 20 after intraplantar injection of *M. tuberculosis* to Lewis rats to induce arthritis, the patches were applied on the right hind paw for the times indicated. Rats were killed, and the right hind paw was removed. PGE_2 was extracted by the method described in Materials and Methods. The PGE_2 content of the inflamed paw exudate was measured by an EIA. The data was calculated as percentage compared to PGE_2 level of the pre-treatment group. Data are expressed as the mean ± SE of the results obtained in 5 animals. **$P < 0.01$, significant difference from the value in the pre-treatment group (Dunnett test).

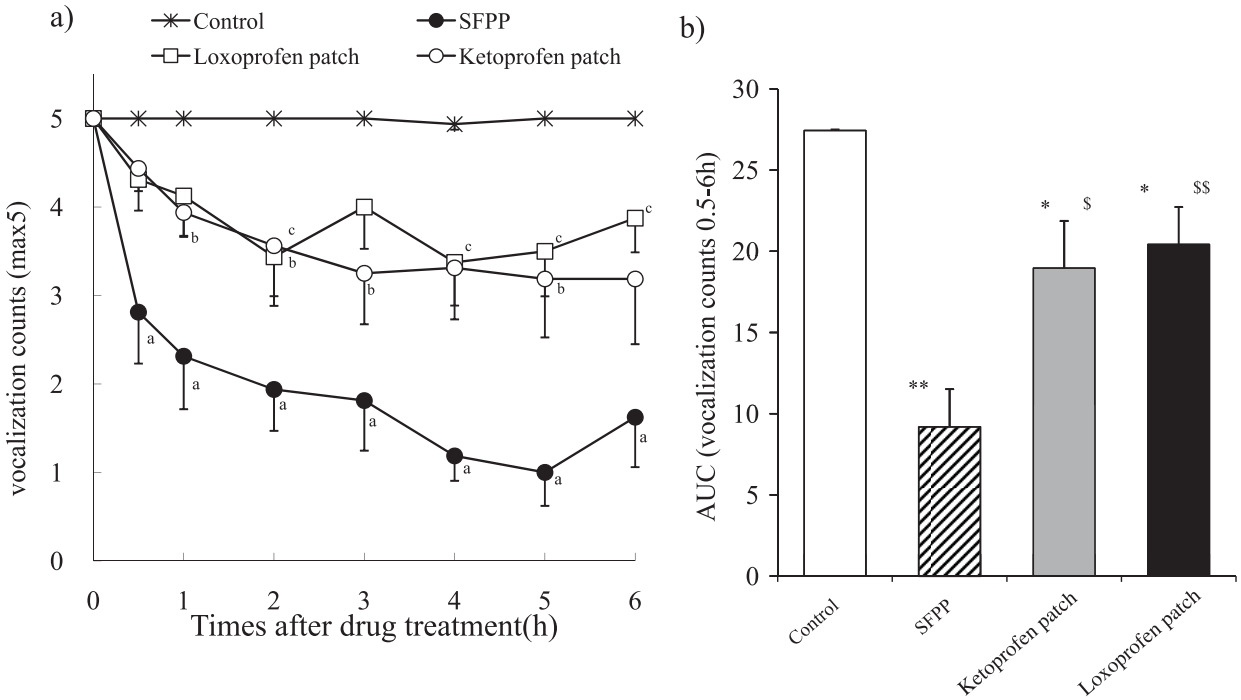

Fig. 4. Effect of the SFPP, ketoprofen patch and loxoprofen patch application on paw hyperalgesia in a Lewis rat AIA model. On day 20 after intraplantar injection of *M. tuberculosis* to induce arthritis, the drugs were applied on the right hind paw for 6 h. Measurements of pain response were performed at each time point indicated after patch application. Each value and bar represents the mean ± SE of the results obtained in 8 animals. (**A**) Time-course of paw hyperalgesia changes after an application of each patch. a: SFPP, b: ketoprofen patch, c: loxoprofen patch, significant difference from the value in the control group, $P < 0.05$, Steel test. (**B**) AUC of paw hyperalgesia changes from 30 min to 6 h. ** $P < 0.01$, * $P < 0.05$, significant difference from the value in the control group (Steel test), \$\$$P < 0.01$, \$$P < 0.05$, significant difference from the value in the SFPP group (Dunnett test).

DISCUSSION

The results of this study show that SFP potently inhibits human COX activity and can penetrates rat skin from patches more efficiently than ketoprofen or loxoprofen from their patches as assessed by its effects in decreasing PGE_2 in paw exudates and alagesic reseponse in the rat AIA model. SFPP application rapidly decreased PGE_2 in the paw exudate in a rat AIA model and had superior analgesic efficacy as compared to application of ketoprofen and loxoprofen patches. These findings indicate that the analgesic effect of the SFPP is attributable both to its potent COX inhibiting activity and improved skin absorption.

The results of our study confirmed the effect of SFP on human COX-1 and COX-2 activity when tested on recombinant proteins, and its IC_{50} value of 8.97 nM for COX-1 and 2.94 nM for COX-2 showed that it has potent inhibitory activity. Since there have been no reports comparing the in vitro human COX-1 and COX-2 inhibitory activity of SFP, ketoprofen and loxoprofen with human recombinant proteins, this is the first report showing that SFP exerts most potent inhibitory activity against human COX-1 and COX-2 compared with ketoprofen and loxoprofen. On the other hand, there is a report of a study that evaluated the inhibitory activity of SFP against COX in vitro by means of intact cell assays [Carabaza et al., 1996] in which the COX-1 isoform of human peripheral blood leukocytes or COX-2 of human monocytes stimulated with LPS, and the reported IC_{50} values for SFP at COX-1 and COX-2 were 2.7 nM and 2.5 nM, respectively. The results of our study were thus consistent with the inhibitory activity reported in previous intact cell assays, thus the recombinant human COX inhibition assays in this study could be useful to screen COX inhibition activity of the compounds as correlating with intact cells assay.

The results of this study indicated that SFPP showed higher drug absorbability through rat skin as assessed by its effects on paw exudate PGE_2 levels and analgesic efficacy as compared to ketoprofen patch and loxoprofen patch and the amount of drug remaining in the patch (Fig. 2). Although FP has higher skin penetrability than other NSAIDs [Yano et al., 1986; Goi et al., 2010], the FP skin absorption from commercially available patches is low (4%)

[Taburet et al., 1995]. Although FP and SFP possess the same lipophilic properties, an important factor influencing skin absorption, application of the SFPP was followed by superior skin absorption as measured by both the residual amount present in the patches (Fig. 2) and its pharmacodynamic effects in this study. It has ben reported that patch formulation with additive agents can enhance drug skin permeation in addition to the lipophilic nature of the active pharmaceutical ingredient (API) [Ma et al., 2010]. The rapid and potent pharmacological effect of SFPP could in part be attributable to the superior skin absorption of SFP, indicating that SFPP is a novel NSAID patch which exerts more enhanced local effect than traditional NSAIDs patch. Due to methodological limitations, the drug absorption study measuring the drug remaining in the patches was conducted 24 h after they were applied. Furthermore, in this study absorption of the three different drugs from their patches was analyzed only in healthy SD rats. Although the results of this study indicate the relative order of permeability of the skin to the drugs in the three patches, we will need to test the skin absorption of the drugs from their patches in the AIA rat in a further permeation study in order to demonstrate direct involvement of their absorption in the analgesic efficacy of the drug patches.

We used a Lewis rat AIA model of chronic inflammation to assess pharmacological activity and observed that a single skin application of the SFPP more rapidly and potently relieved paw hyperalgesia than a ketoprofen patch or loxoprofen patch. The pain response in AIA models is useful for assessing the effects of analgesic compounds on chronic inflammatory pain [Pircio et al., 1975] and the analgesic efficacy of drugs in AIA models is strongly correlated with their clinically efficacy [Dubinsky et al., 1987]. The SFPP can therefore be expected to be a useful NSAID patch for rapid, potent analgesic activity clinically.

The SFPP contains peppermint oil as its base and the other NSAID patches contained menthol, a major constituent of peppermint oil. Since peppermint oil has been reported to possess central and peripheral analgesic effects [Klein et al., 2010; Liu et al., 2013], it may have contributed to the observed analgesic effect of all of the patches applied in this study. Conversely, we demonstrated that the SFPP base does not have a significant analgesic effect in AIA rats (data not shown), suggesting that peppermint oil had only a marginal effect in the experiments conducted in this study. Furthermore, since the purpose of the study was to determine whether SFPP including the patch base exerts analgesic efficacy in vivo, the results for analgesic efficacy suggested the expectation of clinical analgesic efficacy of SFPP comparing the analgesic efficacy of each patch.

It has been reported that PGs produced via COX pathways are the major lipid mediators contributing to inflammatory pain. Of these, PGE_2 has the greatest impact on processing of pain signals and is abundantly produced in inflamed tissues, contributing to the genesis of inflammatory pain [Narumiya et al., 1999]. PGE_2 production is increased in the synovial membranes and synovial fluid of arthritis patients [Amin et al., 2000] and can directly excite nociceptors and potentiate the sensitizing effects of other pain mediators, including ATP, bradykinin, and capsaicin [Wang et al., 2007], inducing hyperalgesia at inflamed sites. In fact, intraplantar injection of PGE_2 in a carrageenan-induced model of inflammation can evoke prolonged tactile allodynia, including nociceptor hypersensitivity and the magnitude of pain response [St-Jacques et al., 2014]. PGE_2 levels in inflamed paws area also increased in AIA model rats [Tateishi et al., 2015]. In this study the SFPP rapidly decreased the PGE_2 level in the inflammatory paw exudate of AIA model rats starting 15 min after application. The rapid suppression of PGE_2 production in inflamed peripheral tissues by SFPP may directly inhibit nociceptors and reduce nociceptor hypersensitivity. The parallel nature of the PGE_2 levels and the pain responses in this study suggested that SFPP exerted PGE_2 inhibition at inflamed sites through direct absorption via the rat skin.

There are reports regarding the analgesic effect of topical NSAID patches using acute inflammatory pain models, but analgesic effects in chronic inflammatory pain model with topical NSAIDs patches have not been reported. Sekiguchi et al., [2008] reported that a loxoprofen patch exerted a more potent analgesic effect in a yeast-induced model of acute inflammation than felbinac, indomethacin, and ketoprofen patches and [Komatsu et al., 2012] reported that a ketoprofen patch had more potent anti-inflammatory and analgesic activity in a carrageenan-induced model of inflammation than diclofenac, flurbiprofen, and piroxicam patches. The differences between the acute inflammation induced by carrageenan and yeast and the chronic inflammation in the AIA model may have influenced the assessment of the analgesic effects of the different NSAID patches. Although the mechanisms underlying the differences between these animal models of pain remains unknown, other reports showed that efficacy of each NSAID patch differed according to each inflammatory model [Kido et al., 1998]. Thus, further studies in several types of animal models of

inflammation will be needed to increase the certainty of the efficacy of the SFPP.

The results of this study showed that, in addition to the potent COX inhibiting activity and high skin permeability of SFP, the SFPP was effective in providing pain relief in an AIA model and superior to ketoprofen and loxoprofen patches suggesting that the SFPP can be expected to be effective as an analgesic patch in the clinic.

ETHICS OF THE ANIMAL EXPERIMENTS

All animal experiments reported here were reviewed and approved by the Institutional Animal Care and Use Committee of Taisho Pharmaceutical Co., Ltd., and were in accordance with the Guidelines for Proper Conduct of Animal Experiments published by Science Council of Japan at 2006.

AUTHOR'S CONTRIBUTIONS

1. Conception and design of the study: M Sugimoto, Y Toda, N Futaki, S Kaku, N Otsuka.
2. Data acquisition or data analysis and interpretation: analgesic efficacy and PG experiments, M Sugimoto, Y Toda, M Hori, T Ichihara, N Futaki; for COX activity assay, A Mitani; skin absorption experiment, T Hirose.
3. Preparation of recombinant human COX proteins: S Sekine.
4. Drafting the article or revising it critically for important intellectual content: M Sugimoto, Y Toda, H Endo, S Kaku, N Otsuka, H Matsumoto.
5. Final approval of the version to be submitted: M Sugimoto, Y Toda, M Hori, A Mitani, T Ichihara, S Sekine, T Hirose, H Endo, N Futaki, S Kaku, N Otsuka, H Matsumoto.

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