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Effects of SKI306X on Arachidonate Metabolism and Other Inflammatory Mediators

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SKI306X was previously reported to have good anti-inflammatory and analgesic efficacy in various studies. To determine its mode of action, an investigation was carried out for some representative mediators. Arachidonic acid metabolism and its products are particularly important in inflammation and pain. The pro-inflammatory cytokines, especially interleukin-1 (IL-1) and tumor necrosis factor (TNF-α), and induced nitric oxide (NO) appear to be most involved in the inflammatory process such as osteoarthritis (OA). Thus SKI306X was tested to determine the effects on enzymes related to arachidonic acid metabolism and the release or synthesis of inflammatory mediators. SKI306X did inhibit the expression of cyclooxygenase-2 (COX-2) enzyme without affecting COX-1 and COX-2 activity. Leukotriene B4 (LTB4) production also was inhibited by SKI306X (IC50 = 98.7 ± 4.26 μg/ml). SKI306X inhibited significantly TNF-α release (IC50 = 97.6 ± 17.8 μg/ml) and NO production (IC50 = 280 ± 17.8 μg/ml). But IL-1α release was not attenuated by SKI306X. This study suggests that inhibition of these mediators by SKI306X may be one of the mechanisms responsible for its anti-inflammatory effects.

Key words SKI306X; cyclooxygenase-2; leukotriene B4; nitric oxide; tumor necrosis factor (TNF)-α; 5-lipoxygenase

Arthritic diseases, such as osteoarthritis (OA) and rheumatoid arthritis (RA) are the most common inflammatory diseases.1) Inasmuch as OA and RA afflict more than 80% of all patients suffering from arthritis, they are major public concerns. Although OA and RA differ fundamentally in several respects, they share some common pathway leading to cartilage degradation, characterized by the gradual and progressive loss of articular cartilage, resulting in erosions to subchondral bone. Symptoms include joint pain, tenderness, limited movement, crepitus and variable degrees of joint inflammation. Current therapeutic strategies for OA alleviate symptoms, particularly pain and inflammation.2)

SKI306X is an extract purified from a mixture of three Oriental herbal medicines, Clematis mandshurica, Trichosanthes kirilowii and Prunella vulgaris which have been widely used for the treatment of inflammatory diseases such as lymphadenitis and arthritis in Far East Asia.3,4) In previous studies, the anti-inflammatory and analgesic activities of this herbal extract were reported in various in vitro and in vivo experimental models. SKI306X had been reported to have protective effects in articular cartilage.5) The double blind, placebo-controlled study also confirmed the clinical efficacy and good tolerability of SKI306X in patients with OA in the knee6) and in another clinical study, this herbal extract was well tolerated and demonstrated clinical efficacy comparable to that of diclofenac SR (slow released). Another clinical study is underway to determine the efficacy of SKI306X in RA patients. But, its mode of action is still to be determined.

Cartilage in OA is a site of the prodigious production of mediators classically associated with inflammation, namely prostaglandins (PGs), nitric oxide (NO) and cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α. These mediators, produced by activated chondrocytes, act in an autocrine/paracrine fashion to exert profound effects on cartilage metabolism in OA. Recent studies showed that IL-1β, TNF-α and inducible nitric oxide synthase (iNOS) are highly expressed in synovial cells, infiltrating leukocytes and endothelial cells in RA.

IL-1 and TNF-α play a central role in many inflammatory processes and appear most involved in the catabolic process of OA.7) These cytokines also enhances the production of 5-lipoxygenase (5-LOX) and prostaglandin E2 (PGE2). It is clearly established that pro-inflammatory cytokines, mainly IL-1 and TNF-α, can activate chondrocytes to enter a catabolic condition and prime a matrix-degrading activity, including protease secretion, radical species production, down-regulation of matrix and protease inhibitor synthesis, inhibition of chondrocyte proliferation and cell death. And NO production by iNOS may reflect the degree of inflammation and provides a measure to assess the effect of drugs on the inflammatory process.8,9) NO, free radical generated from L-arginine by NOS, has been recognized to be an important mediator of cellular communication in several preparations. Several studies have demonstrated that induction of iNOS produces a large amount of NO during endotoxemia and under inflammatory conditions.10) Therefore, drugs that inhibit iNOS expression and/or enzyme activity resulting in decreased NO generation may have beneficial therapeutic effects in the treatment of diseases due to overproduction of NO. Other studies also have suggested that inhibition of NO production may ameliorate arthritis.

Furthermore, cytokine-induced degenerative activities in synovial joint tissues can be potentialized via the biosynthesis of inflammatory eicosanoids by cyclooxygenase (COXs) and 5-LOX. Arachidonic acid is a polyunsaturated fatty acid covalently bound in esterified form in the cell membranes of most body cells. Following irritation or injury, arachidonic acid is released and oxygenated by enzyme systems to the formation of a large group of inflammatory mediators, the eicosanoids “PGs, thromboxanes (TXs) and leukotrienes (LTs)”. The PGs, products of the COXs enzyme pathway, have potent inflammatory properties and PGE2 is readily detectable in equine acute inflammatory exudates.11) PGE2 and PGJ2 markedly enhance edema formation and leukocyte infiltrations...
turation by promoting blood flow in the inflamed region. Consequently, modulation of eicosanoid production, that is arachidonate cascade enzyme modulation, has been a major target of pharmaceutical companies for intervention in the pathogenesis of arthritis.

To determine a molecular basis for potential therapeutic properties associated with SKI306X, we investigated its effects on the expression and activity of arachidonate cascade enzyme and some inflammatory mediators.

MATERIALS AND METHODS

Preparation and Composition of SKI306X SKI306X was prepared by extracting a mixture of three medical herbs (dried root of Clematis mandshurica, dried root of Trichosanthes kirtlo, and dried flower and stem of Chosanthes kirilowii) at 1:2:1 (w/w), respectively with 30% (v/v) ethanol–water. After the extracted solution was filtered and evaporated in vacuo, the residue was partitioned between n-butanol and water. The n-butanol layer was evaporated in vacuo and lyophilized for a complete removal of the residual solvent to yield dark-brown powder. SKI306X was standardized, conforming to the regulations imposed by Korea Food and Drug Administration (KFDA).

Materials Cell culture reagents were obtained from Gibco BRL (ML, U.S.A.). Lipopolysaccharide (LPS), ionophore A23187, arachidonic acid, interferon-γ (IFN-γ), indomethacin, Diclofenac sodium and curcumin were purchased from Sigma (St. Louis, MO, U.S.A.). COX-2 was detected on western blot with murine polyclonal antibody from Cayman, the peroxidase conjugated secondary antibody and some inflammatory mediators.

Preparation of assay (ELISA) (Cayman Enzyme Immunoassay kit); absorbance of the mixture at 550 nm was determined with ELISA plate reader.

For COX-2 expression test, aspirin (500 μM) was added before LPS treatment and were washed three times with phosphate buffered saline. The cells were pre-incubated with test materials for 15 min and were further incubated with or without 1 μg/ml LPS for 16 h. Aliquots were drawn from the supernatant of culture medium and analyzed for PGE2 concentration by ELISA. Cultured RAW 264.7 cells in COX-2 expression test model were washed three times with phosphate buffered saline and were used for western blot analysis.

Western Blot Analysis For total cellular extraction, cells were washed twice and solubilized by addition of Laemmli sample buffer, the composition of which was Tris–HCl 187.5 mM, sodium dodecyl sulfate 6%, glycerol 30%, 2-mercaptoethanol 15%, pH 6.8. The sample was boiled at 95 °C for 5 min. Electrophoresis was performed on 10% acrylamide gels. Proteins were transferred electrically from the gel into Immobilon polyvinylidene difluoride membranes (GVHP Durapore filter: Milipore Corp>Bedford, MA, U.S.A.) by the semi-dry blotting method. The immunoblots were blocked for 3 h with 2% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween 20 (TBST) at 25 °C and incubated with anti-COX-2 antibody (murine) at 1 μg/ml for 1 h at 25 °C. The immunoblots were washed several times and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-murine IgG in TBST containing 2% BSA for 1 h at 25 °C. Blots were developed by using ECL detection reagent and visualized by exposing X-ray film (Sigma).

Measurement of LTB4 Production Freshly venous blood was collected into heparinized tube from healthy volunteers who had not taken any non-steroidal anti-inflammatory drugs (NSAIDs) for at least 2 weeks. 500 μl aliquots of blood were pre-incubated with either 2 μl vehicle or test materials at 37 °C for 1 h. This was followed by incubation of the blood with 5 μM calcium ionophore A23187 for 4 h at 37 °C. After the desired incubation time, the samples were centrifuged at 1500 g at 4 °C for 10 min. Plasma LTB4 concentration was quantified with ELISA.

Measurement of TNF-α Production Heparinized human whole blood was pre-incubated with either vehicle or test materials at 37 °C for 10 min and then further incubated with 1 μg/ml LPS for 12 h. After incubation, the samples were centrifuged at 1500 g at 4 °C for 10 min. Plasma TNF-α concentration was quantified ELISA kit (R & D systems, Minneapolis, U.S.A.).

Nitrite Assay The nitrite concentration in RAW 264.7 cultured medium was measured as an indicator of NO production according to the Griess reaction. One hundred microliters of each supernatant was quantified with ELISA (Cayman Enzyme Immunoassay kit); absorbance of the mixture at 550 nm was determined with ELISA plate reader.

Measurement of iNOS Enzyme Activity For the assay in intact cells, RAW 264.7 cells were plated in 100-mm tissue culture dishes (4×10⁶ cells) and incubated to subculture in 24-well plates and the experiment was performed 2 d after seeding of cells. Test materials were applied to this plate and incubated for 1 h. The cells were washed three times with phosphate buffered saline. The cells were further incubated with or without 1 μg/ml LPS and 10 units IFN-γ for 12 h. After the incubation, aliquots were drawn from the super-
natant of culture medium and analyzed for nitrite concentration by ELISA.

Statistics All data were expressed as mean ± S.E.M. Statistical significances among groups were tested using Sigma Stat (Jandel Co., San Rafael, CA, U.S.A.) by one-way analysis of variance (ANOVA) and Dunnett’s test. Differences were considered significant when $p$ was less than 0.05. All experiments were done at least three times, each time with three or more independent observations.

RESULTS

Effect on PGE$_2$ Production Stimulated by LPS in RAW 264.7 Cells In inhibitory model of induced COX-2, aspirin discriminated basal COX-1 in RAW 264.7 macrophage and treatment of LPS (1 $\mu$g/ml) elevated significantly the medium concentration of PGE$_2$ for 16h. Unstimulated RAW 264.7 macrophages produced a basal amount of PGE$_2$ in the medium (Fig. 1). SKI306X and its component materials reduced PGE$_2$ accumulation by LPS in a concentration-dependent manner. Similarly, with diclofenac and rofecoxib this increase was inhibited by pretreatment of cells with SKI306X and its components. To investigate the cause of this decrease by drugs, the western blot analysis was tested in RAW 264.7 cell. The western blot analysis demonstrated that unstimulated RAW 264.7 macrophages expressed only a small amount of COX-2 proteins and LPS increased COX-2 protein significantly. SKI306X and its components affected the nearly all COX-2 expression, but known COX-2 inhibitor could not inhibit COX-2 expression (Fig. 2). In activity inhibition model of COX-2, diclofenac and rofecoxib inhibited strongly COX-2 activity, whereas SKI306X and its components did not affect the activity of COX-2 (Table 1). These results showed that SKI306X suppressed the PGE$_2$ production in the medium through the inhibition of COX-2 expression in cell. In activity inhibition model of COX-1, diclofenac inhibited strongly COX-1 activity and rofecoxib showed the specificity of COX-1/COX-2 inhibition ratio. But SKI306X and its components did not inhibit the activity of COX-1 (Table 1).

Effect on LTB$_4$ Production Stimulated by Ionophore in Human Whole Blood Calcium ionophore stimulated heparinized human whole blood to produce LTB$_4$ in plasma. MacMillan et al. reported that 5-LOX activity could be measured by inhibition of LTB4 release after stimulation with calcium ionophore A23187, in order to determine the
Fig. 3. Effects of SKI306X, Its Components and Known Compound on LTB4 Production Inhibition by A23187 Stimulated Human Whole Blood

500 μl aliquots of blood were pre-incubated with either 2 μl vehicle or test materials at 37°C for 1 h. This was followed by incubation of the blood with 5 μl A23187 for 4 h at 37°C. Plasma LTB4 concentration was quantified. The values are means of 9 determinations ± S.E.M. * p<0.05 vs. A23187.

Table 2. IC50 Values of SKI306X, Its Components and Curcumin on 5-LO

<table>
<thead>
<tr>
<th>Test materials</th>
<th>IC50</th>
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<tbody>
<tr>
<td>SKI306X</td>
<td>98.7±4.26 μg/ml</td>
</tr>
<tr>
<td>Trichosanthes kirilowii</td>
<td>118.2±9.65 μg/ml</td>
</tr>
<tr>
<td>Clematis mandshurica</td>
<td>144.8±18.3 μg/ml</td>
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<tr>
<td>Prunella vulgaris</td>
<td>&gt;1 mg/ml</td>
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<tr>
<td>Curcumin</td>
<td>8.4±3.25 μM</td>
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</table>

500 μl aliquots of blood were pre-incubated with either 2 μl vehicle or test materials at 37°C for 1 h. This was followed by incubation of the blood with 5 μl A23187 for 4 h at 37°C. Plasma LTB4 concentration was quantified. Each data represents the mean±S.D.

IC50 concentrations of the test materials. Therefore our study was conducted in human whole blood in vitro using a method of MacMillan et al.13) SKI306X was found to inhibit 5-LOX activity as evidenced by the reduction in the formation of LTB4 at final concentrations 30, 100, 300 μg/ml with a 1 h pre-incubation. SKI306X and its components significantly reduced the formation of LTB4 in a concentration-dependent manner (Fig. 3). The SKI306X showed 98.7±4.26 μg/ml in IC50 values for inhibition of LTB4 production and Trichosanthes kirilowii showed 118.2±9.65 μg/ml and Clematis mandshurica showed 144.8±18.3 μg/ml and Prunella vulgaris showed weak inhibitory effect (Table 2).

Effect on TNF-α Production Stimulated by LPS in Human Whole Blood LPS stimulated TNF-α and IL-1α production in heparinized human whole blood.14) SKI306X and its components significantly inhibited TNF-α production (IC50 of SKI306X, 97.6 μg/ml and Clematis mandshurica, 257.3±9.43 μg/ml and Prunella vulgaris 224.7±6.57 μg/ml) but not IL-1α production (data not shown) (Fig. 4, Table 3). In LPS stimulated blood, no significant inhibition was observed for indomethacin. The other 5-LO and COX inhibitors showed no significant inhibitory effect on TNF-α and IL-1α in LPS stimulated blood.

Effect on LPS- and IFN-γ-Induced NO Production in RAW 264.7 Macrophages The effects of SKI306X and its components on LPS- and IFN-γ-induced NO production in RAW 264.7 macrophages were investigated by measuring the accumulated nitrite, which was estimated by the Griess reaction, in the culture medium. SKI306X, Clematis mandshurica, Trichosanthes kirilowii, and Prunella vulgaris at 300 μg/ml did not interfere with the reaction between nitrite and Griess reagents (data not shown). Unstimulated macrophages, after 12 h of incubation in the culture medium, produced background levels of nitrite. When the cells were incubated with the indicated compounds alone, the concentration of nitrite in the medium was maintained. After treatment with LPS for 12 h, nitrite concentrations in the medium increased remarkably by about 20-fold (∼34 μM). When RAW 264.7 macrophages were treated with different concentrations of the indicated compounds, a significant inhibition of nitrite production was detected in the presence of SKI306X and Prunella vulgaris (Fig. 5). The IC50 values of SKI306X and Prunella vulgaris in inhibiting LPS and IFN-γ-induced NO production were 280±17.8 μg/ml and 68.4±11.8 μg/ml (Table 4).

DISCUSSION

Phospholipase A2 (PLA2), activated in response to various stimuli, catalyzes the release of arachidonic acid, the most abundant polyunsaturated fatty acid in the phospholipid component of cell membranes.15) The released arachidonic acid serves as the precursor for the synthesis of the different eicosanoids, mediated through the COXs and 5-LOX.

NSAIDs, which represent the major symptomatic therapy in OA and RA, exert their effects by blocking COXs,16,17) The ability of NSAIDs to reduce the primary symptoms of arthritis (inflammation and pain/hyperalgesia) is established beyond doubt, as is the ability of these agents to produce unwanted and sometimes serious, gastrointestinal side effects. COX is a key enzyme in the biosynthesis of prostanoids from arachidonate. Prostanoids are important mediators of inflammation in arthritis, but also play important roles in normal physiological functions. It is now well established that COXs exist as two isoforms that catalyze the same reaction but differ in terms of regulation of expression. The constitutive isoform COX-1 is responsible for the production of PGs involved in prostanoid-mediated physiological functions. A
second isoform, COX-2, has been identified and has been demonstrated to be highly expressed in response to inflammatory or mitogenic stimuli. Thus, it is proposed that COX-2 is responsible for the production of PGs associated with inflammatory conditions.11) Arachidonic acid is also metabolized to LTs by 5-LOX enzyme. The 5-LOX products are pro-inflammatory mediators that may participate in the process leading to joint destruction. On the other hand, LTs may play a role in the development of gastrointestinal ulceration during long-term treatment with NSAIDs.

In our study on arachidonate metabolism cascade, SKI306X did not show any inhibitory effect on PLA2 (data not shown). SKI306X inhibits the expression of COX-2 enzyme.

5-LOX activity was measured by inhibition of LTB4 release after stimulation with calcium ionophore A23187 in human whole blood. The results demonstrated that SKI306X decreased the production of LTB4 (Fig. 3). SKI306X displayed inhibitory effects of COX-2 expression/5-LOX activity with good selectivity. While novel NSAIDs produced gastric injury, ML-3000, a new well-balanced dual inhibitor of COX/5-LOX produced significantly less gastric injury.18,19)

Table 3. IC50 Values of SKI306X, Its Components and Curcumin on TNF-α

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>SKI306X</td>
<td>97.6±17.8 µg/ml</td>
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<tr>
<td>Trichosanthes kirilowii</td>
<td>&gt;1 mg/ml</td>
</tr>
<tr>
<td>Clematis mandshurica</td>
<td>257.3±9.43 µg/ml</td>
</tr>
<tr>
<td>Prunella vulgaris</td>
<td>224.7±6.57 µg/ml</td>
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<tr>
<td>Curcumin</td>
<td>nd</td>
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Heparinized human whole blood was pre-incubated with either vehicle or test materials at 37 °C for 10 min and then incubated with 1 µg/ml LPS for 12 h. Plasma TNF-α concentration was quantified. Each data represents the mean±S.D. nd: not determined.

Fig. 4. Effects of SKI306X, Its Components and Known Compound on the Release of TNF-α from LPS Stimulated Human Whole Blood for 12 h

Heparinized human whole blood was pre-incubated with either vehicle or test materials at 37 °C for 10 min and then incubated with 1 µg/ml LPS for 12 h. Plasma TNF-α concentration was quantified. The values are means of 5 determinations±S.E.M. * p<0.05, ** p<0.01 vs. LPS.

Table 4. IC50 Values of SKI306X, Its Components and Curcumin on iNOS

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<tr>
<td>SKI306X</td>
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</tr>
<tr>
<td>Trichosanthes kirilowii</td>
<td>&gt;1 mg/ml</td>
</tr>
<tr>
<td>Clematis mandshurica</td>
<td>&gt;1 mg/ml</td>
</tr>
<tr>
<td>Prunella vulgaris</td>
<td>68.4±11.8 µg/ml</td>
</tr>
<tr>
<td>Curcumin</td>
<td>4.75±0.87 µM</td>
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RAW 264.7 cell was pre-incubated with either vehicle or test materials at 37 °C for 10 min and then incubated with 1 µg/ml LPS for 12 h. Plasma nitrite concentration was quantified. Each data represents the mean±S.D.

These results suggest that SKI306X could have improved efficacy and reduced side effects when compared to NSAIDs. SKI306X was also tested to characterize the effects of...
SK1306X on the gastric mucosa and compared to that of diclofenac in the rat model. It showed excellent gastrointestinal tolerability after single and repeated administration in animals and did not cause significant gastric or duodenal erosions, ulcers, or ulcerations at oral doses up to 4 g/kg and at intraperitoneal doses up to 125 mg/kg (submitted).

SK1306X was able to significantly inhibit TNF-α-release in human whole blood (Fig. 4) meanwhile it could not inhibit IL-1α release (data not shown). Currently, infliximab, a chimeric monoclonal antibody to human TNF-α, which binds to both soluble and transmembrane forms of TNF-α, is being used in the treatment of RA. This fact suggests that inhibitory effect of SK1306X on TNF-α release may be helpful to alleviate joint inflammation.

In view of the involvement of iNOS in the inflammatory process,10 we monitored the NO generation in RAW 264.7 macrophages cell exposed to LPS and IFN-γ. This LPS and IFN-γ-induced NO production was inhibited by SK1306X in a time and concentration-dependent manner without notable cytotoxicity.

Several additional experiments were preliminarily carried out to examine the analgesic mechanisms of this drug. We tested the representative analgesic mechanisms such as opioid peptides, GABA and serotonin receptor antagonism. The preliminary results showed that SK1306X has effects on GABA, opiate, tachynin NK, thromboxane A2, serotonin and nicotinic acetylcholine receptors and further studies are now going on with several concentrations and other mechanisms.

The active ingredients of SK1306X such as oleanolic acid, rosmarinic acid and rutin are known to have multifunction including anti-inflammation. Among these compounds, Rosmarinic acid is reported to have excellent activities such as: (i) antioxidant activity and/or biosynthesis of prostacyclin generated in the metabolism of arachidonic acid, and by scavenging the active oxygen generated from polymorphonuclear leukocytes; (ii) anti-inflammatory activity such as the inhibition of inflammatory metabolites and immuno-regulation; (iii) enhancement of blood circulation.20–24 It has also been reported that oleanolic acid has not only remarkable anti-inflammatory and analgesic effects but also an excellent effect for chronic rheumatoid arthritis induced by Mycobacterium butyricum.25–28 These reports suggest that the effects of these ingredients may contribute to the multifunction of SK1306X on inflammation and pain.

In summary, SK1306X inhibits PGE2 production through the inhibition of COX-2 expression in murine macrophages and LTB4 production through the inhibition of 5-LOX activity in human whole blood. These studies showed that SK1306X inhibits LPS-induced TNF-α. Furthermore, LPS and INF-γ-induced NO production through the inhibition of iNOS activity was inhibited by SK1306X. These results strongly suggest that inhibition of these mediators by SK1306X may be one of the mechanisms responsible for its anti-inflammatory effects. More studies are in progress to investigate the action of SK1306X on MMPs and aggregases expression for proving the cartilage protective effects.

REFERENCES