AK106-001616, a Potent and Selective Inhibitor of Cytosolic Phospholipase A2: In Vivo Efficacy for Inflammation, Neuropathic Pain, and Pulmonary Fibrosis

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ABSTRACT

3-[3-Amino-4-(indan-2-yloxy)]-5-(1-methyl-1H-indazol-5-yl)-phenyl]-propionic acid (AK106-001616) is a novel, potent, and selective inhibitor of the cytosolic phospholipase A2 (cPLA2) enzyme. Unlike traditional nonsteroidal anti-inflammatory drugs and selective cyclooxygenase-2 inhibitors, AK106-001616 reduced prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) production by stimulated cells. The suppression of PGE2 and LTB4 production was also confirmed using an air pouch model in rats administered a single oral dose of AK106-001616. AK106-001616 alleviated paw swelling in a rat adjuvant-induced arthritis (AIA) model. The maximum effect of the inhibitory effect of AK106-001616 was comparable with that of naproxen on paw swelling in a rat AIA model. Meanwhile, the inhibitory effect of AK106-001616 was more effective than that of naproxen in the mouse collagen antibody-induced arthritis model with leukotrienes contributing to the pathogenesis. AK106-001616 dose dependently reversed the decrease in paw withdrawal threshold not only in rat carrageenan-induced hyperalgesia, but also in a rat neuropathic pain model induced by sciatic nerve chronic constriction injury (CCI). However, naproxen and celecoxib did not reverse the decrease in the paw withdrawal threshold in the CCI model. Furthermore, AK106-001616 reduced the disease score of bleomycin-induced lung fibrosis in rats. In addition, AK106-001616 did not enhance aspirin-induced gastric damage in fasted rats, increase blood pressure, or increase the thromboxane A2/prostaglandin I2 ratio that is thought to be an underlying mechanism of thrombotic cardiovascular events increased by selective cyclooxygenase-2 inhibitors. Taken together, these data demonstrate that oral AK106-001616 may provide valuable effects for wide indications without attendant gastrointestinal and cardiovascular risks.

Nonsteroidal anti-inflammatory drugs (NSAIDs), which act as both COX-1 and COX-2 inhibitors, are widely prescribed for the relief of the signs and symptoms of rheumatoid arthritis (RA) (Combe et al., 2007) and osteoarthritis (OA) (Hochberg et al., 2012). While the use of traditional NSAIDs (tNSAIDs) is limited due to gastrointestinal (GI) toxicities, the new-generation NSAID COX-2 inhibitors have an improved GI safety profile. However, increases in thrombotic cardiovascular (CV) events have been reported in several clinical studies of COX-2 inhibitors (Bresalier et al., 2005; Nussmeier et al., 2005; Solomon et al., 2005). In addition, many meta-analyses have shown that tNSAIDs also increase the risk of thrombotic CV events (Farkouh and Greenberg, 2009; Trelle et al., 2011). Thus, CV risk needs to be taken into account when prescribing tNSAIDs and COX-2 inhibitors (Trelle et al., 2011).

Abbreviations: AIA, adjuvant-induced arthritis; AK106-001616, 3-[3-amino-4-(indan-2-yloxy)]-5-(1-methyl-1H-indazol-5-yl)-phenyl]-propionic acid; A23187, 5-(methylamino)-2-[(2S,3R,5R,8S,9S)-5,9,10-trimethyl-2-[1-oxo-1-(1H-pyrrol-2-yl)propan-2-yl]-1,7-dioxaspiro[5.5]undecan-8-y]methyl]-1,3-benzoxazole-4-carboxylic acid; CAIA, collagen antibody-induced arthritis; COI, chronic constriction injury; COX, cyclooxygenase; cPLA2, cytosolic phospholipase A2; CV, cardiovascular; GI, gastrointestinal; IPF, idiopathic pulmonary fibrosis; PLA2p, independent phospholipase A2p; LOX, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; LTB4, leukotriene B4; LTE4, leukotriene E4; MC, methyl cellulose; NSAID, nonsteroidal anti-inflammatory drug; OA, osteoarthritis; PAF, platelet-activating factor; PBMC, peripheral blood mononuclear cell; PG, prostaglandin; PGE2, prostaglandin E2; PGE2, prostaglandin E2; PGE2, prostaglandin E2; PGE2, prostaglandin E2; PGE2, prostaglandin E2; PGEM, prostaglandin E metabolite; PGI2, prostaglandin I2; PGI2, prostaglandin I2; PIPLA2, secretory phospholipase A2; tNSAID, traditional nonsteroidal anti-inflammatory drug; TXA2, thromboxane A2; TXB2, thromboxane B2.
At present, there is no clear difference in efficacy between tNSAIDs and COX-2 inhibitors in OA patients (National Clinical Guideline Centre, 2014). Considering each side effect, use of oral tNSAIDs/COX-2 inhibitors at the lowest effective dose for the shortest possible period of time is recommended in OA patients (National Clinical Guideline Centre, 2014). The use of tNSAIDs/COX-2 inhibitors has a small benefit in relieving symptoms for patients with RA (Allen et al., 2018). For these reasons there has been focused research into alternative targets that improve the efficacy and safety of anti-inflammatory drugs.

It has been suggested that LTs, which are another group of mediators derived from cPLA2-released AA, play critical roles in the pain and inflammation of several animal arthritis models (Jain et al., 2001; Anderson et al., 2009; Cortes-Burgos et al., 2009; Yoo et al., 2009; Masferrer et al., 2010). Since both PGs and LTs have been found to play important roles in the pathology of RA (Prete and Gurakar-Osborne, 1997; Mathis et al., 2007) and OA (Lauer, 2003), cPLA2, which controls these mediators, could be a potent therapeutic target in RA and OA. The importance of cPLA2 activity in the pathology of arthritis has been suggested in animal studies using cPLA2-deficient mice (Hegen et al., 2003), antisense oligonucleotides against cPLA2 (Raichel et al., 2008) and a specific inhibitor of cPLA2 (Tai et al., 2010).

Neuropathic pain results from diseases or trauma affecting the nervous system, and is generally resistant to NSAIDs; it is treated with coanalgesics, antidepressants, anticonvulsant drugs, and topical agents. A large-scale meta-analysis indicates that the number needed to treat for a 50% reduction of pain by first-line drugs, antidepressants, and anticonvulsants is 3.5–7.7 (Binder and Baron, 2016). However, considering the value of the number needed to treat, the treatment success rate could be improved. Nerve damage has been shown to alter the neurophysiological properties in the peripheral and central neurons, which leads to neuronal hyperexcitability. The peripheral or central hyperexcitability can be modulated with first-line drugs, resulting in relief from pain (Binder and Baron, 2016). Since activation of cPLA2 in dorsal root ganglion neurons (Tsuda et al., 2007) and the spinal cord (Sung et al., 2007) is involved in the pathogenesis of neuronal hyperexcitability, cPLA2 in both the peripheral nerve and spinal cord could be a potent therapeutic target in neuropathic pain.

Pulmonary fibrosis is an interstitial disorder of the lung parenchyma. Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal form of the disorder characterized by inflammation, fibroblast proliferation, and collagen deposition (Nagase et al., 2002). In 2014, pirfenidone and nintedanib were approved for the treatment of IPF. Although both drugs reduced the progression of IPF and offered survival benefits, no pharmacologic intervention is strongly recommended for patients with IPF (Raghu et al., 2015). The high mortality rate clearly demonstrates the need to develop efficient therapeutic strategies for IPF (Liu et al., 2017). In cPLA2-deficient mice, bleomycin-induced overproduction of thromboxanes and LTs in the lung was reduced, and attenuation of fibrosis was observed (Nagase et al., 2002).

With these observations in mind, we embarked on a medicinal chemistry-directed search and discovered 3-[3-amino-4-(indan-2-yloxy)-5-(1-methyl-1H-indazol-5-yl)-phenyll-propionic acid (AK106-001616) (Fig. 1), a novel selective cPLA2 inhibitor. The preclinical pharmacology of AK106-001616 is described subsequently.

**Materials and Methods**

**Chemicals**

AK106-001616 was synthesized by Asahi Kasei Pharma Co., Ltd. Zileuton and celecoxib were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Naproxen was purchased from Sigma-Aldrich (St. Louis, MO). Nintedanib was purchased from Shanghai Haoyuan Chemexpress (Shanghai, P.R. China). Rofecoxib was purchased from Kemprotec (Cumbria, UK). Bleomycin was purchased from Nippon Kayaku (Tokyo, Japan). Aspirin was purchased from Cayman Chemical (Ann Arbor, MI).

**Animals**

Male Sprague-Dawley (SD) rats and female LEW/CrlCrlj rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Female BALB/c and C57BL/6j mice, female and male SD/slc rats, and male and female WKY/Hos rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). Animal species, strains, and sexes used in each animal model were selected by validation with reference to previous reports. The animals were housed in a room in which the temperature and humidity were controlled to 20–26°C and 30%–70%, respectively, with a 12-hour light-dark cycle and free access to food and water.

All animal experiments were approved by the Institutional Animal Care Committee of the Pharmaceutical Research Center of Asahi Kasei Pharma Corporation, and the experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Animals recommended by the Physiologic Society of Japan (https://www.nips.ac.jp/faops2019/assets/dl/Guiding-principles.pdf).

**In Vitro Enzyme Studies**

**Human cPLA2α.** With reference to the previous report (Sharp et al., 1991), the gene of human cPLA2α was cloned from cDNA of human spleen. This recombinant protein was produced using the expression system combining baculovirus and Sf9 cells (de Carvalho et al., 1993). The activity of human cPLA2α was measured with reference to previous reports (Tojo et al., 1993; Leslie and Gelb, 2004). Using phosphatidylcholine containing AA at the second position as a substrate, the AA concentration produced by human cPLA2α was quantified 60 minutes after the start of the reaction. Quantitation was carried out using high-performance liquid chromatography after fluorescence labeling of AA with 9-anthryldiazomethane (PK-101; Funakoshi, Tokyo, Japan).

**Rat cPLA2α.** With reference to the previous report (Owada et al., 1994), the gene of rat cPLA2α was cloned from cDNA of rat brain. This recombinant protein was produced using the expression system combining baculovirus and Sf9 cells (de Carvalho et al., 1993). The activity of rat cPLA2α was measured with reference to previous reports (Tojo et al., 1993; Leslie and Gelb, 2004). The AA concentration produced by rat cPLA2α was quantified in the same way as for human cPLA2α.

**Human Independent PLA2g.** With reference to the previous report (Larsson et al., 1998), the gene of human Ca2+ independent PLA2g (PLA2g) was cloned from cDNA of human testis. This recombinant protein was produced using the expression system combining
baculovirus and SF9 cells (Wolf and Gross, 1996). The activity of human iPLA2γ was measured with reference to previous reports (Tojo et al., 1993; Jenkins et al., 2002). Using phosphatidylcholine as a substrate, the AA concentration produced by human iPLA2γ was quantified 6 minutes after the start of the reaction. Quantitation was carried out using high-performance liquid chromatography after fluorescence labeling of AA with 9-anthryldiazomethane.

**Bovine Secretory PLA2 Type IB.** Bovine secretory PLA2 (sPLA2) type IB was a commercially available product (Sigma-Aldrich). sPLA2 activity of bovine sPLA2 IB was measured using the sPLA2 Assay Kit (Cayman Chemical).

**Platelet-Activating Factor/Platelet-Activating Factor (PAF)–Acetylhydrolase.** Platelet-activating factor (PAF)–acetylhydrolase (also known as type VII PLA2) is an enzyme that inactivates PAF, an inflammatory mediator (Stafforini et al., 1987). The inhibitory effect on PAF-acetylhydrolase was examined using the PAF Acetylhydrolase Inhibitor Screening Assay Kit (Cayman Chemical).

### Cellular Studies

**AA Release from Rat Basophilic Leukemia 2H3 Cells.** AA released from a rat basophilic leukemia 2H3 (RBL-2H3) mast cell line was measured, as previously described with slight modifications (Glover et al., 1995). Tritium-labeled AA (PerkinElmer) was incorporated into cell membrane phospholipids of RBL-2H3 cells (CRL-2256; American Type Culture Collection) by coculturing overnight. Labeled AA was released by stimulating the cells with Ca2+ ionophore 5-(methylamino)-2-[(2S,3S,5R,SS)-3,5,9-trimethyl-2-[1-oxo-1-1H-pyrrrol-2-yl]propan-2-yl]-1,7-dioxaspiro[5,5]undecan-8-yl)methyl]-1,3-benzoxazo-l-4-carboxylic acid (A23187) (Sigma-Aldrich) at a final concentration of 0.2 μmol/L. Radioactivity of the culture supernatant was measured with a liquid scintillation counter (TRI-CARB 2300 TR; PerkinElmer Japan Co., Ltd.) 30 minutes after the stimulation by A23187. AK106-001616 was added 3 hours before stimulation by A23187 and AK106-001616 was 3 hours after stimulation by A23187.

**Leukotriene B4 Production by RBL-2H3 Cells.** The production of leukotriene B4 (LTB4) by RBL-2H3 cells was measured as previously described with slight modifications (Hamaguchi, 1984; Yamashita et al., 2000). RBL-2H3 cells were stimulated with A23187 (0.4 μmol/L) in a culture solution containing AK106-001616 for 20 minutes, and the concentration of LTB4 in the culture supernatant was measured using the Leukotriene B4 EIA Kit (Cayman Chemical).

**Prostaglandin E2 Production by Human Peripheral Blood Mononuclear Cells.** Prostaglandin E2 (PGF2α) production by human peripheral blood mononuclear cells (PBMCs) was examined by modifying the method of a previous report (Wakitani et al., 1998). After approval of the Research Ethics Committee of Asahi Kasei Pharma Corporation and obtaining the consent of the volunteers, blood was collected from three healthy adults (because the volunteers were anonymous, the gender of each adult was unknown), and human PBMCs were prepared from the blood using Mono-Poly Resolving Medium (Sumitomo Dainippon Pharma). One hour after adding AK106-001616 to the PBMCs, 50 μl/well of lipopolysaccharide (LPS) (L4516; Sigma-Aldrich) solution (0.4 μg/ml) was added, with stimulation for 24 hours. The concentration of PGF2α produced in the culture supernatant was measured using the Prostaglandin E2 EIA Kit-Monoclonal (Cayman Chemical).

### In Vivo Studies

**Air Pouch Model in Rats.** The air pouch model was created, as previously described with slight modifications (Payá et al., 1996, 1997). At the back of a 7-week-old female LEW/Cr1Cr1j rat, filter-sterilized air was injected twice at intervals of 2 days to form an egg-shaped air pouch. Drugs or 1% w/v aqueous methyl cellulose (MC) (vehicle) was administered orally once on day 6 after the first air injection, and 1 hour later a suspension of yzosman (Sigma-Aldrich) was injected into the air pouch. The dose of celexibiz was 1 mg/kg, which sufficiently suppressed the production of PGE2 in the preliminary test. The dose of zileuton was 5 mg/kg, which sufficiently suppressed the production of LTB4 in the preliminary test. The exudate in the air pouch was collected 3 hours after yzosman injection. PGE2 and LTB4 concentrations in the exudate were measured using the Prostaglandin E2 EIA Kit-Monoclonal and Leukotriene B4 EIA Kit, respectively.

**Rat Adjuvant-Induced Arthritis Model.** The adjuvant-induced arthritis (AIA) model was prepared by slightly modifying the method described previously (Tanahashi et al., 1998). Killed Mycobacterium tuberculosis H37Ra organisms (DIFCO Laboratories, Detroit, MI) were suspended at 10 mg/ml in liquid paraffin. Female LEW/Cr1Cr1j rats were injected with 50 μl of the suspension (0.5 mg/paw) in the left hind footpad under anesthesia. Drug or 1% w/v aqueous MC (vehicle) was administered orally once a day from day 13 to 34 after adjuvant injection. The dose of naproxen was within the range where dose response was observed in the preliminary test using the AIA model. Zileuton was administered at 30 mg/kg, which is an excessive dose that suppresses the production of LTB4 in the air pouch model. The swelling volume of the contralateral foot pad was measured as an immune response to self-antigens using a 7140 Plethysmometer (Ugo Basile S.R.L., Varese, Italy) at day 34. After randomizing the order of animals by another person different from the evaluator, measurement was carried out by masking the information about the groups from the evaluator.

To confirm the suppression of LT production by zileuton, another experiment using the AIA model was conducted and urine was collected from day 17 to 18. Urinary leukotriene B4 (LTE4), which is an indicator of systemic production of LTB4 concentration, was measured using the Prostaglandin E Metabolite EIA Kit (Cayman Chemical). Urinary creatinine levels were determined by CRE-EN Kainos (Kainos Laboratories, Inc., Tokyo).

**Mouse Collagen Antibody-Induced Arthritis Model.** The collagen antibody-induced arthritis (CAIA) model was created, as previously described with some modifications (Hutamakalen et al., 2009). Female BALB/c mice received an anti-type II collagen antibody cocktail (Chondrex, Inc., Redmond, WA) (1.5 mg/head) in the tail vein on day 0 and LPS (Wako) (25 μg/head) intraperitoneally on day 3. Drug or 1% w/v aqueous MC (vehicle) was orally administered from day 0 to 12 twice a day. The dose of zileuton was 30 mg/kg, which can be expected to have sufficient effect in the preliminary test using the CAIA model. The dose of naproxen was 30 mg/kg, in which sufficient effect was observed in the AIA model. The degree of swelling of the four limbs was scored cumulatively on day 10 in the blinded manner previously described.

To confirm the suppression of LT production by zileuton, another experiment using the CAIA model was conducted and urine was collected from day 3 to 4 and day 8 to 9. Urinary LTE4 concentration from day 3 to 4 and PGEM concentration from day 8 to 9 were measured using the Leukotriene E4 EIA Kit and the Prostaglandin E Metabolite EIA Kit, respectively. Urinary creatinine levels were determined by CRE-EN Kainos.

**Rat Carrageenan Model: In Vivo Model of Inflammatory Pain.** A rat carrageenan model, which is known as an inflammatory pain model, was used (Yoshino et al., 2005). Male SD rats received 100 μl of 1% carrageenan (Sigma-Aldrich) into the hind paw. Drug or 1% w/v aqueous MC (vehicle) was dosed 1 hour before carrageenan injection. The dose of naproxen was within the range where dose response was observed in the preliminary test. Celecoxib was administered at 3 mg/kg, in which sufficient effect was observed in the preliminary test. Measurement of the hind paw withdrawal thresholds was performed in the blinded manner previously described with a 37215 Analgesy-Meter (Ugo Basile) 4 hours after carrageenan injection.

**Rat Chronic Constriction Injury Model: In Vivo Model of Neuropathic Pain.** The chronic constriction injury (CCI) model rats were made according to the method described previously (Ito et al., 2012). Briefly, male SD rats (7 weeks old) received four loose ligatures.
carried out in the blinded manner described previously. The scoring was the gastric mucosa, which was quantitatively indicated as a gastric lesion induced gastric damage, namely, macroscopic depression and bleeding of been fasted for approximately 24 hours to assess the effects on aspirin-
either AK106-001616 (70 or 200 mg/kg) or celecoxib (70 mg/kg) was determined based on the results of preliminary tests with reference to the previous study (Fiorucci et al., 2003). A mixture of aspirin (70 mg/kg) and

Mouse Pulmonary Fibrosis Model Induced by Bleomycin Administration. A mouse bleomycin-induced pulmonary fibrosis model (Nagase et al., 2002) was modified and used. Female C57BL/ 6j mice were anesthetized with pentobarbital and given a single intratracheal administration of 60 μg of bleomycin (Nippon Kayaku). Bleomycin was dissolved in physiologic saline and administered in a volume of 50 μl. Control animals were treated with saline instead of bleomycin in the same manner. These procedures were performed in a sterile environment. Drug or 1% w/v aqueous MC (vehicle) was administered orally once daily for 14 days from day 7 after bleomycin injection. For nintedanib, a dose was selected in which sufficient effect was observed in the preliminary test. Mice were decapitated on day 21, their lungs were fixed with formalin, and Mattson-Trichrome–stained slices were prepared. According to the method of Ashcroft et al. (1988), the degree of fibrosis was scored for 20 visual fields. The average value was taken as the Ashcroft score of the individual. Evaluation of fibrosis was carried out by masking the information about the slices from the evaluator.

Side-Effect Profiling

Effects of Single Dosing on Aspirin-Induced Gastric Damage in Rats. A rat aspirin-induced gastric mucosal injury model, which is known to enhance the injury produced by a COX-2 inhibitor, was used (Fiorucci et al., 2003). The doses of celecoxib and aspirin were determined based on the results of preliminary tests with reference to the previous study (Fiorucci et al., 2003). A mixture of aspirin (70 mg/kg) and either AK106-001616 (70 or 200 mg/kg) or celecoxib (70 mg/kg) was administered as a single oral dose to male SD rats (12 rats/group) that had been fasted for approximately 24 hours to assess the effects on aspirin-induced gastric damage, namely, macroscopic depression and bleeding of the gastric mucosa, which was quantitatively indicated as a gastric lesion score (sum of the lengths of damage of the mucosa). The scoring was carried out in the blinded manner described previously.

Effects of Repeated Dosing on Blood Pressure in Rats. The systolic blood pressure was measured as previously described with slight modifications (Höcherl et al., 2002). Rofecoxib was used as a control drug because its blood pressure lowering effect was reported in

Fig. 2. Inhibitory effects of AK106-001616 on the enzyme activity of human (A) and rat (B) recombinant cPLA2a. Data are presented as mean ± S.D. (n = 3). The AA concentrations in the absence of AK106-001616, 10.2 μmol/l (A) and 8.1 μmol/l (B) for human and rat, respectively, were taken as 100% activity.
the evaluation, the method of statistical analysis and its sequential procedure, and the sample size were determined before the start of the test. The sequential procedure is described subsequently. However, if pathology preparation or positive target drug was not included, the corresponding analysis was skipped.

Step 1: When the value of the disease group has changed significantly compared with the value of the intact group, the value before pathogenesis preparation, or the value of solvent used for pathogenesis preparation, the following analysis is carried out. (If not, do not analyze steps 2 and 3).

**Fig. 3.** Inhibitory effects of AK106-001616 on the release of AA, PGE\(_2\), and LTB\(_4\). (A) AA release from Ca\(^{2+}\) ionophore A23187-stimulated RBL-2H3 cells. Radioactivity of released tritium labeled AA in the absence of AK106-001616 was 1410 dpm. (B) PGE\(_2\) release from LPS-stimulated human PBMCs. Released PGE\(_2\) in the absence of AK106-001616 was 4322 pg/ml. (C) LTB\(_4\) release from A23187-stimulated RBL-2H3 cells. Each value represents the mean ± S.D. of three data points. Released LTB\(_4\) in the absence of AK106-001616 was 1491 pg/ml.

**Fig. 4.** Effects on PGE\(_2\) (A) and LTB\(_4\) (B) production in an in vivo model. Compounds were administered orally at the indicated dose 1 hour before zymosan injection in the air pouch on the back of the rat. PGE\(_2\) and LTB\(_4\) production in the air pouch was measured 3 hours after zymosan stimulation. In the control group, only air was injected without zymosan injection. Each value represents the mean ± S.E.M. of 10 animals. Significant differences: $*$ $*P < 0.05$ by the Williams test compared with the vehicle group; **$*P < 0.01$ by the t test compared with the control group; ***$*P < 0.005$ by the t test compared with the control group.
Step 2: If a significant effect of a positive control drug is observed, perform the following analysis. (If not, do not analyze step 3).

Step 3: Perform statistical analysis at the endpoint.

For comparison between the two groups, the parametric t test or nonparametric Wilcoxon method was used. The parametric Williams or nonparametric Shirley-Williams test was used for comparison of multiple groups where dose response was assumed based on preliminary test results. The parametric Dunnett or nonparametric Steel test was used for comparison of other multiple groups. Significant differences were determined using the t, Williams, Dunnett, Wilcoxon, and Steel tests using SAS version 8.2 or 9.2 (SAS Institute Inc., Cary, NC) and EXSUS version 7.7.1 (CAC EXICARE, Tokyo, Japan). Differences were considered significant when P < 0.025 (using the Williams test) or P < 0.05 (using other tests).

Results

In Vitro Enzyme Studies. AK106-001616 inhibited human cPLA2 enzyme activity in a concentration-dependent manner with an IC\textsubscript{50} value of 3.8 nmol/l (Fig. 2A). AK106-001616 also inhibited rat cPLA2 with an IC\textsubscript{50} value of 4.3 nmol/l (Fig. 2B), which is the IC\textsubscript{50} value almost identical to that of human cPLA2. In contrast, the inhibitory activity of AK106-001616 for human iPLA2 was approximately 15,000 times weaker (IC\textsubscript{50} = 55.1 μmol/l) than that of cPLA2. The inhibitory activity of AK106-001616 on bovine sPLA2 IB was approximately 26,000 or more times weaker (IC\textsubscript{50} > 100 μmol/l) than that of human cPLA2. AK106-001616 did not affect the activity of PAF-acetylhydrolase (also known as type VII PLA2) at concentrations of up to 500 μmol/l.

Cellular Studies. AK106-001616 suppressed the release of AA from Ca\textsuperscript{2+} ionophore A23187-stimulated rat RBL-2H3 cells with an IC\textsubscript{50} value of 5.5 nmol/l (Fig. 3A). AK106-001616 also suppressed the production of PGE\textsubscript{2} by LPS-stimulated human PBMCs, with an IC\textsubscript{50} value of 5.1 nmol/l (Fig. 3B), and the production of LTB\textsubscript{4} by Ca\textsuperscript{2+} ionophore A23187-stimulated RBL-2H3 cells, with an IC\textsubscript{50} value of 2.6 nmol/l (Fig. 3C). These IC\textsubscript{50} values are consistent with the value of cPLA2 enzyme inhibition reported previously.

In Vivo Air Pouch Model. Suppression of PGE\textsubscript{2} and LTB\textsubscript{4} production was also observed in an in vivo animal model. Zymosan-induced production of PGE\textsubscript{2} and LTB\textsubscript{4} in the air pouch on the back of the rat was reduced when AK106-001616 was administered orally, compared with vehicle treatment (P < 0.005 at 1 or 0.3 mg/kg or more, respectively, by Williams test). The suppressive effects of AK106-001616 on the production of PGE\textsubscript{2} and LTB\textsubscript{4} in this animal study were found to be dose dependent (Fig. 4), and ED\textsubscript{50} values of 1.28 and 0.34 mg/kg, respectively, were observed. In this model, oral administration of celecoxib 1 mg/kg decreased PGE\textsubscript{2} production (P < 0.01 by t test) but increased LTB\textsubscript{4} production (P < 0.01 by t test) compared with the vehicle group (Fig. 4). Zileuton, a 5-LOX inhibitor, suppressed the production of LTB\textsubscript{4} (P < 0.01 by t test) without altering the production of PGE\textsubscript{2} (P > 0.05 by t test) (Fig. 4).

Effect on Arthritis Models. Contralateral paw swelling developed over time in a rat AIA model (data not shown). Repeated administration of AK106-001616 or naproxen starting at day 13 showed dose-dependent inhibition of contralateral paw swelling in the AIA model (P < 0.005 at 1 or 0.3 mg/kg or more, respectively, by Williams test) (Fig. 5A). Zileuton did not inhibit paw swelling even when 30 mg/kg was administered in the rat AIA model (P > 0.05 vs. vehicle group by t test) (Fig. 5A). In another experiment of the rat AIA model, the urinary LTE4 of the zileuton group (1.4 ± 0.1 pg/μg creatinine, n = 5) at days 17 to 18 was significantly lower than that of the vehicle group (4.5 ± 0.2 pg/μg creatinine, n = 5; P < 0.01 by t test), but there was no difference in the PGEM level between the zileuton group (4.4 ± 0.3 pg/μg creatinine, n = 5)
and the vehicle group (4.6 ± 0.3 pg/μg creatinine, n = 5; P > 0.05 by t test).

In a mouse CAIA model, AK106-001616 or zileuton significantly suppressed cumulative swelling score for the limbs at a dose of 30 mg/kg compared with the vehicle group (P < 0.01 by Steel test) (Fig. 5B). Meanwhile, in this model, naproxen did not significantly suppress swelling of the limbs compared with vehicle treatment (P > 0.05 by Steel test) (Fig. 5B). In another experiment of the mouse CAIA model, the urinary LTE4 of the zileuton group (1.6 ± 0.3 pg/μg creatinine, n = 4) at days 3 to 4 was significantly lower than that of the vehicle group (6.4 ± 0.3 pg/μg creatinine, n = 4; P < 0.01 by t test), but there was no difference in the PGEM level between the zileuton group (2.7 ± 0.2 pg/μg creatinine, n = 4) and the vehicle group (2.7 ± 0.2 pg/μg creatinine, n = 5; P > 0.05 by t test).

Analgesic Effects in a Rat Carrageenan-Induced Inflammatory Pain Model. As shown in Fig. 6A, carrageenan induced mechanical hyperalgesia, as demonstrated by a decrease in the paw withdrawal threshold. AK106-001616 administered on a single occasion dose dependently reversed the decrease in the paw withdrawal threshold in carrageenan-induced hyperalgesia compared with vehicle treatment (P < 0.005 at 3 and 30 mg/kg by the Williams test) (Fig. 6A). Naproxen also improved the paw threshold reduction in a dose-dependent manner compared with vehicle treatment (P < 0.005 at 3 and 30 mg/kg by the Williams test) (Fig. 6B). Celecoxib reversed the decrease in threshold induced by carrageenan at a dose of 3 mg/kg (P < 0.01 by t test compared with the vehicle group) (Fig. 6A).

Effects on Bleomycin-Induced Lung Fibrosis in Mice. Intratracheal injection of bleomycin induced a significant increase in the Ashcroft score of lung fibrosis in all mice (P < 0.01 by the Wilcoxon test compared with the control group) (Fig. 8). Nintedanib significantly reduced the Ashcroft score compared with the vehicle group (P < 0.01 by the Wilcoxon test) (Fig. 8). AK106-001616 at 30 mg/kg significantly reduced the Ashcroft score in mice (P < 0.05 by the Steel test compared with the vehicle group), but dose dependency was not confirmed (Fig. 8).

Effects of Single Dosing on Aspirin-Induced Gastric Damage in Rats. Aspirin alone produced marked macroscopic gastric damage in all 12 individuals (Fig. 9). Coadministration of celecoxib and aspirin produced a significant increase

Fig. 6. (A and B) Analgesic effects in a rat carrageenan-induced inflammatory pain model. Compounds were administered orally at the indicated dose 1 hour before carrageenan injection into a hind paw. Paw withdrawal thresholds were measured before dosing and 4 hours after carrageenan injection using the Randall-Selitto method. Each value represents the mean ± S.E.M. of 10 animals. Significant differences: **P < 0.005 by the Williams test compared with the vehicle group; $$P < 0.01 by the t test compared with the prevalues in the vehicle group; ##P < 0.01 by the t test compared with the vehicle group.
in the mean lesion score compared with aspirin alone ($P < 0.01$ by $t$ test) (Fig. 9). Coadministration of AK106-001616 and aspirin did not produce a significant increase in the mean lesion score compared with aspirin alone ($P > 0.05$ by the Dunnett test) (Fig. 9).

**Effects of Repeated Dosing on Blood Pressure in Rats.** The systolic blood pressure of the vehicle-treated group increased with time (Fig. 10A). Repeated administration of rofecoxib produced a significant increase in systolic blood pressure on day 28 of dosing compared with the vehicle control group ($P < 0.01$ by the $t$ test) (Fig. 10A). Repeated administration of AK106-001616 did not produce a significant increase in systolic blood pressure ($P > 0.05$ by the Dunnett test compared with the vehicle group) (Fig. 10A).

**Effects on LPS-Induced Production of TXA2 and PGI2 in the Rat.** Rofecoxib suppressed the production of 6-keto PGF$_{1\alpha}$ dose dependently, with significant suppression at 3 and 30 mg/kg ($P < 0.005$ by the Williams test compared with the vehicle group) (Fig. 10B). On the other hand, rofecoxib did not produce any significant change in the plasma level of TXB$_2$ ($P > 0.05$ by the Dunnett test compared with the vehicle group) (Fig. 10C). The ratios of the TXB$_2$ level to the 6-keto
PGF₁α level were significantly higher in the rofecoxib groups (3 and 30 mg/kg) than in the vehicle-treated group ($P < 0.01$ by the Dunnett test) (Fig. 10D). AK106-001616 produced a significant decrease at 3 and 30 mg/kg in the levels of both TXB₂ and 6-keto PGF₁α ($P < 0.005$ by the Williams test compared with the vehicle group) (Fig. 10, B and C), but it did not increase the ratio of TXB₂ to 6-keto PGF₁α ($P > 0.05$ by the Dunnett test) (Fig. 10D).

### Discussion

AK106-001616 specifically inhibited cPLA₂, and no difference between humans and rats was observed in its enzyme inhibitory activity. In cell-based assays, AK106-001616 inhibited the secretion of AA and the production of PGs and LTs to the same extent, and its inhibitory concentration was consistent with the enzyme inhibitory activity. AK106-001616 suppressed the production of PGs and LTs to the same extent in the in vivo test. From the results in the rat AIA model, the dose of AK106-001616 was 0.3–30 mg/kg, with associated plasma free concentrations ($C_{max}$) of 79–3040 nmol/l (Table 1). This in vivo concentration range was included in the concentration range specifically inhibiting the activity of cPLA₂ (Fig. 2). In a clinical phase IIa trial multicenter study with a randomized and double-blind design, AK106-001616 (100 mg twice daily over a 28-day treatment period) inhibited the production of PGs and LTs, and was efficacious and well tolerated by patients with RA (Yamanishi et al., 2013). These results confirmed the concept that AK106-001616 reduces PGs and LTs by inhibiting cPLA₂.

cPLA₂ is the first regulatory enzyme that releases AA, which is a precursor for diverse bioactive lipid metabolites that can have opposing effects. Therefore, it is possible that AK106-001616 suppresses the production of not only inflammatory mediators but also anti-inflammatory mediators. In this study, since AK106-001616 eventually suppressed inflammation of...
zileuton decreased urinary LTE4 levels, but did not inhibit paw swelling in the CAIA model. Meanwhile, in a rat AIA model, which is an indicator of systemic production of LTs, and paw inflammatory pain caused by carrageenan, and its action in the present study, zileuton decreased the urinary levels of LTE4, but not after peripheral inflammation produced by complete Freund s adjuvant (Tsuda et al., 2007). It has also been reported that cPLA2 is expressed in the spinal cord, predominantly in dorsal horn neurons and oligodendrocytes (Kim et al., 2008). In addition to peripheral inhibition, the central inhibition by AK106-001616 to decrease inflammatory mediators may contribute to mitigate the neuropathic pain seen in CCI rats.

It is well known that downstream mediators of cPLA2 are involved in the pathology of IPF. LTBs4 (Wilborn et al., 1996), leukotriene C4 (Wilborn et al., 1996), and prostaglandin F2α (Oga et al., 2009) are involved in IPF. In mice overexpressing leukotriene C4, bleomycin-induced pulmonary fibrosis was more severe than in wild-type mice (Hirata et al., 2013). Knockout of the 5-LO gene (Peters-Golden et al., 2002) and pharmacological inhibition of LTs (Failla et al., 2006) alleviate the pathology in the mouse bleomycin model. Prostaglandin F2α is involved in transforming growth factor β–independent pulmonary fibrosis (Oga et al., 2009). Therefore, AK106-001616, as a cPLA2 inhibitor, may inhibit pulmonary fibrosis by inhibiting the production of these mediators. Further examination is necessary to prove this hypothesis.

Hypertension is considered one of the risk factors causing CV events. In clinical trials, taking selective COX-2 inhibitors or nonsteroidal anti-inflammatory analgesics caused hypertension (Justice and Carruthers, 2005). COX-2 inhibition increases the vasoconstrictor action and platelet aggregation of TXA2 by suppressing PGII, which has vasodilating activity.

### TABLE 1
Pharmacokinetic parameters of AK106-001616 based on free plasma concentration after repeated oral administration

| Disease Model | Dose | Cmax | AUC0–24h | n |
|---------------|------|------|----------|---|
| Rat AIAa      | 0.3  | 79 ± 88 | 276 ± 904 | 3 |
|               | 1    | 82 ± 16 | 1010 ± 360 | 3 |
|               | 3    | 301 ± 21 | 3870 ± 490 | 3 |
|               | 10   | 976 ± 117 | 11800 ± 2200 | 3 |
| Mouse CAIAb   | 30   | 3040 ± 660 | 33500 ± 100 | 3 |
| Rat CCIc      | 0.3  | 10 ± 5 | 808 ± 263 | 4 |
|               | 3    | 179 ± 64 | 1000 ± 320 | 4 |
|               | 30   | 1340 ± 260 | 11400 ± 3100 | 4 |
|               | 300  | 3990 ± 1040 | 30800 ± 14500 | 4 |

**AUC0–24h** area under the curve from 0 to 24 hours.

**a** AK106-001616 was administered orally again to the animals for collection of blood samples on day 34, which was the next day of the endpoint for inhibitory effects on swelling.

**b** AK106-001616 was still administered orally to the animals twice a day until day 12, and then the compound was administered orally again for collection of blood samples on day 13.

### TABLE 2
Pharmacokinetic parameters after repeated oral administration in the rat AIA and mouse CAIA models

| Drug       | Dose | Cmax     | AUC0–24h  |
|------------|------|----------|----------|
|            |      | (n = 3)  | (n = 3)  |
| Rat AIA    |      | µmol/l   | µmol h/l |
| Mouse CAIA |      | µmol/l   | µmol h/l |
| AK106-001616 | 30   | 217 ± 47 | 213 ± 13 |
|            | 30   | 317 ± 66 | 280 ± 21 |
| Naproxen   |      | 207 ± 24 | 2123 ± 436 |
| Zileuton   |      | 54 ± 2   | 143 ± 29 |
|            | 30   | 143 ± 29 | 2123 ± 436 |

**AUC0–24h** area under the curve from 0 to 24 hours.

**a** AK106-001616 was administered orally again to the animals for collection of blood samples on day 34, which was the next day of the endpoint for inhibitory effects on swelling.

**b** Compounds were administered orally again to the animals twice a day until day 12, and then the compounds were administered orally again for collection of blood samples on day 13.
and platelet aggregation inhibitory actions; as a result, it increases CV risk (Konstantinopoulos and Lehmann, 2005). In the present study, rofecoxib produced a significant increase in systolic blood pressure in WKY/Hos rats, whereas AK10-001616 did not. In addition, blood pressure is normal in cPLA2 knockout mice, and hypertension is not induced even when nitric oxide inhibitor or angiotensin II is administered to cPLA2 knockout mice (Khan et al., 2015). Rofecoxib increased the thromboxane of TXA2 to PGI2, which is thought to be a risk factor in thrombotic CV events (Vainio et al., 2004), whereas AK10-001616 had no effect on this ratio. These results suggest that AK10-001616 does not increase CV risk.

Low-dose aspirin used clinically for CV risk reduction has a risk of causing gastric mucosal disorders. COX-2 inhibitors that do not induce gastric mucosal injury themselves enhance aspirin-induced gastric mucosal injury (Fiorucci et al., 2003). Furthermore, it has been suggested that COX-2 inhibitors increase production of LTB4 by suppressing other substrates of LOX in aspirin-administered animals (Fiorucci et al., 2003). In the present study, celecoxib enhanced aspirin-induced gastric mucosal injury and increased LTB4 production. On the other hand, licoferone, a COX/LOX inhibitor, suppressed LTB4 production and did not exacerbate aspirin-induced gastric mucosal injury (Fiorucci et al., 2003). AK10-001616 also decreased LTB4 and did not exacerbate aspirin-induced gastric mucosal injury. Therefore, the increase in LTB4 may be involved in the exacerbation of aspirin-induced gastric mucosal injury. Since this study is a single dose study, it is necessary to further investigate repeated administration of aspirin and AK10-001616.

The GI risk when AK10-001616 was repeatedly administered to RA patients for 28 days was evaluated in the phase IIa trial. The incidence of GI-related treatment-emergent adverse events by AK10-001616 (100 mg twice daily) tended to be less than that of naproxen. AK10-001616 did not change from baseline in the mean number of mucosal breaks without hemorrhage; however, naproxen significantly increased it. These results suggest that AK10-001616 has a better GI profile compared with naproxen.

In this research, there was the limitation of the repeated dose period. The small intestines of the cPLA2 knockout mice had numerous small ulcers (Takaku et al., 2000). Several patients with inherited cPLA2 deficiency had abnormalities in the GI tract (Leslie, 2015), and catastrophic GI disease was also included (Brooke et al., 2014). Furthermore, long-term inhibition of cPLA2 in chronic dosing may have a serious effect on membrane remodeling. Therefore, in the future, risks due to chronic administration of AK10-001616 should be evaluated and an appropriate administration period should be found.

Preclinical Features of AK10-001616, a cPLA2 Inhibitor

In summary, AK10-001616 is a potent and orally available inhibitor of cPLA2. In rodents, AK10-001616 inhibited inflammation, inflammatory and neuropathic pain, and pulmonary fibrosis. On the other hand, AK10-001616 did not enhance aspirin-induced gastric damage and did not increase blood pressure and the TXA2/PGI2 ratio. The present data demonstrate that AK10-001616 may provide valuable effects in a wide range of indications without the attendant GI and CV risks.

Authorship Contributions

Participated in research design: Shimizu, Nakamura, Ito, Kuriyama.
Conducted experiments: Nakamura, Shimizu, Ito, Sakurada, Tanaka, Komatsu, Takeda, Endo, Saito, Kozaki.
Contributed new reagents or analytic tools: Shoda.
Performed data analysis: Shimizu, Nakamura, Ito.
Wrote or contributed to the writing of the manuscript: Ito, Shimizu.

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TABLE 3

| Concentration Value | Free plasma concentration (nmol/l) | CSF concentration (nmol/l) | Kp (CSF/plasma concentration ratio) |
|---------------------|-----------------------------|-----------------------------|-----------------------------------|
| Free plasma concentration (nmol/l) | 1340 ± 259 | 500 ± 559 | 0.37 |

CSF, cerebrospinal fluid.

In summary, AK10-001616 is a potent and orally available inhibitor of cPLA2. In rodents, AK10-001616 inhibited inflammation, inflammatory and neuropathic pain, and pulmonary fibrosis. On the other hand, AK10-001616 did not enhance aspirin-induced gastric damage and did not increase blood pressure and the TXA2/PGI2 ratio. The present data demonstrate that AK10-001616 may provide valuable effects in a wide range of indications without the attendant GI and CV risks.
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