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AK106-001616, a Potent and Selective Inhibitor of Cytosolic Phospholipase A_2: 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-1*H*-indazol-5-yl)-phenyl]-propionic acid (AK106-001616) is a novel, potent, and selective inhibitor of the cytosolic phospholipase A_2 ($cPLA_2$) enzyme. Unlike traditional nonsteroidal anti-inflammatory drugs and selective cyclooxygenase-2 inhibitors, AK106-001616 reduced prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) production by stimulated cells. The suppression of PGE_2 and LTB_4 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 A_2/ prostaglandin I_2 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.

Introduction

Phospholipase A_2 (PLA_2) enzymes recognize the sn-2 acyl bond of glycerophospholipids and hydrolyze the bond. The cytosolic PLA_2 ($cPLA_2$) enzyme is activated by various inflammatory stimuli and preferentially cleaves arachidonic acid (AA) from membrane phospholipid (Kudo and Murakami, 2002; Ghosh et al., 2006). AA is then converted into prostaglandins (PGs) and thromboxanes by cyclooxygenase (COX) and leukotrienes (LTs) by lipoxygenase (LOX). Consequently, an inhibitor of $cPLA_2$ enzyme would be expected to inhibit the production of both PGs and LTs.

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).

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ABBREVIATIONS: AA, arachidonic acid; AIA, adjuvant-induced arthritis; AK106-001616, 3-[3-amino-4-(indan-2-yloxy)-5-(1-methyl-1*H*-indazol-5-yl)-phenyl]-propionic acid; A23187, 5-(methylamino)-2-[[(2*S*,3*R*,5*R*,8*S*,9*S*)-3,5,9-trimethyl-2-[1-oxo-1-(1*H*-pyrrol-2-yl)propan-2-yl]-1,7-dioxaspiro[5.5]undecan-8-yl]methyl]-1,3-benzoxazole-4-carboxylic acid; CAIA, collagen antibody-induced arthritis; CCI, chronic constriction injury; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; CV, cardiovascular; GI, gastrointestinal; IPF, idiopathic pulmonary fibrosis; $iPLA_{2\beta}$, independent phospholipase $A_{2\beta}$; LOX, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; LTB_4, leukotriene B_4; LTE_4, leukotriene E_4; MC, methyl cellulose; NSAID, nonsteroidal anti-inflammatory drug; OA, osteoarthritis; PAF, platelet-activating factor; PBMC, peripheral blood mononuclear cell; PG, prostaglandin; PGE_2, prostaglandin E_2; PGEM, prostaglandin E metabolite; $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$; PGI_2, prostaglandin I_2; PLA_2, phospholipase A_2; RA, rheumatoid arthritis; RBL-2H3, rat basophilic leukemia 2H3; SD, Sprague-Dawley; $sPLA_2$, secretory phospholipase A_2; tNSAID, traditional nonsteroidal anti-inflammatory drug; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2.

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 $cPLA_2$-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 (Laufer, 2003), $cPLA_2$, which controls these mediators, could be a potent therapeutic target in RA and OA. The importance of $cPLA_2$ activity in the pathology of arthritis has been suggested in animal studies using $cPLA_2$-deficient mice (Hegen et al., 2003), antisense oligonucleotides against $cPLA_2$ (Raichel et al., 2008) and a specific inhibitor of $cPLA_2$ (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 $cPLA_2$ 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, $cPLA_2$ 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 $cPLA_2$-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-1*H*-indazol-5-yl)-phenyl]-propionic acid (AK106-001616) (Fig. 1), a novel selective $cPLA_2$ inhibitor. The preclinical pharmacology of AK106-001616 is described subsequently.

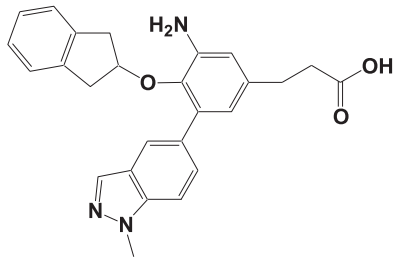

Fig. 1. Chemical structure of AK106-001616.

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 $cPLA_{2\alpha}$. With reference to the previous report (Sharp et al., 1991), the gene of human $cPLA_{2\alpha}$ 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 $cPLA_{2\alpha}$ 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 $cPLA_{2\alpha}$ 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 (FK-101; Funakoshi, Tokyo, Japan).

Rat $cPLA_{2\alpha}$. With reference to the previous report (Owada et al., 1994), the gene of rat $cPLA_{2\alpha}$ 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 $cPLA_{2\alpha}$ was measured with reference to previous reports (Tojo et al., 1993; Leslie and Gelb, 2004). The AA concentration produced by rat $cPLA_{2\alpha}$ was quantified in the same way as for human $cPLA_{2\alpha}$.

Human Independent $PLA_{2\beta}$. With reference to the previous report (Larsson et al., 1998), the gene of human Ca^{2+} independent $PLA_{2\beta}$ ($iPLA_{2\beta}$) 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 iPLA$_{2\beta}$ 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 iPLA$_{2\beta}$ 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 PLA$_2$ Type IB. Bovine secretory PLA$_2$ (sPLA$_2$) type IB was a commercially available product (Sigma-Aldrich). PLA$_2$ activity of bovine sPLA$_2$ IB was measured using the sPLA$_2$ Assay Kit (Cayman Chemical).

Platelet-Activating Factor/Acetylhydrolase. Platelet-activating factor (PAF)–inactivating enzyme PAF-acetylhydrolase (also known as type VII PLA$_2$) 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 Ca^{2+} ionophore 5-(methylamino)-2-[[(2*S*,3*R*,5*R*,8*S*,9*S*)-3,5,9-trimethyl-2-[1-oxo-1-(1*H*-pyrrol-2-yl)propan-2-yl]-1,7-dioxaspiro[5.5]undecan-8-yl]methyl]-1,3-benzoxazole-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 to examine its inhibitory effect on AA release.

Leukotriene B$_4$ Production by RBL-2H3 Cells. The production of leukotriene B$_4$ (LTB$_4$) by RBL-2H3 cells was measured as previously described with slight modifications (Hagmann, 1994; 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 LTB$_4$ in the culture supernatant was measured using the Leukotriene B$_4$ EIA Kit (Cayman Chemical).

Prostaglandin E$_2$ Production by Human Peripheral Blood Mononuclear Cells. Prostaglandin E$_2$ (PGE$_2$) 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 PGE$_2$ produced in the culture supernatant was measured using the Prostaglandin E$_2$ 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 zymosan (Sigma-Aldrich) was injected into the air pouch. The dose of celecoxib was 1 mg/kg, which sufficiently suppressed the production of PGE$_2$ in the preliminary test. The dose of zileuton was 5 mg/kg, which sufficiently suppressed the production of LTB$_4$ in the preliminary test. The exudate in the air pouch was collected 3 hours after zymosan injection. PGE$_2$ and LTB$_4$ concentrations in the exudate were measured using the Prostaglandin E$_2$ EIA Kit-Monoclonal and Leukotriene B$_4$ 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 LTB$_4$ 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 E$_4$ (LTE$_4$), which is an indicator of systemic production of LT concentration, was measured using the Leukotriene E$_4$ EIA Kit (Cayman Chemical). Urinary PG E metabolite (PGEM), which is an indicator of systemic production of PG 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 (Hutamekalin 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 LTE$_4$ concentration from day 3 to 4 and PGEM concentration from day 8 to 9 were measured using the Leukotriene E$_4$ 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

placed on the sciatic nerve with 4-0 suture silk thread (Nicchо Industry Co. Ltd., Tokyo, Japan) under isoflurane anesthesia on day 0. In sham-operated rats, the sciatic nerve was exposed without ligation. Drug or 1% w/v aqueous MC (vehicle) was administered orally once daily for 7 days from day 8. The doses of celecoxib and naproxen were selected within the range that efficacy was observed in the carrageenan model and tolerated by repeated administration. Measurement of the paw withdrawal threshold was performed in the blinded manner previously described with an analgesy-meter 2 hours after dosing (day 14).

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 the previous study (Höcherl et al., 2002). For rofecoxib, a dose was selected in which decrease of blood pressure was observed in the preliminary test. Drug or 1% w/v aqueous MC (vehicle) was repeatedly administered orally once a day for 28 days to male WKY/Hos rats (10 rats/group) by gavage to assess the effects on systolic blood pressure measured under conscious conditions using a noninvasive blood pressure monitor (model MK-2000; Muromachi Kikai Co. Ltd., Tokyo, Japan). Measurement of blood pressure was carried out in the blinded manner described previously.

Effects on LPS-Induced Production of Thromboxane A_2 and Prostaglandin I_2 in the Rat. The concentrations of plasma prostaglandin I_2 (PGI_2) and thromboxane A_2 (TXA_2) were assessed as previously described with slight modifications (Baba, 1988). The dose range of rofecoxib was defined as the range including the dose that lowered blood pressure. Drug or 1% w/v aqueous MC (vehicle) was orally administered to female SD rats (six rats per group) 1 hour before intravenous LPS injection (B5; Sigma-Aldrich) to assess the effects on the systemic production of TXA_2 and PGI_2. Plasma levels of thromboxane B_2 (TXB_2), a stable metabolite of TXA_2, and 6-keto prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), a stable metabolite of PGI_2, were measured as indicators of TXA_2 and PGI_2, respectively, 1 hour after LPS injection using the TXB_2 EIA Kit (Cayman Chemical) and the 6-k $PGF_{1\alpha}$ EIA Kit (Cayman Chemical).

Plasma and Cerebrospinal Fluid Concentration Levels

AK106-001616 or other compounds were administered orally to the AIA model rat on day 34 after measurement of the swelling volume of the foot pad was completed. After an additional dosing of AK106-001616 or other compounds, plasma samples were collected predosing and at the following time intervals: 0.5, 1, 2, 4, 8, 12, and 24 hours postdose.

AK106-001616 or other compounds were administered orally to the CAIA model mouse on day 13. Compounds were still administered orally to the CAIA model mouse twice a day until day 12 after evaluation of the degree of swelling of the limbs was completed, and then compounds were administered again for collection of blood samples on day 13. After an additional dosing of AK106-001616 or other compounds, plasma samples were collected predosing and at the following time intervals: 0.5, 1, 2, 4, 6, 8, and 24 hours postdose.

AK106-001616 was administered orally to the CCI model rat on day 15 after measurement of paw withdrawal threshold was completed. After an additional dosing of AK106-001616, plasma samples were collected predosing and at the following time intervals: 1, 2, 4, 6, 8, and 24 hours postdose.

AK106-001616 concentrations were determined using liquid chromatography–tandem mass spectrometry analysis. Free plasma concentrations of AK106-001616 were calculated using the unbound ratio in rat or mouse plasma, 1.4% or 1.8%, respectively (unpublished in-house data). Pharmacokinetic parameters were calculated by non-compartmental analysis using Phoenix WinNonlin (Certara).

Statistical Analysis

All in vivo tests were performed prospective and confirmatory with reference to the method based on the interim analysis of the clinical trial (Maïofiss-Dullin et al., 2007). Therefore, based on the results of the preliminary tests, the criteria for establishing the test, the endpoint of

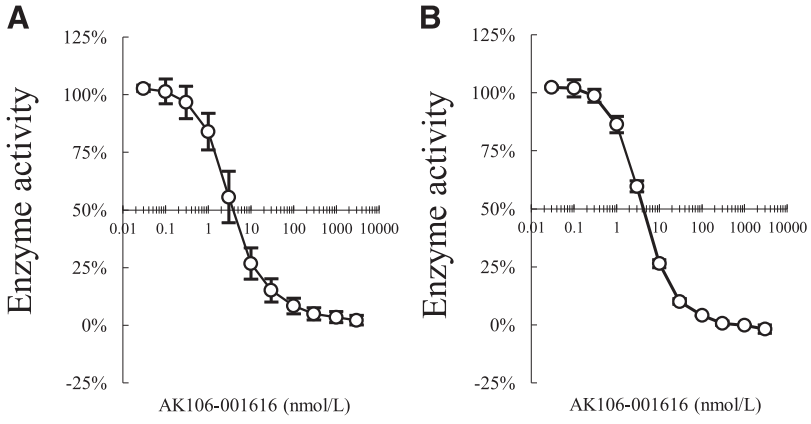

Fig. 2. Inhibitory effects of AK106-001616 on the enzyme activity of human (A) and rat (B) recombinant $cPLA_{2\alpha}$. Data are presented as mean ± S.D. (n = 3). The AA concentrations in the absence of AK106-001616, 10.2 μmol/l (A) and 8.1 μmol/l (B) for human and rat, respectively, were taken as 100% activity.

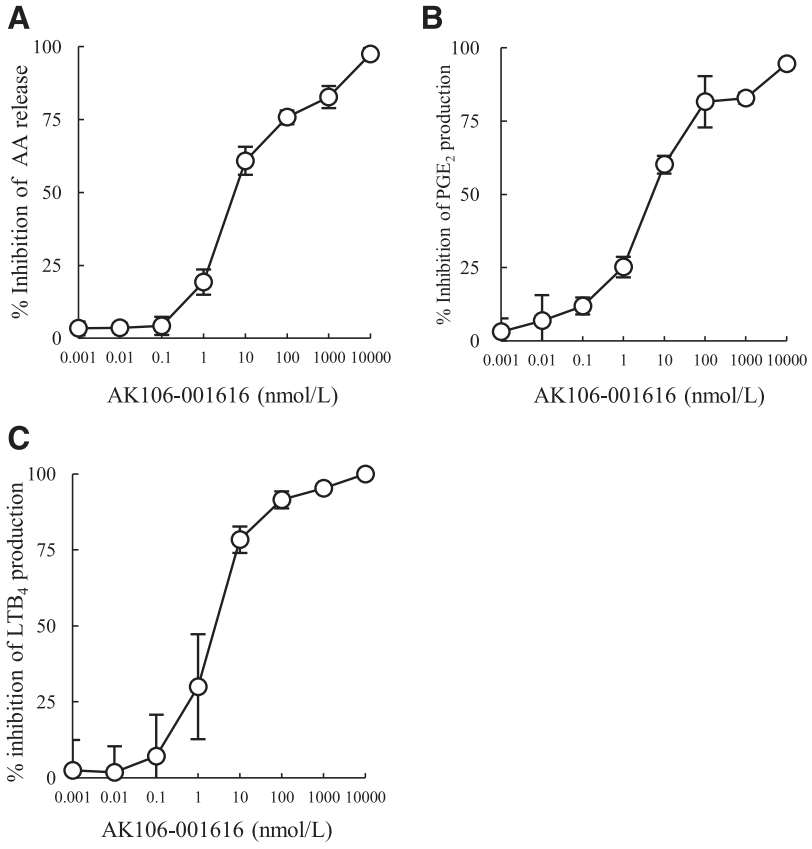

Fig. 3. Inhibitory effects of AK106-001616 on the release of AA, PGE_2, and LTB_4. (A) AA release from Ca^{2+} ionophore A23187-stimulated RBL-2H3 cells. Radioactivity of released tritium labeled AA in the absence of AK106-001616 was 1410 dpm. (B) PGE_2 release from LPS-stimulated human PBMCs. Released 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.

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).

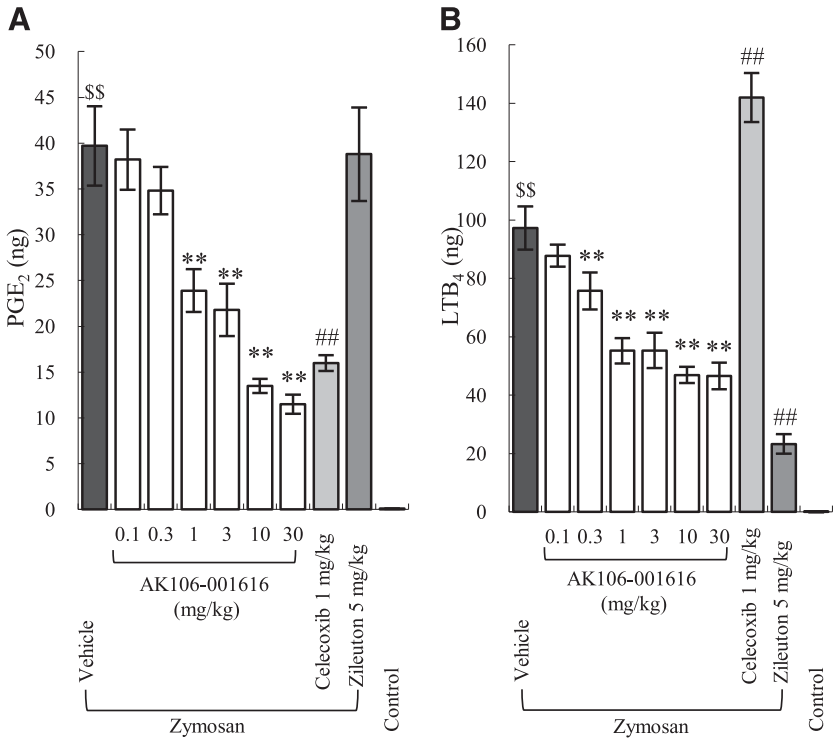

Fig. 4. Effects on PGE_2 (A) and LTB_4 (B) production in an in vivo model. Compounds were administered orally at the indicated dose 1 hour before zymosan injection in the air pouch on the back of the rat. PGE_2 and LTB_4 production in the air pouch was measured 3 hours after zymosan stimulation. In the control group, only air was injected without zymosan injection. Each value represents the mean ± S.E.M. of 10 animals. Significant differences: $^{**}P < 0.005$ by the Williams test compared with the vehicle group; $^{\$\$}P < 0.01$ by the t test compared with the control group; $^{\#\#}P < 0.01$ by the t test compared with the vehicle 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 cPLA$_2$ enzyme activity in a concentration-dependent manner with an IC$_{50}$ value of 3.8 nmol/l (Fig. 2A). AK106-001616 also inhibited rat cPLA$_2$ with an IC$_{50}$ value of 4.3 nmol/l (Fig. 2B), which is the IC$_{50}$ value almost identical to that of human cPLA$_2$. In contrast, the inhibitory activity of AK106-001616 for human iPLA$_2$ was approximately 15,000 times weaker (IC$_{50}$ = 55.1 μmol/l) than that of cPLA$_2$. The inhibitory activity of AK106-001616 on bovine sPLA$_2$ IB was approximately 26,000 or more times weaker (IC$_{50}$ > 100 μmol/l) than that of human cPLA$_2$. AK106-001616 did not affect the activity of PAF-acetylhydrolase (also known as type VII PLA$_2$) at concentrations of up to 500 μmol/l.

Cellular Studies. AK106-001616 suppressed the release of AA from Ca^{2+} ionophore A23187-stimulated rat RBL-2H3 cells with an IC$_{50}$ value of 5.5 nmol/l (Fig. 3A). AK106-001616 also suppressed the production of PGE$_2$ by LPS-stimulated human PBMCs, with an IC$_{50}$ value of 5.1 nmol/l (Fig. 3B), and the production of LTB$_4$ by Ca^{2+} ionophore A23187-stimulated RBL-2H3 cells, with an IC$_{50}$ value of 2.6 nmol/l (Fig. 3C). These IC$_{50}$ values are consistent with the value of cPLA$_2$ enzyme inhibition reported previously.

In Vivo Air Pouch Model. Suppression of PGE$_2$ and LTB$_4$ production was also observed in an in vivo animal model. Zymosan-induced production of PGE$_2$ and LTB$_4$ 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$_2$ and LTB$_4$ in this animal study were found to be dose dependent (Fig. 4), and ED$_{50}$ values of 1.28 and 0.34 mg/kg, respectively, were observed. In this model, oral administration of celecoxib 1 mg/kg decreased PGE$_2$ production ($P < 0.01$ by t test) but increased LTB$_4$ production ($P < 0.01$ by t test) compared with the vehicle group (Fig. 4). Zileuton, a 5-LOX inhibitor, suppressed the production of LTB$_4$ ($P < 0.01$ by t test) without altering the production of PGE$_2$ ($P > 0.05$ 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 LTE$_4$ 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, $n = 5$)

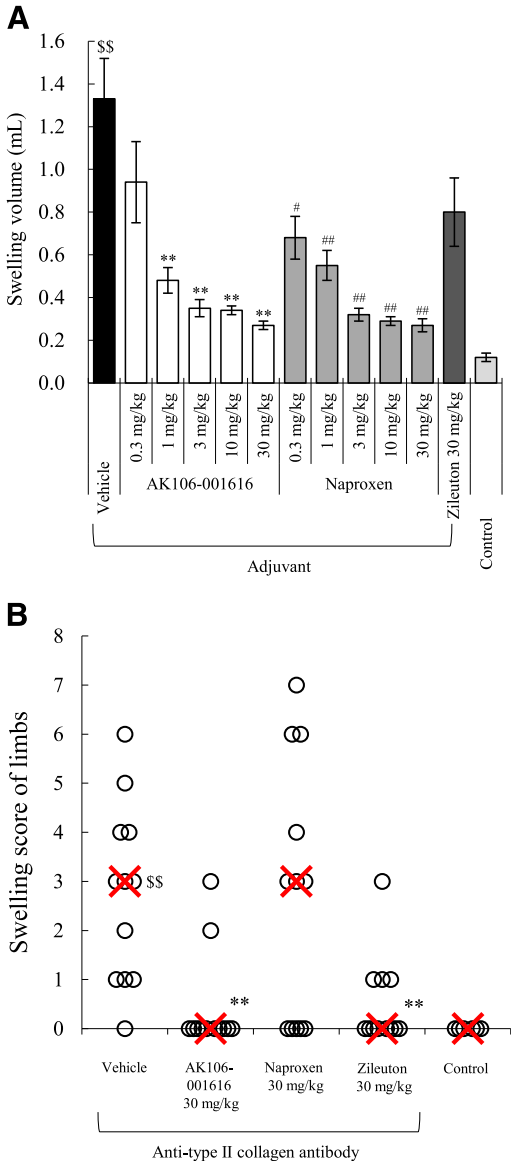

Fig. 5. Inhibitory effects on swelling in the rat AIA (A) and mouse CAIA (B) models. (A) Compounds were administered orally at the indicated dose once a day from day 13 to 34 after adjuvant injection. Swelling volumes of contralateral foot pads were measured at day 34. Each value represents the mean ± S.E.M. of six animals. Control: intact group. Significant differences: $^{**}P < 0.005$ by the Shirley-Williams test compared with the vehicle group; $^{\$\$}P < 0.01$ by the Wilcoxon test compared with the control group; $^{\#\#}P < 0.005$; $^{\#}P < 0.025$ by the Shirley-Williams test compared with the vehicle group. (B) The mouse CAIA model was made by the injection of anti-type II collagen antibody on day 0 and LPS on day 3. Compounds were administered orally at the indicated dose twice a day from day 0 to 10. Cumulative swelling scores of the four limbs were assessed on day 10. Each circle plot and X represents an individual score and median, respectively. Control: intact group. Significant differences: $^{**}P < 0.01$ by the Steel test compared with the vehicle group; $^{\$\$}P < 0.01$ by the Wilcoxon test compared with the control group.

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 LTE_4 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).

Analgesic Effects in a Rat CCI-Induced Neuropathic Pain Model. As shown in Fig. 7, mechanical hyperalgesia, a decrease in the paw withdrawal threshold, developed over time on the ipsilateral hind paw in CCI model rats. The threshold of the vehicle group on day 14 was confirmed to be significantly lower than that of the vehicle group 1 day before CCI surgery ($P < 0.01$ by t test). Repeated administration once daily for 7 days from day 8 of AK106-001616 dose dependently reversed the decrease in the paw withdrawal threshold in CCI rats, and its effect was significant at 3 mg/kg or more on day 14 ($P < 0.005$ by the Williams test compared with the vehicle group) (Fig. 7A). However, repeated administration of effective doses of naproxen or celecoxib in the carrageenan model did not recover the paw withdrawal threshold on day 14 in CCI rats ($P > 0.025$ by the Williams test compared with the vehicle group) (Fig. 7B).

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

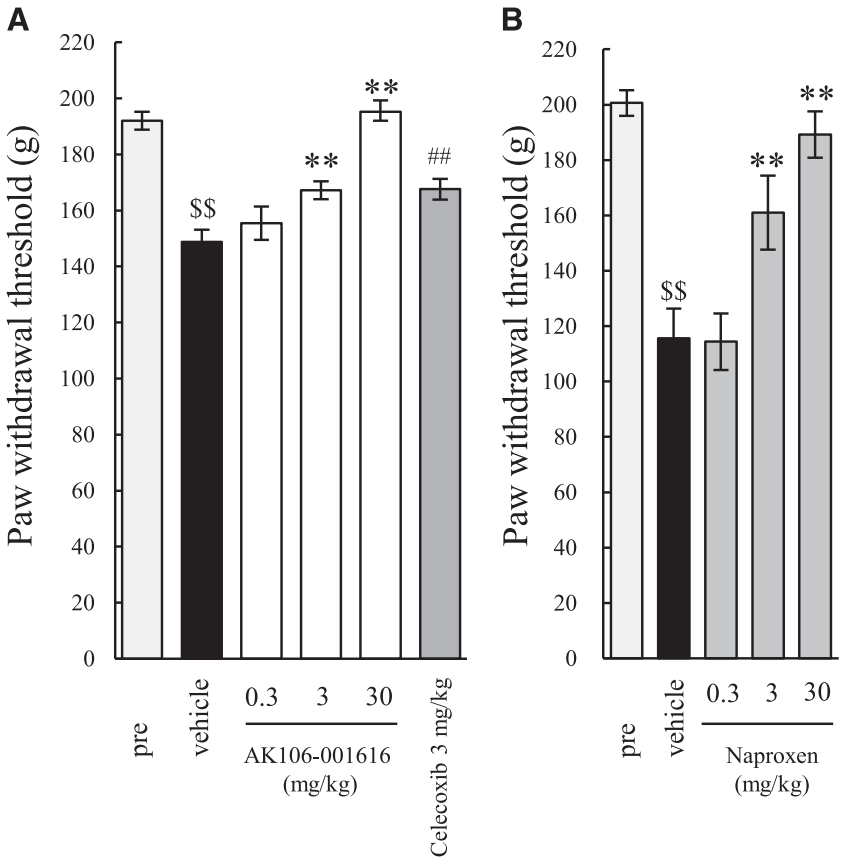

Fig. 6. (A and B) Analgesic effects in a rat carrageenan-induced inflammatory pain model. Compounds were administered orally at the indicated dose 1 hour before carrageenan injection into a hind paw. Paw withdrawal thresholds were measured before dosing and 4 hours after carrageenan injection using the 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.

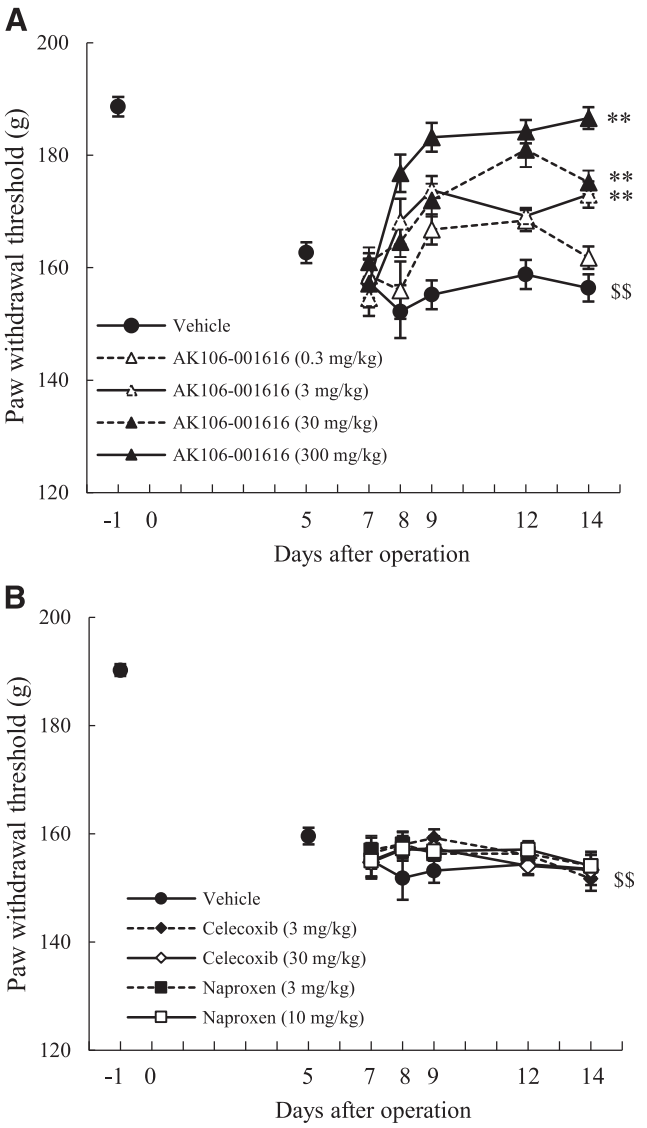

Fig. 7. (A and B) Analgesic effects in a rat neuropathic pain model induced by sciatic nerve CCI. Fifty-six CCI rats were prepared, and 50 animals were selected on day 7 and divided into five groups (n = 10/group). Compounds were administered orally at the indicated dose once a day from day 8 to 14 after surgery. Paw withdrawal thresholds were measured using the Randall-Selitto method. Each value represents the mean ± S.E.M. Statistical testing was performed at day 14. 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. There were no significant differences in the celecoxib and naproxen groups compared with the vehicle group; $P > 0.05$ by the Dunnett test.

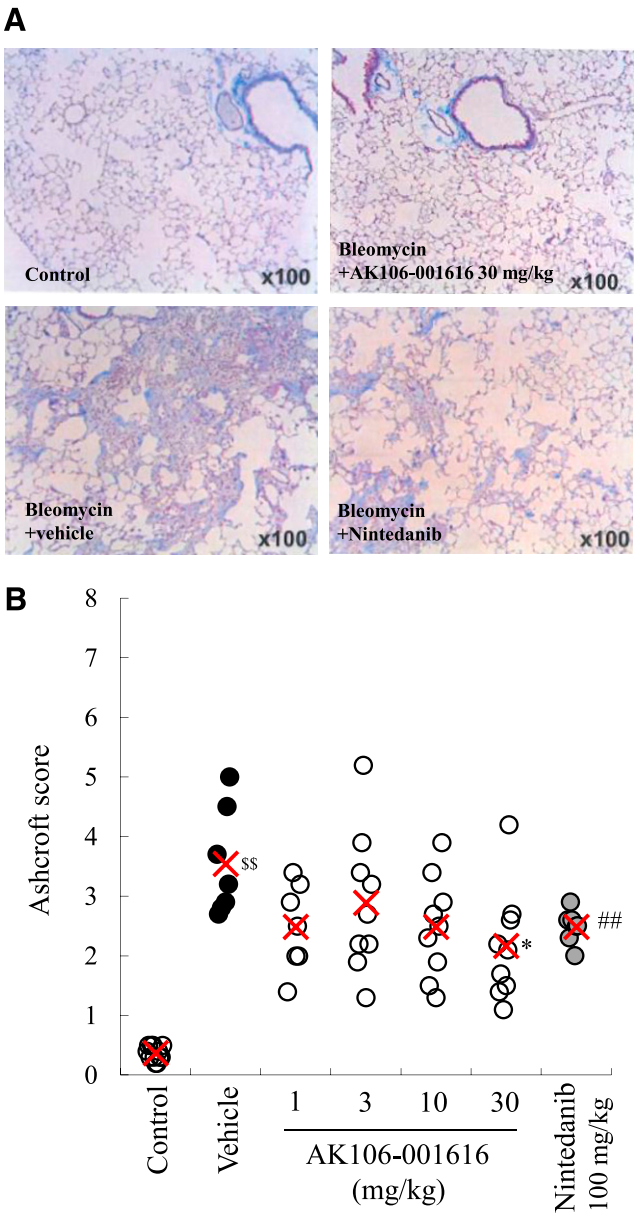

Fig. 8. Effects on bleomycin-induced lung fibrosis in mice. AK106-001616 and nintedanib were administered orally twice and once a day, respectively, from day 7 to 21 after intratracheal bleomycin administration. (A) Representative photomicrographs of Masson's trichrome-stained lung sections. (B) Ashcroft score. Each circle plot and X represents an individual score and median (n = 7–12), respectively. Control: intact group. Significant differences: $^{*}P < 0.05$ by the Steel test compared with the vehicle group; $^{\$\$}P < 0.01$ by the Wilcoxon test compared with the control group; $^{\#\#}P < 0.01$ by the Wilcoxon 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 Dumnett test compared with the vehicle group) (Fig. 10A).

Effects on LPS-Induced Production of TXA_2 and PGI_2 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

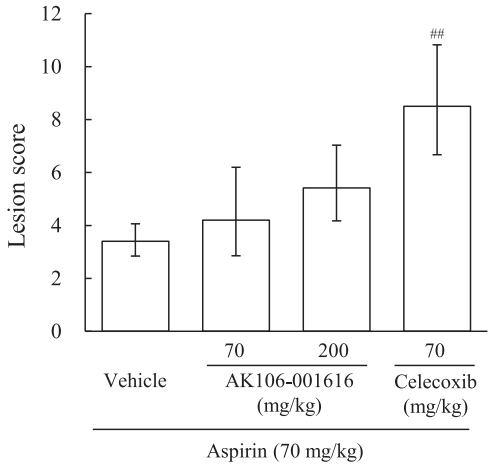

Fig. 9. Effects of single dosing on aspirin-induced gastric damage in rats. A mixture of aspirin and either AK106-001616 or celecoxib was administered as a single oral dose 6 hours before assessment of the gastric lesion score (sum of the lengths of damage of the mucosa). Each value represents the mean ± S.E.M. of 12 animals. Significant differences: $^{\#\#}P < 0.01$ by the t test compared with the vehicle group. There were no significant differences in AK106-001616 groups compared with the vehicle group; $P > 0.05$ by the Dunnett test.

$PGF_{1\alpha}$ 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 TXB_2 and 6-keto $PGF_{1\alpha}$ ($P < 0.005$ by the Williams test compared with the vehicle group) (Fig. 10, B and C), but it did not increase the ratio of TXB_2 to 6-keto $PGF_{1\alpha}$ ($P > 0.05$ by the Dunnett test) (Fig. 10D).

Discussion

AK106-001616 specifically inhibited cPLA$_2$, 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 cPLA$_2$ (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 cPLA$_2$.

cPLA$_2$ 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

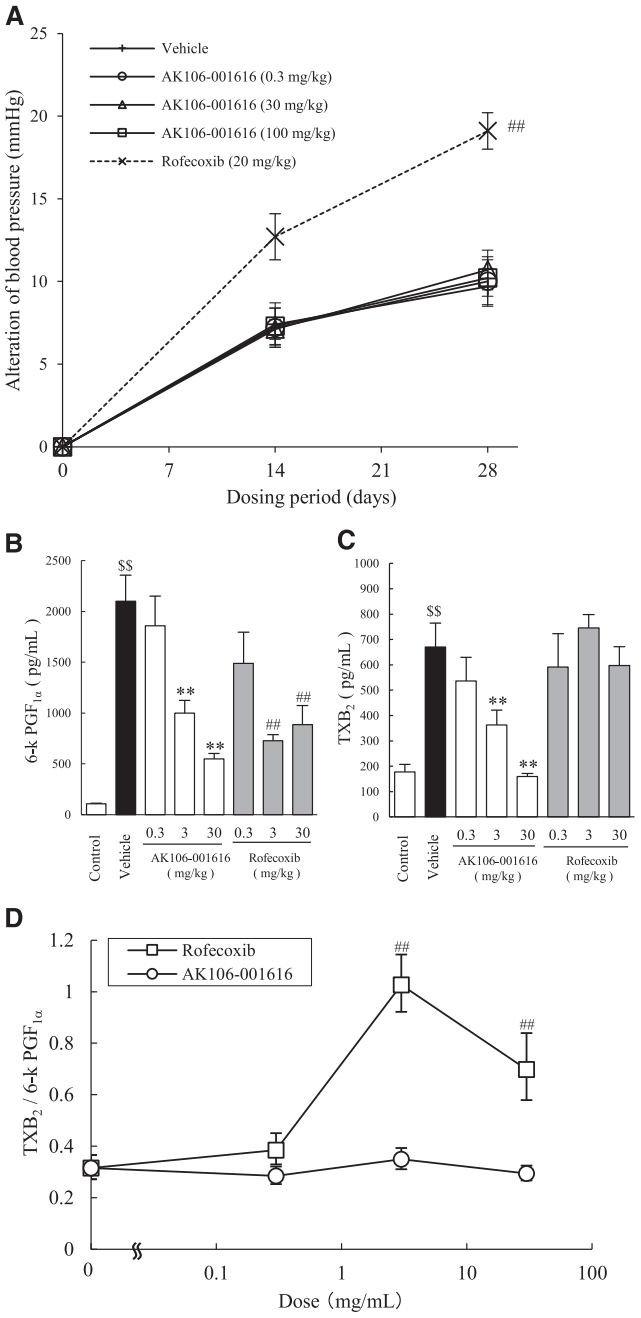

Fig. 10. (A) Effects on systolic blood pressure in rats. Compounds were administered orally at the indicated dose once a day for 28 days. Each value represents the mean ± S.E.M. of 10 animals. Statistical testing was performed at day 28. Significant differences: $^{\#\#}P < 0.01$ by the t test compared with the vehicle group. There were no significant differences in the AK106-001616 groups compared with the vehicle group; $P > 0.05$ by the Dunnett test (B). (C) Inhibition of LPS-induced production of PGI_2 and TXA_2 in rats. Compounds were administered orally at the indicated dose 1 hour before LPS injection. Plasma levels of 6-keto $PGF_{1\alpha}$ (B) and TXB_2 (C), which are stable metabolites of PGI_2 and TXA_2, respectively, were measured 1 hour after LPS injection. In the control group, saline was injected without LPS injection. Each value represents the mean ± S.E.M. of six animals. Significant differences: $^{**}P < 0.005$; $^{\#\#}P < 0.005$ by the Williams test compared with the vehicle group; $^{\$\$}P < 0.01$ by the t test compared with the control group. (D) The ratios of the TXB_2 level to the 6-keto $PGF_{1\alpha}$ level. Each value represents the mean ± S.E.M. of six animals. Significant differences: $^{\#\#}P < 0.01$ by the Dunnett test compared with the vehicle group.

TABLE 1
Pharmacokinetic parameters of AK106-001616 based on free plasma concentration after repeated oral administration
Each value represents the mean ± S.D.

Disease Model	Dose	C_{max}	AUC_{0-24}[a]	n
	mg/kg	*nmol/l*	*nmol·h/l*	
Rat AIA[b]	0.3	79 ± 88	276 ± 904	3
	1	82 ± 16	1010 ± 360	3
	3	301 ± 21	3870 ± 490	3
	10	976 ± 117	11,800 ± 2200	3
	30	3040 ± 660	33,500 ± 100	3
Mouse CAIA[c]	30	3830 ± 230	17,100 ± 1300	3
Rat CCI[d]	0.3	10 ± 5	808 ± 263	4
	3	179 ± 64	1000 ± 320	4
	30	1340 ± 260	11,400 ± 3100	4
	300	3090 ± 1040	30,800 ± 14,500	4

AUC_{0-24}, area under the curve from 0 to 24 hours.
[a]AUC_{0-24} was calculated after repeated administration.
[b]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.
[c]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.
[d]AK106-001616 was administered orally again to the animals for collection of blood samples on day 15, which was the next day of the endpoint for analgesic effects.

arthritis models, it probably strongly suppressed inflammatory mediators rather than anti-inflammatory mediators.

A mouse CAIA model has been used to show that LTs are required in the pathogenesis of arthritis (Mathis et al., 2007). In the present study, zileuton decreased the urinary levels of LTE_4, which is an indicator of systemic production of LTs, and paw swelling in the CAIA model. Meanwhile, in a rat AIA model, zileuton decreased urinary LTE_4 levels, but did not inhibit paw swelling. These results indicate that LTs contribute to the pathogenesis in the CAIA model, but not in the AIA model. AK106-001616, which inhibits productions of PGs and LTs, suppressed paw swelling in both the AIA and the CAIA models. Naproxen, which suppresses PG production by inhibiting COX, is effective in the AIA model but ineffective in the CAIA model. Since there were no differences in the pharmacokinetic profiles of AK106-001616 and naproxen between the rat AIA and mouse CAIA models (Table 2), the change in the effect of naproxen cannot be accounted for by the difference in the area under the curve between the models. This suggests that PGs contribute to the pathogenesis of the AIA model and but not the CAIA model.

Single oral administration of AK106-001616 inhibited inflammatory pain caused by carrageenan, and its action intensity was equivalent to naproxen and celecoxib. In the CCI model, effective doses of naproxen and celecoxib in the carrageenan model were ineffective, while AK106-001616 was effective. Since celecoxib did not suppress neuropathic pain despite suppressing PGs, suppression of mediators other than PGs was thought to be involved in the action of AK106-001616 in the neuropathic pain model. It is known that the mediators downstream from $cPLA_2$, LTs (Noguchi and Okubo, 2011) and PAF (Hasegawa et al., 2010; Okubo et al., 2012; Shindou et al., 2017), are involved in neuropathic pain. This suggests that $cPLA_2$ inhibitors may have a broader analgesic effect than COX-1 and -2 inhibitors.

The free plasma concentration after administration of 30 mg/kg AK106-001616 in the CCI model was 1340 nmol/l at 2 hours (Table 3). The AK106-001616 level in the cerebrospinal fluid was 500 nmol/l at 2 hours (Table 3). These concentrations were sufficient to inhibit $cPLA_2$ (Fig. 2). In the peripheral sensory nerve in vivo, the number of $cPLA_2$-activated neurons was increased after peripheral nerve injury, but not after peripheral inflammation produced by complete Freund's adjuvant (Tsuda et al., 2007). It has also been reported that $cPLA_2$ 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 $cPLA_2$ are involved in the pathology of IPF. LTB_4 (Wilborn et al., 1996), leukotriene C_4 (Wilborn et al., 1996), and prostaglandin $F_{2\alpha}$ (Oga et al., 2009) were elevated in the bronchoalveolar lavage fluid of patients with IPF. In mice overexpressing leukotriene C_4, 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 $F_{2\alpha}$ is involved in transforming growth factor β–independent pulmonary fibrosis (Oga et al., 2009). Therefore, AK106-001616, as a $cPLA_2$ 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 action of TXA_2 by suppressing PGI_2, which has vasodilating

TABLE 2
Pharmacokinetic parameters after repeated oral administration in the rat AIA and mouse CAIA models
Each value represents the mean ± S.D.

Drug	Dose	C_{max}		AUC_{0-24}[a]	
		Rat AIA Model[b] (n = 3)	Mouse CAIA Model[c] (n = 4)	Rat AIA Model[b] (n = 3)	Mouse CAIA Model[c] (n = 4)
	mg/kg	*μmol/l*	*μmol/l*	*μmol·h/l*	*μmol·h/l*
AK106-001616	30	217 ± 47	213 ± 13	2390 ± 10	949 ± 71
Naproxen	30	317 ± 66	280 ± 21	2123 ± 436	766 ± 105
Zileuton	30	54 ± 2	143 ± 29	413 ± 117	281 ± 42

AUC_{0-24}, area under the curve from 0 to 24 hours.
[a]AUC_{0-24} was calculated after repeated administration.
[b]Compounds were 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.
[c]Compounds were still administered orally 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.

TABLE 3

Free plasma and cerebrospinal fluid concentration of AK106-001616 2 h after repeated oral administration at a dose of 30 mg/kg in a rat CCI model

Each value represents the mean ± S.D. (n = 3 to 4).

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

CSF, cerebrospinal fluid.

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 AK106-001616 did not. In addition, blood pressure is normal in cPLA$_2$ knockout mice, and hypertension is not induced even when nitric oxide inhibitor or angiotensin II is administered to cPLA$_2$ knockout mice (Khan et al., 2015). Rofecoxib increased the ratio of TXA$_2$ to PGI$_2$, which is thought to be a risk factor in thrombotic CV events (Vainio et al., 2004), whereas AK106-001616 had no effect on this ratio. These results suggest that AK106-001616 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 LTB$_4$ 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 LTB$_4$ production. On the other hand, licofelone, a COX/LOX inhibitor, suppressed LTB$_4$ production and did not exacerbate aspirin-induced gastric mucosal injury (Fiorucci et al., 2003). AK106-001616 also decreased LTB$_4$ and did not exacerbate aspirin-induced gastric mucosal injury. Therefore, the increase in LTB$_4$ 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 AK106-001616.

The GI risk when AK106-001616 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 AK106-001616 (100 mg twice daily) tended to be less than that of naproxen. AK106-001616 did not change from baseline in the mean number of mucosal breaks without hemorrhage; however, naproxen significantly increased it. These results suggest that AK106-001616 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 cPLA$_2$ knockout mice had numerous small ulcers (Takaku et al., 2000). Several patients with inherited cPLA$_2$ 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 cPLA$_2$ in chronic dosing may have a serious effect on membrane remodeling. Therefore, in the future, risks due to chronic administration of AK106-001616 should be evaluated and an appropriate administration period should be found.

In summary, AK106-001616 is a potent and orally available inhibitor of cPLA$_2$. In rodents, AK106-001616 inhibited inflammation, inflammatory and neuropathic pain, and pulmonary fibrosis. On the other hand, AK106-001616 did not enhance aspirin-induced gastric damage and did not increase blood pressure and the TXA$_2$/PGI$_2$ ratio. The present data demonstrate that AK106-001616 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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