A Novel Inhibitor of I-KappaB Kinase Beta Ameliorates Experimental Arthritis through Downregulation of Proinflammatory Cytokines in Arthritic Joints

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Inhibitor of kappaB (IκB) kinase beta (IKKβ) plays a critical role in nuclear factor-kappaB (NF-κB) activation and production of proinflammatory cytokines in various inflammatory diseases including rheumatoid arthritis. We previously reported a novel IKKβ inhibitor Compound D, 4-[6-(cyclobutylamino)-imidazo[1,2-b]pyridazin-3-yl]-2-fluoro-N-[(2S,4R)-4-fluoropyrrolidin-2-yl]methyl]benzamide, which is efficacious in experimental arthritis models. In the present study, we characterized the pharmacological properties of Compound D and investigated the mechanisms of the anti-arthritic effect. Compound D inhibited IKKβ kinase activity with 160-fold selectivity against IKKα. The cellular analyses revealed that Compound D selectively blocked NF-κB promoter activity among major cellular signaling pathways, such as the activator protein-1 pathway, consistent with inhibition of the NF-κB signaling pathway including phosphorylation of IκBα. In addition, Compound D inhibited NF-κB-driven production of tumor necrosis factor alpha (TNFα) and interleukin-6 comparably. The correlation between inhibitory effect on TNFα production and plasma concentration of the compound was observed in vivo. Consecutive administration of Compound D decreased gene expression of proinflammatory cytokines and inflammatory mediators in the paws of arthritic mice with attenuation of paw swelling. Notably, Compound D was rapidly distributed to the arthritic paws, rather than healthy paws, and where it decreased the gene expression of proinflammatory cytokines by a single oral administration. Furthermore, Compound D completely inhibited arthritis progression even when treatment occurred after disease development. These data suggest that the downregulation of proinflammatory cytokines in local inflamed joints is one of the mechanisms underlying the anti-arthritic effect of the IKKβ inhibitor, Compound D.

Key words inhibitor of kappaB kinase beta; inhibitor; arthritis; nuclear factor-kappaB; tumor necrosis factor alpha

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is characterized by the chronic inflammation of joints, leading to synovial hyperplasia, infiltration of leukocytes, and progressive destruction of cartilage and bone.1) Many studies have indicated that proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin (IL)-1 and IL-6, contribute to the development of RA.2,3) These cytokines induce recruitment, differentiation, and proliferation of inflammatory cells and amplify inflammation by further production of various inflammatory mediators.1,2) Many reports demonstrated that these cytokines were detected at high levels in the synovial fluid and serum of RA patients.4–5) Therefore, proinflammatory cytokines are believed to be promising targets for RA therapeutics. Supporting this, some biologics targeting proinflammatory cytokines have improved the effectiveness of RA therapy.6–7)

Most proinflammatory cytokines associated with RA are regulated by the nuclear factor-kappaB (NF-κB). NF-κB plays an important role in immune response, inflammation, cell differentiation, proliferation and survival.8–10) Dysregulation of NF-κB is observed in various diseases, such as cancer and inflammatory diseases including RA.2,11–13) In resting cells, NF-κB exists as an inactive form through association with inhibitor of kappaB (IκB) in the cytoplasm. In the canonical pathway, a variety of stimuli such as TNFα and lipopolysaccharide (LPS) induces phosphorylation and subsequent polyubiquitination of IκB, leading to degradation through the S26 proteasome pathway. NF-κB released from IκB translocates into the nucleus and initiates the transcription of target genes, including TNFα, IL-1 and IL-6.12–17)

The phosphorylation of IκB is catalyzed by the IκB kinase (IKK) complex, which consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NF-κB essential modulator.17,18) Ample evidence indicates that IKKβ, but not IKKα, is required for NF-κB activation in response to inflammatory stimuli,18–19) suggesting that IKKβ is a key factor in the production of proinflammatory cytokines in inflammatory conditions. Therefore, further understanding the mechanisms of NF-κB regulation by IKKβ is of great interest for RA therapeutics. Several small molecule inhibitors of IKKβ have been identified, and the efficacy of each inhibitor has been clearly demonstrated in arthritis models.20–23) However, studies using diverse chemical classes of IKKβ inhibitors with characteristic mechanisms of action are still required to validate IKKβ as a therapeutic target for RA. In fact, there is no report where IKKβ inhibitors are analyzed from the perspective of the relationship between the tissue distribution of the compounds and the regulation of proinflammatory cytokines in the arthritic joints, which are the primary target tissues of RA.


The authors declare no conflict of interest.
D, 4-[6-(cyclobutylamino)imidazo[1,2-b]pyridazin-3-yl]-2-fluoro-N-[(2S,4R)-4-fluoropyrrolidin-2-yl]methyl]benzamide, which shows potent IKK\(\beta\) inhibitory activity, favorable physiological properties, good pharmacokinetics and efficacy in collagen-induced arthritis (CIA) models.\(^{25}\) In the present study, we characterized the pharmacological properties of Compound D and investigated mechanisms of the anti-arthritis effect.

MATERIALS AND METHODS

**Animals** Male BALB/c mice and female DBA/1J mice were purchased from Charles River (Tokyo, Japan) and Japan SLC (Shizuoka, Japan), respectively. All mice were used at the age of 7–10 weeks. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

**Materials** Compound D was synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). LPS (Escherichia coli O111:B4) and Freund’s complete adjuvant containing Mycobacterium butyricum were purchased from Difco (Detroit, MI, U.S.A.). PathDetect cis-reporter luciferase plasmids for NF-\(\kappa\)B, activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT), serum response element (SRE), cAMP response element (CRE) and interferon-stimulated response element (ISRE), were purchased from Agilent Technologies (Santa Clara, CA, U.S.A.). Fugene HD, ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail, Pefablock, and leupeptin were purchased from Roche (Basel, Switzerland).

**Kinase Assay** Kinase selectivity was assessed using a commercial kinase profiling service (Carna Biosciences, Kobe, Japan). Kinase activities of IKK\(\beta\) and IKK\(\alpha\) were measured by IMAP\(^{\text{TM}}\) assay using ATP at the concentrations around \(K_m\) for each kinase.

**Reporter Assay** Reporter assay was performed as previously reported,\(^{29}\) with some modifications. In brief, 293T cells (ATCC) grown in a 10 cm dish in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (FBS), 50 units/mL of penicillin and 50 \(\mu\)g/mL of streptomycin were transfected with 10 \(\mu\)g of reporter luciferase plasmids using Fugene HD. The following day, transfected cells were harvested and plated at a density of 2\(\times\)10\(^4\) cells/well in a poly-L-lysine 96-well plate (BD Bioscience). The cells were pretreated with various concentrations of Compound D just before the stimulation as follows: 3 ng/mL of TNF\(\alpha\) for NF-\(\kappa\)B, 5 ng/mL of phorbol 12-myristate 13-acetate for AP-1 and SRE, 5 ng/mL of phorbol 12-myristate 13-acetate and 1 \(\mu\)g/mL of of ionomycin for NFAT, 5 \(\mu\)M forskolin for CRE, 4000 units/mL of interferon beta for ISRE. After incubation for 6 h (TNF\(\alpha\)) or 24 h (the others), the cells were lysed with Bright-Glo (Promega) and their luciferase activities were measured using ARVO-MX (PerkinElmer, Inc.). For evaluation of cytotoxicity, untransfected 293T cells were plated at a density of 2\(\times\)10\(^5\) cells/well in a poly-L-lysine 96-well plate. The cells were pretreated with various concentrations of Compound D and incubated for 21 h. The cells were treated with WST-8 (Cell counting kit-8, Dojindo) and incubated for 3 h. The absorbance at 450 nm was measured.

**IkB\(\alpha\) Phosphorylation and Degradation Assays** HeLa cells (ATCC) grown in a 24-well plate in RPMI-1640 medium supplemented with 10% (v/v) FBS, 50 units/mL of penicillin and 50 \(\mu\)g/mL of streptomycin were starved with serum-free medium for 1 d. The cells were pretreated with various concentrations of Compound D for 15 min, followed by stimulation with 10 ng/mL of TNF\(\alpha\) for 10 min (IkB\(\alpha\) phosphorylation) or 15 min (IkB\(\alpha\) degradation). After washing with Hank’s balanced salt solutions, the cells were lysed with 50 \(\mu\)M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4), 150 mM NaCl, 30 mM Na\(_2\)PO\(_4\), 50 mM NaF, 1 mM Na\(_3\)VO\(_4\), 1% (v/v) Triton X-100, and EDTA-free protease inhibitor cocktail by freeze-thawing. The supernatant was obtained by centrifugation (13000 \(\times\) g rpm at 4°C for 5 min) and boiled with Laemmli sample buffer. The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Blots were blocked with 5% (w/v) bovine serum albumin in Tris(hydroxymethyl)aminomethane-buffered saline with 0.1% (v/v) Tween 20 and incubated with polyclonal anti-phosphorylated IkB\(\alpha\) antibody (Cell Signaling) or anti-IkB\(\alpha\) antibody (Upstate), and subsequently incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (GE Healthcare). Enhanced chemiluminescence (GE Healthcare) was used for detection.

**NF-\(\kappa\)B-DNA Binding Assay** NF-\(\kappa\)B-DNA binding was detected by electrophoretic mobility shift assay. HeLa cells grown in a 24-well plate were starved with serum-free medium for 1 d. The cells were pretreated with various concentrations of Compound D for 15 min, followed by stimulation with 10 ng/mL of TNF\(\alpha\) for 30 min. The cells were harvested and washed with Buffer H (20 mM HEPES, pH 7.9, 1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid, 2 mM MgCl\(_2\), 1 mM Na\(_2\)VO\(_4\), 20 mM NaF, 1 mM dithiothreitol, 0.1 mM Pefablock and 10 \(\mu\)g/mL of leupeptin). After removal of the cytosolic fraction by Buffer H containing 0.2% (v/v) Nonidet P-40, a nuclear pellet was vigorously mixed with Buffer H containing 420 \(\mu\)g NaCl and 20% (v/v) glycerol for 2 h. The supernatant was obtained by centrifugation (13000 \(\times\) g at 4°C for 5 min) as nuclear extracts. The nuclear extracts were incubated with 2 units/mL of poly(di-dC) in 10 mM HEPES, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol and 0.1% (v/v) Nonidet P-40 for 30 min, followed by incubation for 30 min with 2 pmol/mL of biotinylated oligonucleotides of consensus DNA-binding sites for NF-\(\kappa\)B as follows: sense, 5'-AGCTCA AACAGGGG GCTTCTCTCCTCTC-3'; antisense, 5'-AGCTGAGGAG GGAAGCCCCGTGTGG-3'. The samples were separated using Tris(hydroxymethyl)aminomethane–borate–EDTA gel electrophoresis and transferred onto nylon membranes, subsequently crosslinked by UV irradiation. The binding of NF-\(\kappa\)B to the oligonucleotides was detected using a Chemiluminescent Nucleic Acid Detection Module (Piece) according to the manufacturer’s instructions.

**LPS-Induced Cytokine Production Assay Using Human Blood Cells** Human blood cells were obtained from the venous blood of healthy adult volunteers in accordance with the ethical approval guidelines of the Daiichi Sankyo Co., Ltd., Ethics Board. The blood was suspended to 2\(\times\)10\(^6\) cells/mL in RPMI-1640 containing 10% FBS, 25 mM HEPES, 50 units/mL of penicillin and 50 \(\mu\)g/mL of streptomycin. The cells were plated in a 96-well plate at a volume of 100 \(\mu\)L/well and pretreated with various concentrations of Compound D just before the stimulation with 1 \(\mu\)g/mL of LPS. After 4 h (TNF\(\alpha\)) or 24 h (IL-6) of incubation at 37°C, the culture supernatants.
were harvested by centrifugation (400 x g at 4°C for 5 min). The concentrations of TNFα and IL-6 in the supernatants were measured using an OptEIA ELISA kit (BD Bioscience) according to the manufacturer’s instructions.

**LPS-Induced TNFα Production Assay in Mice** BALB/c mice were orally given Compound D or vehicle (0.5% methyl cellulose). After 30 min, 0.02 mg/kg of LPS or saline was administered intravenously. After 1 h, blood was collected in the presence of heparin and the plasma was obtained by centrifugation (1000 x g at 4°C for 30 min). The concentration of TNFα in the plasma was measured using a Quantikine ELISA kit (R&D Systems) according to the manufacturer’s instructions. Plasma concentrations of Compound D were determined by the LC/MS/MS method.

**Induction and Assessment of CIA in Mice** DBA/1J mice were immunized by an intradermal injection at the base of the tail with 0.1 mL of emulsion containing 1.5 mg/mL of bovine type II collagen (Collagen Gijutsu-Kenshukai, Tokyo, Japan) in Freund’s complete adjuvant. Twenty-one days later, the mice were secondarily immunized by an intradermal injection at the abdomen with a total of 0.1 mL of the emulsion. Compound D was orally administered once daily beginning with the day of the 2nd immunization for 14 d and all paws of the mice were obtained for measurement of gene expression 24 h after final administration at 14 d after the 2nd immunization. Alternatively, for the evaluation of the efficacy of the compound administered after disease development, Compound D was orally administered once daily from 5 d after the 2nd immunization while the compound weakly inhibited promoter activities of other pathways, showing over 100-fold selectivity compared to the kinase selectivity of Compound D, we determined the IC50 values against IKKβ and related kinase IKKα. Compound D inhibited kinase activities of IKKβ and IKKα with an IC50 of 0.037 and 6.1 µM, respectively, demonstrated 164-fold selectivity (Fig. 1B).

To examine the selectivity of Compound D in cellular signaling pathways, we performed the reporter assays that represent major signaling pathways using 293T cells. Compound D inhibited NF-xB promoter activity with an IC50 of 0.15 µM, while the compound weakly inhibited promoter activities of other pathways, showing over 100-fold selectivity compared to the NF-xB pathway (Table 1). In addition, the compound did not inhibit viability.

### RESULTS

**Compound D Is a Selective Inhibitor of IKKβ** Compound D (Fig. 1A) was identified as a potent small molecule inhibitor of IKKβ from the chemical optimization of a series of imidazo[1,2-b]pyridazine derivatives discovered by high-throughput screening of chemical libraries.24,26,27 To evaluate the kinase selectivity of Compound D, we determined the IC50 values against IKKβ and related kinase IKKα. Compound D inhibited kinase activities of IKKβ and IKKα with an IC50 of 0.037 and 6.1 µM, respectively, demonstrated 164-fold selectivity (Fig. 1B).

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### Table 1. Signaling Pathway Selectivity of Compound D in Reporter Assay

<table>
<thead>
<tr>
<th>Target</th>
<th>IC50 (µM)</th>
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<tr>
<td>NF-xB</td>
<td>0.15</td>
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<tr>
<td>AP-1</td>
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<tr>
<td>NFAT</td>
<td>&gt;20</td>
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<tr>
<td>SRE</td>
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</tr>
<tr>
<td>CRE</td>
<td>&gt;20</td>
</tr>
<tr>
<td>ISRE</td>
<td>16</td>
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<tr>
<td>Viability</td>
<td>&gt;20</td>
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Fig. 1  **Compound D Is a Selective Inhibitor of IKKβ**  
(A) Chemical structure of Compound D. (B) Inhibition of kinase activities of IKKβ and IKKα measured by IMAP™ assay using ATP at the concentrations around Kα for each kinase. The data are expressed as the mean percentage of inhibition over the vehicle control group.
not affect the cell viability within 24 h (Table 1).

**Compound D Inhibits the NF-κB Signaling Pathway**

To confirm whether Compound D inhibits the IKK-mediated signal transduction to NF-κB, we performed IκBα phosphorylation and degradation assays and an NF-κB-DNA binding assay using HeLa cells. Compound D inhibited IκBα phosphorylation and subsequent degradation induced by TNFα in a concentration-dependent manner (Fig. 2A). Additionally, Compound D inhibited NF-κB-DNA binding as well (Fig. 2B). The IC_{50} values against these signals were in the range of 0.1 to 1 µM, which were comparable to that against NF-κB promoter activity.

**Compound D Inhibits NF-κB-Driven Cytokine Production**

LPS is known to induce NF-κB-driven production of proinflammatory cytokines such as TNFα and IL-6. Compound D inhibited LPS-induced TNFα production in mouse blood cells with an IC_{50} of 0.23 µM as described in our previous report. To examine whether Compound D inhibits NF-κB-driven production of proinflammatory cytokines other than TNFα, we performed LPS-induced IL-6 production assay using human blood cells. Compound D inhibited the productions of TNFα and IL-6 comparably with IC_{50} of 0.22 and 0.27 µM, respectively (Fig. 3A). Additionally, Compound D had no species difference between human and mouse in its inhibitory activity on LPS-induced TNFα production.

**The Inhibition of TNFα Production Was Correlated with the Plasma Concentration of Compound D in Vivo**

To examine the relationship between the effect on cytokine production and plasma concentration of Compound D, we performed an LPS-induced TNFα production assay in vivo. The mice were pretreated with Compound D for 30 min, followed by LPS injection, and 1 h later, blood was collected to measure TNFα and Compound D levels. Orally administered Compound D inhibited LPS-induced TNFα production in a dose-dependent manner (Fig. 3B). Compound D inhibited 30% of TNFα production with a dose of 10 mg/kg, and completely inhibited it with a dose of 100 mg/kg. The concentrations of Compound D in the plasma 1.5 h after administration were 0.39, 2.9 and 9.4 µM at doses of 10, 30 and 100 mg/kg, respec-
tively, showing dose-dependent elevation. Thus, the inhibition of TNFα production was correlated with the plasma concentration of Compound D in vivo.

**Compound D Inhibits Expression of Proinflammatory Cytokines in Arthritic Paws by Consecutive Treatment**

Compound D is efficacious in experimental arthritis models as described previously. To investigate mechanisms of the anti-arthritic effect of Compound D, we analyzed the arthritic paws of the compound-administered CIA mice in detail. First, to confirm the efficacy of Compound D, multiple dosages of Compound D or vehicle were administered once daily beginning with the day of the 2nd immunization for 14 d and the severity of arthritis was scored periodically. As shown in Fig. 4A, Compound D significantly decreased the mean arthritis scores in a dose-dependent manner. The repeated administration of the compound did not affect body weight gain (data not shown). Considering the inhibitory effect of LPS-induced TNFα production in vivo by Compound D, it is suggested that one of the mechanisms of its efficacy in CIA is downregulation of proinflammatory cytokines in the arthritic paws. To examine this, we measured the gene expression levels of proinflammatory cytokines and other inflammatory mediators associated with RA in the arthritic paws. The paws of CIA mice were harvested 24 h after final administration at 14 d after the 2nd immunization and gene expressions for proinflammatory cytokines, TNFα, IL-1β and IL-6, and various mediators related to the destruction of bone and cartilage, receptor activator of NF-κB ligand (RANKL), matrix metalloproteinase (MMP)-3 and MMP-9, were measured by quantitative PCR. The arthritic mice administered with only vehicle displayed higher levels of the mRNA expressions of the proinflammatory cytokines and inflammatory mediators compared to disease-free normal mice (Table 2). In contrast, the mRNA expression levels of Compound D-treated mice tended to be low compared to the vehicle-treated mice, although they did not reach statistical significance (Table 2). The expression levels of the genes, except for TNFα, were markedly reduced by Compound D treatment even at 30 mg/kg. These data suggested that the consecutive treatment of Compound D inhibited the gene expression of proinflammatory cytokines and inflammatory mediators in the arthritic paws of CIA mice.

**Compound D Is Distributed to Arthritic Paws and Downregulates Proinflammatory Cytokines** We investigated the relationship between pharmacokinetics and the inhibition of gene expression by Compound D in arthritic mice. Compound D levels in plasma and paws of normal and arthritic mice were measured at the indicated time after oral administration of the compound at a dosage of 30 mg/kg. In the normal mice, Compound D levels in both plasma and paw tissue decreased from 1 h after administration and disappeared at 24 h. In contrast, Compound D in the arthritic paws was detected at a higher level compared to plasma, which remained at more than 1000 pmol/g at 24 h, while the plasma compound level of arthritic mice exhibited only slight elevation compared to normal mice (Fig. 4B). These data indicated that Compound D was distributed to the arthritic paws rather than healthy paws.

We further investigated whether Compound D directly functions in the arthritic paws because the consecutive treatment has a potential to affect various cells and organs. To examine the direct effect of Compound D on gene expression of proinflammatory cytokines in arthritic paws, we measured the mRNA levels of TNFα and IL-1β in the paws of CIA mice 2 h after a single oral administration of the compound. The gene expression levels of TNFα and IL-1β in the arthritic paws were elevated compared to normal mice, and Compound D decreased the gene expression levels of TNFα and IL-1β at a dose of 100 mg/kg (Fig. 4C). The Compound D levels in the paws 2 h after administration at dosages of 30 and 100 mg/kg were 1166±554 and 2472±3594 pmol/g (means±S.D.), respectively, showing rapid distribution of the compound into the arthritic paws in a dose-dependent fashion. These data indicated that Compound D was rapidly distributed to the arthritic paws and where the compound directly downregulated the gene expression of proinflammatory cytokines.

**Compound D Inhibits Arthritis Progression by the Treatment after Disease Development** Finally, to evaluate the efficacy of Compound D administered after disease development, the compound was orally administered once daily from 5 d after the 2nd immunization for 12 d and arthritis scores were periodically measured. As shown in Fig. 4D, Compound D at a dosage of 100 mg/kg completely inhibited the progression of arthritis under the treatment during this experiment.

**DISCUSSION**

We have identified a novel IKKβ inhibitor, Compound D, which has shown potent IKKβ inhibitory activity, favorable physical properties, good pharmacokinetics and efficacy in experimental arthritis models. In the present study, we validated the selectivity of Compound D in the inhibition of IKKβ kinase activity and the blockade of the NF-κB signaling pathway. In addition, we confirmed the concentration-dependent inhibition of NF-κB-driven production of proinflammatory cytokines by Compound D in vitro and in vivo. Furthermore, we demonstrated that Compound D is distributed to arthritic paws rather than healthy paws and where it down-

<table>
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<th>Genes</th>
<th>Normal</th>
<th>Arthritis + Vehicle</th>
<th>Arthritis +30mg/kg Compound D</th>
<th>Arthritis +100mg/kg Compound D</th>
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<td>TNFα</td>
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The experiment was performed as described in the figure legend to Fig. 4A. Gene expressions in the paws of CIA mice 14 d after the 2nd immunization are shown as relative expression values against each glyceraldehyde-3-phosphate dehydrogenase expression level. The data are expressed as means±S.E. of seven mice in each group.
regulates proinflammatory cytokotyes in the articular joints. The results of the in vitro analyses demonstrate that Compound D is a selective inhibitor of IKKβ. Compound D inhibited IKKβ kinase activity with an IC_{50} of 0.037 μM with 160-fold selectivity against related kinase IKKα. Studies using genetically manipulated mice implied that IKKβ, but not IKKα, was a key driver of NF-κB activation\(^1\),\(^2\),\(^3\),\(^4\),\(^5\),\(^6\), therefore, high selectivity for IKKβ over IKKα should be profitable for the downregulation of proinflammatory cytokines in inflammatory conditions where NF-κB is preferentially activated. The NF-κB pathway mediated by IKKβ is activated in response to inflammatory stimuli, such as TNFα and IL-1, leading IκB phosphorylation, subsequent degradation, NF-κB nuclear translocation and its DNA binding.\(^7\),\(^8\),\(^9\) Compound D inhibited TNFα-induced IκBα phosphorylation, degradation and NF-κB-DNA binding in HeLa cells in a concentration-dependent manner with comparable inhibitory potency against NF-κB promoter activity as well as NF-κB signaling in HeLa cells. Consistent with this, Compound D inhibited production of IL-6, one of the proinflammatory cytokines regulated by NF-κB, as potent as TNFα in human blood cells. These findings suggest that the inhibition of the cytokine production is based on the blockade of the NF-κB pathway via IKKβ inhibition. These inhibitory profiles of Compound D should contribute its efficacy in inflammatory conditions such as arthritis where multiple proinflammatory cytokines are simultaneously elevated.\(^7\),\(^8\),\(^9\)

Compound D displayed a unique pharmacokinetic feature manifested as rapid distribution to arthritic paws rather than healthy paws. Previous studies have shown that vascular permeability increased preferentially in the arthritic joints.\(^1\),\(^2\),\(^3\),\(^4\),\(^5\),\(^6\) This enhanced vascular permeability has been associated with mast cell, neutrophil and endothelial cells in response to immune complexes in the joints.\(^1\),\(^2\),\(^3\),\(^4\),\(^5\),\(^6\) These findings suggest that Compound D may preferentially distribute to the arthritic paws by immune complex-triggered alterations in the local joint environment. Notably, Compound D was retained in the
arthritic paws at a higher level even though the compound disappeared from plasma. The retention period of Compound D seems to be much longer in comparison with other small-molecular agents, such as methotrexate.\textsuperscript{32} This finding suggests that the mechanisms of the characteristic tissue localization of Compound D would depend on its chemical property. It is probable that the compound binds to arthritic joint contents such as joint structural components, inflammation-induced proteins and immune complexes.

The consecutive treatment of Compound D inhibited the gene expression of proinflammatory cytokines and inflammatory mediators in the paws of CIA mice, accompanied with the dose-dependent efficacy to the disease, although the compound exhibited weaker inhibition against TNF\( \alpha \) compared to the other genes. On the other hand, Compound D reduced the gene expression of TNF\( \alpha \) as well as IL-1\( \beta \) in the established arthritic paws after 2h of single administration. It is known that TNF\( \alpha \) plays an important role only in the development of CIA in an early stage and its expression is reduced in late stages of the disease.\textsuperscript{3,35} Indeed, the TNF\( \alpha \) mRNA level was low at the time point used in our present study, consistent with previous reports.\textsuperscript{36,37} These observations imply that the elevation of TNF\( \alpha \) expression might be too low to evaluate the inhibitory effect of the compound even though the expression is higher than unimmunized normal mice. Alternatively, it is possible that TNF\( \alpha \) expression was recovered to the vehicle-treated level at 24h after administration due to the decline of the concentration of the compound in the paw even though the expression was inhibited just after administration as observed in the present study; because TNF\( \alpha \) is expressed more rapidly than other cytokines by proinflammatory stimulation in the paws.\textsuperscript{38–41} Nevertheless, the efficacy of Compound D has been observed despite upregulation of the TNF\( \alpha \) expression. In contrast, the other genes may be continuously downregulated at least until 24h after administration and thereby markedly suppressed by the consecutive treatment. Consistent with this, Compound D completely inhibited arthritis progression even when treatment occurred after disease development, suggesting that the downregulation of proinflammatory cytokines in the arthritic joints contributes to the anti-arthritic effect.

Compound D reduced gene expression of inflammatory mediators involved in the destruction of bone and cartilage, such as RANKL, MMP-3 and MMP-9, as well as proinflammatory cytokines in the paws of CIA mice. MMP-3 and MMP-9, which are overexpressed as a result of aspects of joint inflammation such as proliferation of synovial cells and infiltration of inflammatory cells, degrade the extracellular matrix and cartilage.\textsuperscript{42,43} MMPs were detected at high levels in the synovial fluid or serum of RA patients.\textsuperscript{3,44} RANKL is known to contribute to bone erosion and destruction by differentiation and activation of osteoclasts.\textsuperscript{45} Suppression of bone destruction by Compound D was observed in the rat arthritis model, accompanied with delayed onset and relieved symptoms of paw swelling.\textsuperscript{24} Based on these findings, Compound D has a potential to suppress the destruction of bone and cartilage in RA.

CIA is commonly used as an RA model because of its similarities in both etiology and pathology.\textsuperscript{46} In the joint tissue of CIA mice, synovial hyperplasia and infiltration of inflammatory cells have been observed.\textsuperscript{46} These synovial cells secrete proinflammatory cytokines, which amplify inflammation through further generation of various inflammatory mediators. Involvement of proinflammatory cytokines in the pathogenetic process of CIA was evident by the effectiveness of neutralizing antibodies against TNF\( \alpha \), IL-1\( \beta \) and IL-6.\textsuperscript{14,47,48} Additionally, NF-\( \kappa \)B activation has been demonstrated in the synovial tissue of mice in the development of CIA.\textsuperscript{15,22} A previous report demonstrated that the intra-articular gene transfer of wild-type IKK\( \beta \) into the joints of normal rats caused paw swelling and synovial inflammation, while the transfer of a dominant-negative IKK\( \beta \) ameliorated the severity of adjuvant arthritis.\textsuperscript{19} These findings suggest that IKK\( \beta \) plays an important role in synovial inflammation in arthritic joints. The role of IKK\( \beta \) in inflammatory diseases has been demonstrated in multiple disease-relevant cells and animal models using selective IKK\( \beta \) inhibitors.\textsuperscript{20–23,49} However, the mechanisms of the action of IKK\( \beta \) inhibitors are complicated because NF-\( \kappa \)B plays an important role in immune response, inflammation, cell differentiation, proliferation and survival in various cells and tissues.\textsuperscript{8–10} An IKK\( \beta \) inhibitor could induce a variety of functional alterations in various cells through suppression of the NF-\( \kappa \)B signaling as contribution to the efficacy or otherwise side effects, in addition to inhibiting proinflammatory cytokine productions in arthritic joints. In this regard, Compound D may be a useful agent to understand the mechanisms of the anti-arthritic effect of IKK\( \beta \) inhibitors in arthritic joints because Compound D may function in local inflammatory sites due to its preferential distribution in the arthritic paws compared to healthy ones. Supporting this, Compound D did not affect the body weight gain of arthritic mice by consecutive treatment, implying the compound has less systemic toxicity. The inhibitory activity of Compound D against generation of proinflammatory cytokines in the arthritic joint tissue could be led by a blockade of the NF-\( \kappa \)B pathway through IKK\( \beta \) inhibition and break the amplification loop of inflammation in CIA. Taking these findings together, one of the mechanisms underlying the anti-arthritic effect of Compound D should be the direct downregulation of proinflammatory cytokines in the arthritic joints.

In summary, we have characterized a novel small molecule IKK\( \beta \) inhibitor, Compound D, exhibiting selective inhibition of the IKK\( \beta \) kinase activity and NF-\( \kappa \)B signaling pathway. Furthermore, we have demonstrated that Compound D was rapidly distributed to the arthritic paws, rather than healthy paws, and where it directly downregulated the gene expression of proinflammatory cytokines, thereby exhibiting the anti-arthritic effect. These findings suggest that regulation of IKK\( \beta \) in local inflamed joints may be a possible approach for the therapeutics of inflammatory diseases including RA.

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REFERENCES

2) Brown KD, Claudio E, Siebenlist U. The roles of the classical and alternative nuclear factor-kappaB pathways: potential implications


18) Podolini PL, Callahan JF, Bolognese BJ, Li YH, Carlson K, Davis TG, Mellor GW, Evans C, Roshak AK. Attenuation of murine collagen-induced arthritis by a novel, potent, selective small molecule inhibitor of IkappaB kinase 2, TPICa-1 (2-(taminocarboxylaminol)-5-(4-fluorophenyl)-3-thiophenecarboxamide), occurs via reduction of proinflammatory cytokines and antigen-induced T cell prolifera-

19) Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, Johnson TG, Mellor GW, Evans C, Roshak AK. Attenuation of murine col-

20) Cloutier N, Pare A, Fardnale RW, Schumacher HR, Nigrovic PA, Binstadt BA, Patel PR, Alencar H, Nigrovic PA, Lee DM, McNagny KM. Loss of CD34 leads to exacerbated autoim-


30) Blanchet MR, Gold M, Maltby S, Bennett J, Petri B, Kubes P, Lee DM, McNagny KM. Loss of CD34 leads to exacerbated autoim-


34) Williams RO, Feldmann M, Maini RN. Anti-tumor necrosis factor beta inhibitor blocks nuclear factor kappaB-mediated in-


36) Podolini PL, Callahan JF, Bolognese BJ, Li YH, Carlson K, Davis TG, Mellor GW, Evans C, Roshak AK. Attenuation of murine collagen-induced arthritis by a novel, potent, selective small molecule inhibitor of IkappaB kinase 2, TPICa-1 (2-(taminocarboxylaminol)-5-(4-fluorophenyl)-3-thiophenecarboxamide), occurs via reduction of proinflammatory cytokines and antigen-induced T cell prolifera-


