Pharmacological Properties of JTE-952, an Orally Available and Selective Colony Stimulating Factor 1 Receptor Kinase Inhibitor

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Received August 19, 2019; accepted November 7, 2019

Colony stimulating factor 1 (CSF1) receptor (CSF1R) is a receptor protein-tyrosine kinase specifically expressed in monocyte-lineage cells, such as monocytes and macrophages. In this study, we characterized the pharmacological properties of an azetidine compound, JTE-952 ((2S)-3-[[2-[(3-[[4-(4-cyclopropylbenzyloxy)-3-methoxyphenyl]azetidin-1-yl]carbonyl]pyrithidi-4-yilmethoxy]propane-1,2-diol], which is a novel CSF1R tyrosine kinase inhibitor. JTE-952 potently inhibited human CSF1R kinase activity, with a half maximal inhibitory concentration of 11.1 nmol/L, and inhibited the phosphorylation of CSF1R in human macrophages and the CSF1-induced proliferation of human macrophages. It also inhibited human trophomysin-related kinase A activity, but only at concentrations 200-fold higher than that required to inhibit the activity of CSF1R in inducing the proliferation of human macrophages. JTE-952 displayed no marked inhibitory activity against other kinases. JTE-952 potently inhibited lipopolysaccharide-induced proinflammatory cytokine production by human macrophages and in whole blood. JTE-952 (≥3 mg/kg given orally) also significantly attenuated the CSF1-induced priming of lipopolysaccharide-induced tumor necrosis factor-alpha production in mice and arthritis severity in a mouse model of collagen-induced arthritis. Taken together, these results indicate that JTE-952 is an orally available compound with potent and specific inhibitory activity against CSF1R, both in vitro and in vivo. JTE-952 is a potentially clinically useful agent for various human inflammatory diseases, including rheumatoid arthritis.

Key words JTE-952; kinase inhibitor; colony stimulating factor 1 receptor; macrophage colony-stimulating factor; rheumatoid arthritis

INTRODUCTION

Colony stimulating factor 1 (CSF1) receptor (CSF1R) is a receptor protein–tyrosine kinase of the platelet-derived growth factor receptor (PDGFR) family. CSF1, also known as macrophage colony-stimulating factor, binds to its receptor to induce cell signaling.3 CSF1R is specifically expressed in osteoclasts and myelomonocytic-lineage cells, such as monoocytes and macrophages, and the activation of CSF1R signaling promotes the proliferation or differentiation of these cells. It also promotes the production of inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL6).2–4 The phenotype of mice lacking functional CSF1R ligands or CSF1R includes osteopetrosis, reduced numbers of macrophages, and reduced inflammatory cytokine production, which indicate the diverse functions of CSF1R signaling.5–6

The role of CSF1R in rheumatoid arthritis (RA) is evident in the fact that CSF1 levels are higher in the blood and synovial fluid of RA patients than in those of healthy subjects or patients with osteoarthritis.7 Synovial fibroblasts from RA patients also produce high levels of CSF1,8 suggesting that CSF1 signaling is upregulated and the activation of monocyte-lineage cells might be important in the pathogenesis of RA. Exogenous CSF1 exacerbated collagen-induced arthritis (CIA) and inhibition of the CSF1R signal reduced the severity of arthritis in models of CIA, collagen antibody-induced arthritis, and K/BxN-serum-transfer-induced arthritis.9,10 Thus, CSF1R signal inhibitors might be useful in the treatment of RA and other inflammatory diseases in which monocyte-lineage cells and osteoclasts are pathogenic. Recently, IL34 was identified as a second ligand of CSF1R.11,12 IL34 is expressed in the synovial tissues of RA patients,13 but its role in arthritis is unclear. Therefore, the pathophysiological mechanisms causing CSF1R activation have not been fully defined and further studies are required to understand the diverse mechanisms arising from CSF1R activation in inflammatory diseases.

Several small molecules have CSF1R-inhibiting activity in vitro and in vivo. However, at similar concentrations, most also have broad inhibitory activity against other kinases, especially PDGFR kinases, such as stem cell factor receptor (KIT), fms-like tyrosine kinase 3 (FLT3), and PDGFR. Imatinib inhibits CSF1R, ABL oncogene 1 receptor tyrosine kinase (ABL1), KIT, and PDGFR kinases, and inhibited joint inflammation in a mouse model of CIA.14 JNJ-12831214 inhibits CSF1R, KIT, tropomysin-related kinase A (TRKA), and FLT3 kinases and inhibits tumor growth.15 However, the discovery of GW2580 as the first orally active and selective

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CSFIR inhibitor has significantly advanced our understanding of the pharmacology and function of CSFIR. JTE-952 is an orally available and highly selective CSFIR tyrosine kinase inhibitor, both in vitro and in vivo, and the aim of this study was to determine its pharmacological properties, focusing on its effects on RA.

MATERIALS AND METHODS

Compounds JTE-952, (2S)-3-[(2-[3-[4-(4-cyclopropylbenzoyloxy)-3-methoxyphenyl]azetidine-1-yl]carbonyl)pyridin-4-yl]methoxy)propane-1,2-diol (Fig. 1), was chemically synthesized at Central Pharmaceutical Research Institute, Japan Tobacco Inc. (Osaka, Japan). For biochemical and cell-based experiments, JTE-952 (purity 97.5%) and K-252a (Enzo Life Sciences International, Inc., Farmingdale, NY, U.S.A.) were dissolved in dimethyl sulfoxide and diluted in culture medium immediately before use. The final concentration of dimethyl sulfoxide was less than 0.1%. For animal studies, JTE-952 and methotrexate hydrate (MTX; Sigma-Aldrich Co., St. Louis, MO, U.S.A.) were suspended in 0.5% (w/v) aqueous JTE-952 and methotrexate hydrate (MTX; Sigma-Aldrich Co., St. Louis, MO, U.S.A.) were suspended in 0.5% (w/v) aqueous solution of methylcellulose.

Human Kinase Assays To evaluate human CSFIR kinase activity, assays were performed using the homogeneous time-resolved fluorescence (HTRF) technology, as previously described.16 Briefly, a mixtures of various concentrations of JTE-952 and the intracellular domain of human CSFIR (amino acids 538–972, encompassing the tyrosine kinase domain, maintained in-house) were preincubated at room temperature for 5 min in 96-well half-area plates. ATP (Sigma-Aldrich Co.) and poly-(Glu, Tyr)-biotinylated peptide (CIS Bio International S.A., Codolet, France) as its substrate, were added to the mixture, which was incubated at room temperature for 10 min to allow the enzymatic reaction. The composition of the reaction system was: 20 mmol/L 3-(N-morpholino)propanesulfonic acid (pH 7.0), 10 mmol/L MgCl$_2$, 1 mmol/L dithiothreitol, 0.01% bovine serum albumin, 25 ng/mL human CSFIR, 100 µmol/L ATP, and 4 µg/mL poly-(Glu, Tyr)-biotinylated peptide. To stop the enzymatic reaction and allow the phosphorylated substrate to be detected, ethylenediaminetetraacetic acid, an anti-phosphotyrosine monoclonal antibody labeled with europium cryptate (CIS Bio International S.A.), and XL665-conjugated streptavidin (CIS Bio International S.A.) were added, and the mixture incubated at room temperature for 2 h. The intensity of the fluorescence obtained with excitation at 320 nm was measured at 665 and 620 nm with an HTRF microplate reader (model K-101, Kyoritsu Radio Service Co., Ltd., Tokyo, Japan). The HTRF read-out was expressed as the ratio: fluorescence intensity at 665 nm/fluorescence intensity at 620 nm.

Table 1. Effect of JTE-952 on the Activities of Various Kinases in Humans

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<th>Kinase</th>
<th>IC$_{50}$ (nmol/L)</th>
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The evaluation was performed by the Millipore KinaseProfiler™ Assay Service. Results are expressed as the mean IC$_{50}$ value of two experiments. ABL, product of ABL proto-oncogene; Aurora, Aurora kinase; CDK, cyclin-dependent protein kinase; CHK, checkpoint kinase; CK, casein kinase; KIT, product of KIT proto-oncogene; CSK, C-terminal SRC kinase; c-RAF, cellular product of RAF proto-oncogene; c-SRC, cellular product of SRC oncogene; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FLT, fms-like tyrosine kinase; FYN, product of FYN proto-oncogene; GSK, glycogen synthase kinase; IGF1R, insulin-like growth factor-1 receptor; IKK, IKB kinase; IR, insulin receptor; JNK, c-Jun N-terminal kinase; KDR, kinase insert domain receptor; LCK, lymphocyte kinase; Lyn, Lyn/Yes-related tyrosine kinase; MAPK, mitogen-activated protein kinase; MAPKAP-K, MAPK-activated protein kinase; MEK, MAPK/ERK kinase; MET, product of MET proto-oncogene; MSK, mitogen- and stress-activated protein kinase; p70S6K, p70 ribosomal protein S6 kinase; PDGFR, platelet-derived growth factor receptor; PDK, 3-phosphoinositide-dependent protein kinase; PKA, 3′,5′-AMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; PLK, polo-like kinase; ROCK, Rho-dependent protein kinase; SAPK, stress-activated protein kinase; SGK, serum- and glucocorticoid-induced protein kinase; SYK, spleen tyrosine kinase; TIE, tyrosine kinase with Ig- and EGF-homology domains; TRKA, product of TRK gene; ZAP70, 70kDa TCR ε-chain-associated protein.

The effects of 1 µmol/L JTE-952 on 51 human kinases (Table 1) were evaluated by the Millipore KinaseProfiler™ Assay Service (Millipore UK Ltd., Watford, U.K.).

Preparation of Bone-Marrow-Derived Macrophages (BMDMs) Human bone-marrow-derived mononuclear cells (BMMCs) were supplied by Sankō Junyaku Co., Ltd., (Tokyo, Japan). BMMCs from DBA/1J mice and Lewis rats were collected by flushing the lumens of their femurs and tibias with Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) from a syringe inserted into the epiphysis. The cells were suspended in DMEM supplemented with 10% fetal calf serum (FCS) and seeded on a cell culture dish for 2 h at 37°C under 5% CO$_2$ in a water-saturated atmosphere to remove the stromal fibroblasts. The nonadherent BMMCs were cultured in DMEM supplemented with 10% FCS and 100 ng/mL recombinant CSF1 (human

**Western Blotting** Human BMDMs were seeded at a density of 5 × 10⁵ cells/35-mm dish and cultured in DMEM supplemented with 10% FCS and 100 ng/mL recombinant human CSF1 for 24 h. The adherent cells were starved of CSF1 for 24 h to upregulate the expression of CSFIR. Various concentrations of JTE-952 were added to the cells, which were then incubated for 30 min. The cells were then stimulated with 100 ng/mL recombinant human CSF1 at room temperature. After 30 s, the cells were rinsed with ice-cold phosphate-buffered saline and lysed with lysis buffer. The cell lysates were boiled for 5 min at 95°C and stored at −80°C. The proteins in the cell lysates were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were then probed with a specific antibody directed against the CSFIR protein (C-20, Santa Cruz Biotechnology, Inc., TX, U.S.A.), phosphorylated CSFIR protein (14723, Cell Signaling Technology Inc., MA, U.S.A.), or the internal control, α-tubulin (B-7, Santa Cruz Biotechnology, Inc.). The membranes were then probed with a horseradish-peroxidase-coupled secondary antibody (GE Healthcare UK Ltd., Buckinghamshire, U.K.).

The bands of these proteins were visualized with an ECL Plus Western blotting Detection System (GE Healthcare UK Ltd.) and a luminescent image analyzer (LAS-3000, FUJIFILM Co., Tokyo, Japan).

**In Vitro Cell Proliferation Assay** Human BMDMs were seeded at a density of 1 × 10⁴ cells/well in 96-well flat-bottomed plates and cultured in DMEM supplemented with 10% FCS and 100 ng/mL recombinant human CSF1 for 24 h at 37°C under 5% CO₂. The adherent cells were starved of CSF1 for 24 h, and then stimulated with 100 ng/mL recombinant human CSF1 and cultured for 24 h. 5-Bromo-2′-deoxyuridine (BrdU; EMD Chemicals Inc., Gibbstown, NJ, U.S.A.) was added and the cells were incubated further for 6 h. To evaluate the nerve growth factor (NGF)-dependent proliferative responses in TF-1 cells (a human erythroleukemia cell line; American Type Culture Collection, Manassas, VA, U.S.A.), an assay was performed as previously reported. In brief, TF-1 cells were starved of NGF and incubated overnight. The cells (1 × 10⁴ cells/well) were stimulated with NGF (R&D Systems, Inc.) for 42 h, and then with BrdU for 6 h. The BrdU labeling was detected with an enzyme immunoassay and the cells’ proliferative capacity was determined with optical density (OD) measurements (OD₄₅₀ − OD₅₅₀). Various concentrations of JTE-952 or the reference compound K-252a were added 30 min before stimulation with CSF1 or NGF.

**In Vitro Cytokine Production Assays** Human, rat, or mouse BMDMs were seeded in 96-well flat-bottomed plates (human: 1 × 10⁴ cells/well; rat and mouse: 5 × 10⁴ cells/well) and cultured in DMEM supplemented with 10% FCS and 100 ng/mL recombinant CSF1 for 24 h. The adherent cells were starved of CSF1 for 24 h. Various concentrations of JTE-952 were then added to the cells, which were incubated for 30 min. The cells were then stimulated with 100 ng/mL recombinant CSF1. The cells were cultured for 24 h, and then 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich Co.) was added and the cells were incubated for 6 h. The concentrations of TNF-α and IL6 in the culture supernatants were measured with Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc.).

**Cytokine Production Assay in Human Whole Blood** Human peripheral blood was collected from healthy in-house volunteers in tubes containing citric acid and were used for experiments on the same day. After the whole blood (70 μL) was placed in assay tubes, it was incubated with various concentrations of JTE-952 for 30 min, and then 100 ng/mL recombinant human CSF1 was added. After incubation for 24 h at 37°C under 5% CO₂, 500 ng/mL LPS was added as a stimulant, and the blood was incubated for 4 h. The plasma was collected by centrifugation, and the TNF-α and IL6 levels in the plasma were measured with Quantikine ELISA kits (R&D Systems, Inc.). All procedures in this study were approved by the Ethics Committee for the Use of Human-Derived Samples at Japan Tobacco Inc., and according to the Declaration of Helsinki.

**Animals** Male DBA/1J mice and female Lewis rats were supplied by Charles River Laboratories Japan, Inc. (Yokohama, Japan) when 6–7 weeks old. All the animals were housed under specific-pathogen-free conditions at a room temperature of 23 ± 3°C and air humidity of 55 ± 15% under a 12-h light/dark cycle. They were given access to standard laboratory chow diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. All procedures related to the use of animals in this study were reviewed and approved according to the Institutional Animal Care and Use Committee guidelines at Japan Tobacco Inc., and were performed in accordance with standards published by the National Research Council (Guide for the Care and Use of Laboratory Animals, NIH OACU), the National Institutes of Health Policy on Human Care and Use of Laboratory Animals.

**In Vivo TNF-α Production and Pharmacokinetic Study** Recombinant mouse CSF1 was injected intraperitoneally into DBA/1J mice at a dose of 100 μg/kg, except for those in the sham group. After 2 h, LPS was injected intraperitoneally into all the mice at a dose of 5 μg/kg. At 1 h after LPS injection, the peripheral blood was collected under anesthesia. JTE-952 was orally administered at a dose of 1, 3, or 10 mg/kg at 1 h before the CSF1 injection. The plasma samples were collected by centrifugation and the TNF-α levels in the plasma were measured with a Quantikine ELISA kit (R&D Systems, Inc.). For the pharmacokinetic study, JTE-952 was orally administered at a dose of 1, 3, or 10 mg/kg in a volume of 10 mL/kg. Serial blood samples were collected at 0.5, 1, 3, 5, 8, and 24 h after dosing. The blood samples were centrifuged and the plasma was collected. The JTE-952 concentrations in the plasma were analyzed with LC-tandem mass spectrometry.

**Induction of CIA in Mice** CIA was induced in DBA/1J mice aged 10 weeks according to a previously reported method. Type II collagen (Collagen Research Center, Kiyose, Japan) derived from bovine articular cartilage was dissolved to a concentration of 4 mg/mL in 0.01 mol/L acetic acid, and then emulsified with an equal volume of Freund’s complete adjuvant containing 2 mg/mL heat-killed Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI, U.S.A.). Arthritis was induced with an intradermal injection of 100 μL of 2 mg/mL type II collagen emulsion at the base of the tail on days 1 and 22. All groups were immunized, except the normal group. The severity of arthritis was scored for the digits of each limb using a three-point scale ranging from 0 to 2 (score
0, normal; score 1, swelling of one finger; score 2, swelling of two or more fingers) on days 22, 27, 30, 34, and 36. For the carpus and tarsus of each limb, the severity of arthritis was scored on a three-point scale ranging from 0 to 2 (score 0, normal; score 1, moderate; score 2, severe). The total arthritis score for each mouse was expressed as the sum of the scores for the four limbs (maximum possible score: 16). Scoring was performed blind for each group. JTE-952 was orally administered at a dose of 1, 3, or 10 mg/kg once a day for 14 d, from day 22 onwards. Methotrexate (MTX), a disease-modifying antirheumatic drug, was used as the reference compound and orally administered at dose of 3 mg/kg in the same manner as JTE-952. This experiment was repeated three times.

**IC₅₀ Determination**  
IC₅₀ was calculated from the concentration of JTE-952 and the residual activity (%) with a logistic function in the SAS software (SAS Institute Japan Ltd., Tokyo, Japan).

**Statistical Analysis**  
All statistical analyses were performed with SAS System version 8.2 and SAS preclinical package version 5.0 (SAS Institute Japan Ltd.). To evaluate TNF-α production in mice, Welch’s t-test was used to compare the sham and vehicle-treated groups, and the Steel test was used to compare the vehicle- and JTE-952-administered groups. To evaluate the arthritis scores in the mouse CIA model, the following statistical analyses were performed with a closed testing procedure. The Wilcoxon rank-sum test was used to compare the normal and vehicle groups, and the vehicle and MTX groups. The Steel test was used to compare the vehicle- and JTE-952-administered groups. A two-tailed p value < 0.05 was considered statistically significant.

**RESULTS**

**Effects of JTE-952 on Human CSF1R and Other Kinases**  
JTE-952 inhibited human CSF1R tyrosine kinase activity in a concentration-dependent manner, with an IC₅₀ value of 11.1 ± 2.2 nmol/L (Fig. 2A). The effect of JTE-952 on CSF1R phosphorylation in human BMDMs treated with the indicated concentrations of JTE-952, followed by incubation with CSF1. Phosphorylation levels of CSF1R in cell lysates were examined with Western blotting. a, high-molecular-weight CSF1R; b, low-molecular-weight CSF1R.

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To determine the kinase selectivity of JTE-952, we investigated the effects of JTE-952 on 51 types of human kinases other than CSF1R. All of them were selected based on the report of Graczyk. JTE-952 inhibited human TRKA activity with an IC₅₀ value of 261 nmol/L (Table 1). The IC₅₀ values for the other 50 kinases were all greater than 1000 nmol/L. The strongest inhibitory activity of JTE-952 against the 50 kinases was on human KIT (36% inhibition at 1000 nmol/L). To
determine the effect of JTE-952 on TRKA-related cell functions, we examined NGF-dependent proliferation of TF-1 cells (a TRKA-expressing human cell line). JTE-952 suppressed the proliferative capacity of TF-1 cells induced by the addition of NGF, with an IC₅₀ value of 4000 nmol/L (Fig. 3). The reference compound K-252a, a TRK tyrosine kinase inhibitor, had an IC₅₀ value of 4000 nmol/L, similar to the IC₅₀ value of 13 nmol/L in a previous report. 23)

Effects of JTE-952 on Macrophage Functions The effects of JTE-952 on the functions of CSF1-stimulated monocye-lineage cells were examined. The addition of CSF1 increased the proliferative activity of human BMMDMs, which was inhibited by JTE-952 in a concentration-dependent manner. The IC₅₀ value for JTE-952 on the proliferative activity of BMMDMs was 21.7 ± 5.8 nmol/L (Fig. 4A). CSF1 also enhanced the LPS-induced production of TNF-α and IL6 by BMMDMs. The IC₅₀ values for JTE-952 on TNF-α production by human, rat, and mouse BMMDMs were 7.9 ± 3.8, 30.7 ± 5.5, and 10.2 ± 2.3 nmol/L, respectively (Figs. 4B–D, circles). The IC₅₀ values for JTE-952 on IL6 production by human,
JTE-952 inhibited human CSF1R kinase activity with an IC_{50} value of 11.1 nmol/L and attenuated TNF-α production in a dose-dependent manner. Statistically significant differences were observed at doses ≥3 mg/kg, and the percentage inhibition on day 36 was 19.8, 25.9, or 37.1% after a dose of 1, 3, or 10 mg/kg, respectively. The reference compound MTX (3 mg/kg) also significantly suppressed the increase in the arthritis score in a dose-dependent manner. Statistically significant differences were observed at doses ≥3 mg/kg, and the percentage inhibition on day 36 was 19.8, 25.9, or 37.1% after a dose of 1, 3, or 10 mg/kg, respectively. The reference compound MTX (3 mg/kg) also significantly suppressed the increase in the arthritis score.

**DISCUSSION**

In this study, we examined the pharmacological properties of JTE-952, a novel CSF1R tyrosine kinase inhibitor, both in vitro and in vivo. JTE-952 inhibited human CSF1R kinase activity with an IC_{50} value of 11.1 nmol/L and attenuated
CSF1R phosphorylation in human macrophages at concentrations ≥10 nmol/L. On Western blotting, the CSF1R protein was detected as two major bands in our human macrophages. The protein at the lower molecular weight was considered to be an immature glycosylated precursor of the mature-type CSF1R, which was observed at the higher molecular weight (170 kDa). The 170-kDa CSF1R was predominantly detected in CSF1-unstimulated cells, which is thought to indicate that the differentiation of BMMCs progressed during culture and that fully mature adherent macrophages were generated. It has been reported that the mature form of CSF1R is expressed on the cell surface, and the phosphorylated CSF1R detected in our study was considered to be the phosphorylated CSF1R on the cell surface.

In terms of its effects on cellular functions, JTE-952 inhibited the CSF1-induced proliferation of human macrophages, with an IC_{50} value of 21.7 nmol/L. JTE-952 also inhibited the increase in LPS-induced production of TNF-α and IL6 by human, rat, and mouse macrophages, with IC_{50} values of 7.9–31.2 nmol/L, suggesting that there are no species differences in the inhibitory effects of JTE-952. However, the IC_{50} values for JTE-952 on the kinase activity of 50 other kinases, including KIT, FLT3, and PDGFR, were all >1 μmol/L, which is approximately 100-fold higher than that for CSF1R. Although JTE-952 inhibited human TRKA kinase activity at a concentration approximately 20-fold higher than that at which it inhibited CSF1R activity, its IC_{50} value for the NGF-dependent proliferation of TF-1 cells was 4 μmol/L, which is approximately 200-fold higher than that for inhibition of the CSF1-dependent proliferation of human macrophages. The reference compound K-252a, a TRK tyrosine kinase inhibitor, inhibited the NGF-dependent proliferative activity of TF-1 cells (IC_{50} 23 nmol/L). K-252a reportedly inhibited NGF-stimulated NFAT activation with an IC_{50} of 13 nmol/L in a cell line transfected with human TRKA. In the present study, K-252a had an inhibitory effect similar to that previously reported. This suggests that the assay system used in this study was suitable for the detection of the inhibitory effect of JTE-952 on TRKA in a cell system. These results show that JTE-952 has potent CSF1R-inhibiting activity and is a selective CSF1R inhibitor, and does not inhibit other PDGFR family members.

To establish the pharmacodynamics of JTE-952, we examined its effects on the functions of primary human whole blood in vitro. JTE-952 markedly inhibited the LPS-induced increase in TNF-α and IL6 production in all donors examined, with a mean IC_{50} value of 0.6 μmol/L. Consistent with the results of our cell-based study, the oral administration of JTE-952 to mice significantly suppressed the CSF1-induced priming of the LPS-induced increase in TNF-α production in a dose-dependent manner, with an ED_{50} of 1.8 mg/kg. The suppressive effect of JTE-952 on cytokine production in vivo increased as the plasma concentration of JTE-952 increased, suggesting that the effect of JTE-952 correlates with its plasma concentration. In the mouse CIA model, JTE-952 significantly inhibited joint inflammation at doses ≥3 mg/kg. The C_{max} value after a single oral dose of 3 mg/kg JTE-952 was 0.54 μmol/L, and this was consistent with the IC_{50} value (0.6 μmol/L) for primary human whole blood. Therefore, the effective plasma concentration of JTE-952 in RA patients was estimated to be higher than 0.54 μmol/L, based on the results of human whole blood and animal studies. Regarding the difference between the IC_{50} of in vitro CSF1R kinase activity and the effective plasma concentration of JTE-952 in vivo, we found that the effective binding activity of JTE-952, in blood mainly, influences the effective concentration of JTE-952 in vivo. The protein binding activity of JTE-952 was 99.3% in humans and 99.8% in mice, and there was no apparent difference between them.

In RA patients, the plasma concentrations of CSF1 and the monocyte counts in the blood are significantly higher than those in healthy subjects, and monocytes derived from the peripheral blood of RA patients produce higher levels of TNF-α when stimulated with LPS than those from control subjects. Monocytes derived from RA patients also show greater adhesion than normal monocytes. These findings suggest that JTE-952 inhibits the elevated CSF1R signals in inflammatory cells, such as monocytes, that lead to the production of TNF-α and IL6 at inflammatory sites.

The effect of a neutralizing anti-CSF1R antibody in the mouse CIA model has previously been demonstrated. In that report, although a high-dose anti-CSF1R antibody fully inhibited bone destruction, the arthritis score was only partly reduced. The effect of the anti-CSF1R antibody on the arthritis score was thought to reflect the maximum possible efficacy of CSF1R inhibition on joint inflammation in the mouse CIA model. Specifically, the inhibitory effect of small-molecule synthetic inhibitors with poor kinase selectivity is more likely to include effects on off-target-related response in addition to the inhibitory effect by suppressing the intended target. The inhibitory effect of JTE-952 (10 mg/kg) on the arthritis score in our study was also partial, although its efficacy was considered similar to that of the anti-CSF1R antibody. Based on these findings, we consider that JTE-952 effectively suppresses the contribution of CSF1R to joint inflammation in the mouse CIA model. Further studies with other known inhibitors are required to clarify the effectiveness of CSF1R inhibition.

JTE-952 completely inhibited the TNF-α production at a dose of 10 mg/kg in vivo, although the suppressive effect was partial on arthritis score in the mouse CIA model. In addition, agents that neutralize soluble TNF-α such as anti-TNF-α antibody also suppress the clinical score in the mouse CIA model, although the effect of anti-TNF-α antibody is partial, and its efficacy seems to be similar to that of JTE-952. These results suggest that the contribution of TNF-α to joint inflammation in the mouse CIA model is partial and JTE-952 sufficiently suppresses the involvement of TNF-α in the mouse CIA model. As for the arthritis that is not sufficiently suppressed by JTE-952, it is possible that inflammatory cytokines from T-cells (which rarely express CSF1R) may be involved. Indeed, MTX has been reported to inhibit production of cytokines such as interferon-gamma from pathogenic T-cells and to suppress arthritis in the mouse CIA model. In contrast, in clinical settings, anti-TNF-α biological agents such as infliximab have marked anti-inflammatory activity that reduces RA activity, and its efficacy has been reported to be more efficacious and better tolerated than MTX. From these findings, JTE-952, which inhibits completely TNF-α production from monocyte lineage cells, may have a greater anti-inflammatory effect than MTX on human RA pathology where TNF-α is considered to be relatively highly involved. In addition, the dose of MTX used in our mouse CIA model is far more than
the dose calculated from the clinical dose. Thus, we consider that further comparative studies with appropriate dose of reference compounds are needed to reveal the usefulness of JTE-952 in human RA.

GW2580 was previously reported to be a highly selective inhibitor of CSF1R kinase activity, with an IC_{50} of 30nmol/L, and it inhibits cellular functions with an IC_{50} of 100–500nmol/L.\(^{16,17}\) This suggests that JTE-952 has a three-fold more potent effect on the kinase activity of CSF1R and a 30-fold more potent effect on cellular functions than GW2580. Moreover, the effective dose of GW2580 required to inhibit LPS-induced TNF-α production in vivo in mice was >20mg/kg, 10mg/kg in a rat adjuvant-induced arthritis model, and 30mg/kg in a mouse CIA model.\(^{10,16,17}\) In our in vivo study, the ED_{50} value for GW2580 on mouse TNF-α production was 12.9mg/kg (data not shown). These results suggest that the potency of JTE-952 is approximately 5–10-fold greater than that of GW2580 in vivo.

In conclusion, we have demonstrated that JTE-952 has potent CSF1R-inhibiting activity, with great selectivity vis-à-vis other kinases. Furthermore, JTE-952 suppresses CSF1R-related cell functions in monocyte-lineage cells, including in primary human whole blood. Moreover, orally delivered JTE-952 is sufficiently efficacious to abolish the effects of CSF1, even in an inflammatory disease. These results indicate that JTE-952 can be used to clarify the role of CSF1R in vitro and in vivo and is a potentially clinically useful agent for the treatment of a variety of human inflammatory diseases, including RA.

Acknowledgments We thank Noboru Furukawa, Kayoko Takagi, Hiromi Yoshiuchi, Yuichi Naka, Tomokazu Kanehisa, Chika Oki, and Yusuke Kadota for their excellent technical assistance and helpful advice in the preparation of this article.

Conflict of Interest The authors declare no conflict of interest.

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