RESEARCH ARTICLE

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In Vitro and In Vivo Pharmacological Profiles of DS-1211, a Novel Potent, Selective, and Orally Bioavailable Tissue-Nonspecific Alkaline Phosphatase Inhibitor

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ABSTRACT

Inhibition of tissue-nonspecific alkaline phosphatase (TNAP) may prevent ectopic soft tissue calcification by increasing endogenous pyrophosphate (PPi). DS-1211 is a potent and selective novel small molecule TNAP inhibitor with well-characterized pharmacokinetics (PKs) in rodent and monkey. Herein, we report a comprehensive summary of studies establishing the pharmaceutical profile of DS-1211. In vitro studies characterized the mode of inhibition and inhibitory effects of DS-1211 on three human alkaline phosphatase (ALP) isozymes—TNAP, human intestinal ALP, human placental ALP—and on ALP activity across species in mouse, monkey, and human plasma. In vivo PK and pharmacodynamic (PD) effects of a single oral dose of DS-1211 in mice and monkeys were evaluated, including biomarker changes in PPi and pyridoxal 5′-phosphate (PLP). Oral bioavailability (BA) was determined through administration of DS-1211 at a 0.3-mg/kg dose in monkeys. In vitro experiments demonstrated DS-1211 inhibited ALP activity through an uncompetitive mode of action. DS-1211 exhibited TNAP selectivity and potent inhibition of TNAP across species. In vivo studies in mice and monkeys after single oral administration of DS-1211 showed linear PKs, with dose-dependent inhibition of ALP activity and increases in plasma PPi and PLP. Inhibitory effects of DS-1211 were consistent in both mouse and monkey. Mean absolute oral BA was 73.9%. Overall, in vitro and in vivo studies showed DS-1211 is a potent and selective TNAP inhibitor across species. Further in vivo pharmacology studies in ectopic calcification animal models and clinical investigations of DS-1211 in patient populations are warranted. © 2022 Daiichi Sankyo, Inc. *Journal of Bone and Mineral Research* published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: ALKALINE PHOSPHATASE; ECTOPIC CALCIFICATION; PYROPHOSPHATE; TISSUE-NONSPECIFIC ALKALINE PHOSPHATASE INHIBITION; DS-1211

Introduction

The family of alkaline phosphatase (ALP) enzymes controls dephosphorylation and transphosphorylation of numerous substrates in vitro.[1] In humans, three ALP isozymes exhibit predominant expression in specific tissues (intestinal ALP [IAP], placental ALP [PLAP], and germ cell ALP) and one tissue-nonspecific ALP (TNAP) that exhibits wide distribution in bone, liver, and kidney tissues.[1,2] Therapies that change activity of these ALP isozymes may affect distinct disease mechanisms and cellular processes.[3]

One biological function of TNAP is the regulation of extracellular matrix calcification in skeletal and dental tissues.[4] The endogenous substrates of TNAP include pyrophosphate (PPi), pyridoxal 5′-phosphate (PLP; vitamin B6), and phosphoethanolamine (PEA).[5,6] TNAP regulates skeletal and dental mineralization through hydrolysis of extracellular PPi to maintain a balanced inorganic phosphate to PPi ratio.[1,4]

In nonskeletal tissue, TNAP is important in maintaining proper PPi levels to prevent ectopic vascular and soft tissue calcification.[1] PPi, an endogenous mineralization inhibitor, is tightly regulated in a narrow physiological range through the release of its precursors and the subsequent generation of PPi.[7,8] Elevated TNAP expression reduces PPi levels and, consequently, leads to increased soft tissue calcification.[8-10] This PPi metabolic pathway dysfunction is linked to several monogenetic ectopic calcification diseases, such as pseudoxanthoma elasticum (PXE) and generalized arterial calcification of infancy (GACI).[7] Inhibiting

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TNAP to increase PPi levels has emerged as a potential therapeutic strategy to prevent ectopic soft tissue calcification.[11-14]

Pharmacological activities of existing inhibitors of ALP isozymes, like levamisole and theophylline, are described in the literature. However, these compounds are not TNAP-specific and show only weak inhibition on TNAP.[15,16] Nonclinical studies show the prototype TNAP-specific inhibitor, SBI-425, reduces ectopic calcification in animal models of vascular calcification and PXE, further supporting TNAP inhibition as a promising therapeutic mechanism.[1,7,17-19] The TNAP inhibitor reported here, DS-1211, shows characteristics suitable for clinical application and has advanced to studies in humans.[20] DS-1211 is an orally administered, potent, and highly specific small molecule TNAP inhibitor. Two first-in-human, phase 1 studies on single and multiple ascending doses of DS-1211 found healthy subjects appear to tolerate a wide range of doses of DS-1211; pharmacodynamic (PD) data suggest DS-1211 inhibits TNAP activity.[20] There is a need for a comprehensive understanding of the pharmacological properties of DS-1211, such as its mechanism of action in vitro and in vivo, to assess its applicability in clinical practice.

Here we report a comprehensive summary of studies establishing the pharmaceutical profile of DS-1211. In vitro studies determined the inhibition mode and inhibitory effects of DS-1211 on human TNAP, human IAP, human PLAP, and ALP activity in mouse, monkey, and human plasma. Additionally, the in vivo pharmacokinetic (PK) and PD effects of a single oral dose of DS-1211 in mice and monkeys were evaluated, including changes in PPi and PLP.

Materials and Methods

Study design

The pharmaceutical profile of DS-1211 was investigated through five in vitro and four in vivo experiments. In vitro studies characterized the mode of inhibition of DS-1211; evaluated the inhibitory effects of DS-1211 on human TNAP, human IAP, human PLAP; examined the effect of DS-1211 on ALP activity in mouse, monkey, and human plasma to study potency across animal species; analyzed the pharmacological actions of DS-1211 on a total of 88 receptors, channels, transporters, and enzymes; and investigated plasma protein binding of DS-1211 in mice, rats, monkeys, and humans. In vivo studies investigated ALP inhibition and PK in mice with a single dose of DS-1211; analyzed changes in ALP activity, PPi, and PLP in mice after single-dose DS-1211 administration; evaluated the effects of a single oral dose of DS-1211 on ALP inhibition, PK, and PPi in monkeys; and determined the oral bioavailability of DS-1211 in monkeys. Animals were allocated to treatment groups as shown in Supplemental Table S1. Experimental sample sizes were determined from previously conducted in-house data taking into account planned statistical analyses. Investigators were not blinded during allocation, animal handling, and endpoint measurements; however, appropriate records were conducted with respect to actual experimental operations to not include arbitrariness.

Materials

The test substance used in the in vitro and in vivo studies is DS-1211.[21] The molecular formula of the compound is $C_{15}H_{14}FN_3O_5S$, with a molecular weight of 367.35 g/mol (Fig. 1).

TNAP, IAP, and PLAP enzymes were obtained by an in-house recombinant system; mouse, monkey, and human plasma for the in vitro experiments on the inhibitory effects of DS-1211 were purchased. Mouse plasma (Charles River Laboratories Japan, Inc., Yokohama, Kanagawa, Japan) was obtained from the heart of male C57BL/6J mice 8 weeks of age. Cynomolgus monkey plasma and human plasma were obtained from KAC Co., Ltd. (Kyoto, Japan). Monkey plasma samples were collected from several healthy male and female adult cynomolgus monkeys; samples were pooled plasma. Human plasma was acquired from one healthy female adult donor. All assays for enzyme activity and measurement of plasma ALP activity in monkey and human were conducted in 96-well plates (half-well type, code no. 3695, Corning Inc., Corning, NY, USA), and measurement of plasma ALP activity in mice were conducted in 384-well plates (code no. 3702, Corning Inc.).

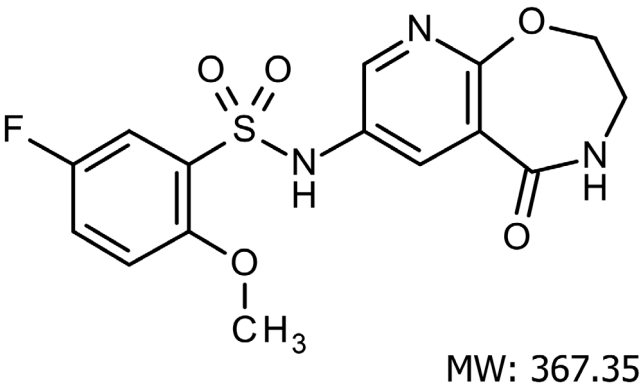

Fig. 1. Chemical structure of DS-1211. MW = molecular weight.

To obtain platelet-free plasma in blood samples for measurement of plasma PPi concentration, cut-off filters (Ultrafree-MC-VV Centrifugal Filters, Merck Millipore, Ltd., Watford, UK; UFC30VV00) were used to remove platelets from plasma immediately after collection of blood.

Reagents used in the studies in this report are as follows: DMSO (Sigma-Aldrich, St. Louis, MO, USA), pNPP (ProteoChem, Hurricane, UT, USA), $MgCl_2$ (Wako Pure Chemical Industries, Osaka, Japan), 0.1-mol/L $ZnCl_2$ solution (Kanto Chemical, Tokyo, Japan), UltraPure 1 M Tris–HCl pH 7.5 (1 mol/L Tris, Thermo Fisher Scientific, Waltham, MA, USA), 1-M HEPES buffer (Thermo Fisher Scientific), adenosine 5′-phosphosulfate sodium salt (Sigma-Aldrich), adenosine triphosphate (ATP) sulfurylase (New England Biolabs Japan, Tokyo, Japan), Otsuka distilled water (Otsuka Pharmaceutical Factory, Naruto, Japan), and CellTiter-Glo (CTG) reagent (CellTiter-Glo Buffer and CellTiter-Glo Substrate, Promega, Madison, WI, USA). All reagents were high-pressure liquid chromatography reagent grade, guaranteed reagent grade, or equivalent grade.

Animals

Four in vivo experiments in mice and monkeys were conducted to further understand the inhibitory effects of DS-1211 and its PK properties. Animal experiments were performed in Japan and were conducted in accordance with guidelines approved by the Institutional Animal Care and Use Committee (Sekisui Medical Co., Ltd., Ibaraki, Japan, and Daiichi Sankyo Co., Ltd., Tokyo, Japan, respectively). All animal studies are reported in

compliance with the ARRIVE 2.0 guidelines for reporting experiments involving animals.[22]

Seven-week-old male C57BL/6J mice (Charles River Laboratories Japan, Inc.) and 4-year-old male cynomolgus monkeys (Shin Nippon Biomedical Laboratories, Ltd., Tokyo, Japan) were used in the in vivo PK and TNAP inhibition studies. Mice were acclimatized for 1 week before testing. The mouse and monkey species were selected for studies investigating inhibitory effects of DS-1211 on TNAP function, and PKs in these species are assumed to be similar to human.[23,24] Oral administration of DS-1211 to animals was selected based on the intended clinical route.

In vitro models for enzyme inhibition and selectivity

To characterize the inhibition mode and determine the ability of DS-1211 to inhibit enzymatic activity in vitro, recombinant enzymes were incubated with the substrate *p*-nitrophenylphosphate (pNPP) in the presence of DS-1211 using methods previously described.[16,25] To study the mechanism of inhibition, Lineweaver-Burk plots were created to show TNAP activity versus substrate concentration in the presence of DS-1211; inhibition parameters were extracted graphically.[16,25]

Preparation of recombinant enzymes and DS-1211 solutions

Human TNAP, IAP, PLAP enzymes were expressed and purified using published methods.[15,26] Expression plasmids containing a secreted epitope-tagged TNAP, IAP, or PLAP were transfected into COS-1 cells for transient expression. After 7 to 8 hours, the medium was replaced with Dulbecco's Modified Eagle Medium (10% fetal calf serum, 1% antibiotics) followed by serum-free OPTI-MEM (1% antibiotics) 15 to 16 hours later. The serum-free medium containing the secreted proteins was collected 96 hours after transfection. The conditioned medium was concentrated through a cellulose filter (Amicon Ultra-15, 10 KDa, Merck Millipore) and dialyzed against 50 mM Tris containing 200 mM sodium chloride, 1 mM magnesium chloride ($MgCl_2$), and 20 μM zinc chloride ($ZnCl_2$). Purification condition was confirmed by western blotting using Flag-tag. Protein concentration was quantified after enzyme purification, and the resulting enzyme was diluted with assay buffer as needed to prepare the enzyme solutions.

DS-1211 was prepared in powder as a free form, sodium salt, and potassium salt, and reconstituted in 100% dimethylsulfoxide (DMSO) to a 10-mmol/L stock solution. Stepwise dilutions of the stock solution with DMSO were performed as needed to achieve solutions with the desired experimental concentrations of DS-1211.

Inhibition mode of DS-1211 on the recombinant enzyme of human TNAP

To characterize the mode of inhibition of DS-1211, ALP activity was measured with various concentrations of DS-1211. The assay buffer consisted of 0.88 M Tris–HCl (pH 9.8), 1 M diethanolamine, 1 mM $MgCl_2$, 0.02 mM $ZnCl_2$, and 0.01% Tween 20. The TNAP enzyme solution was prepared by diluting the prepared recombinant enzyme 3000 times with the assay buffer. Seven concentrations of the substrate pNPP solution (top concentration: 0.58 mg/mL) were prepared through serial dilution. The original pNPP solution was prepared at a concentration of 1.15 mg/mL. This original solution was diluted 1.5 times and the obtained solution was further diluted 1.5 times. This procedure was repeated 6 times, resulting in the 7 final concentrations.

In a 96-well plate, 1 μL of each DS-1211 solution was mixed with 50 μL of the enzyme solution. A control group with no DS-1211 was also included. Final experimental concentrations of DS-1211 were 0, 5, 10, and 20 nM. Next, 50 μL of pNPP solution was added to each well as the substrate and absorbance was measured at 405 nm (A_{405}) using a plate reader (sunrise RAINBOW, Tecan Japan Co., Ltd., Kawasaki, Japan) before and after a 30-minute incubation at 37°C protected from light. ALP activity was calculated by subtracting preincubation A_{405} from postincubation A_{405}. Each group was performed in triplicate.

Lineweaver-Burk plots were created from resulting ALP activity and substrate concentration data; the maximum velocity of the reaction (V_{max}) and Michaelis–Menten constant (Km) values were calculated from the plots using the following equations:

x-intercept $= -1/\mathrm{Km}$ and y-intercept $= 1/V_{max}$.

Inhibition of human TNAP, IAP, PLAP enzymes

Inhibition effects of DS-1211 on the enzymatic response on human TNAP, IAP, and PLAP enzymes were evaluated in vitro. DMSO was the experimental control and the assay buffer consisted of 1 mmol/L $MgCl_2$, 0.02 mmol/L $ZnCl_2$, 0.005% Tween 20, purified water, and 0.1 mol/L Tris–HCl (pH 7.5). The substrate solution (1.15 mg/mL pNPP solution; final concentration, 0.58 mg/mL) and recombinant enzyme solutions (enzyme with assay buffer) were prepared just before use. Assay conditions were optimized for each enzyme to ensure reactions were linear.

The DS-1211 test solutions were added to 96-well plates in combination with TNAP (0–100 μM/L), IAP (0–5000 μM/L), or PLAP (0–5000 μM/L). Control wells contained DMSO and the respective enzyme solution (TNAP, IAP, or PLAP) or DMSO and assay buffer. The pNPP solution (50 μL/100 μL of total assay volume in a well) was added to all wells and all solutions were tested in triplicate. Well plates were protected from light and incubated at 37°C for 1 hour (TNAP and IAP) or 5 hours (PLAP); absorbance was measured at 405 nm.

Inhibition rate was calculated for each enzyme and plotted on the y-axis against the log of DS-1211 concentrations on the x-axis. Inhibition rates were calculated following 100 – response rate, where response rate $= [(B - N) / (B_0 - N)] \times 100\%$; B, individual absorbance of test substance well; B_0, mean absorbance of control well; N, mean absorbance of well without enzymes.

The half maximal inhibitory concentration (IC_{50}) values were estimated using nonlinear regression according to the following logistic model: $Y = Y_{min} + (Y_{max} - Y_{min})/(1 + 10^{[-\beta \times (X - ED0.5)]})$; β, slope (hill coefficient); ED0.5, common logarithm of IC_{50}; X, common logarithm of test substance concentration; Y, inhibition rate. Y_{min} and Y_{max} were assumed to be 0 and 100, respectively.

ALP inhibition in mouse, monkey, and human plasma

The effect of DS-1211 on TNAP inhibition in mouse, monkey, and human plasma were investigated to examine potency across animal species. DMSO without DS-1211 was the negative experimental control and the assay buffer consisted of 1 mmol/L $MgCl_2$, 0.02 mmol/L $ZnCl_2$, and 50 mmol/L Tris–HCl (pH 7.5). The substrate solution was a 4.6-mg/mL pNPP solution (final concentration, 0.575 mg/mL) and prepared just before use.

Samples of mouse plasma were purchased and used without filtration. Monkey and human plasma samples were purchased and thawed at 37°C and filtered with gauze. Next, these plasmas

were placed into a syringe and put through a 0.45-μm filter (STERIVEX-HV, Merck, Kenilworth, NJ, USA). The filtered plasma was divided into 2-mL aliquots and stored frozen until use.

For mouse plasma experiments, DS-1211 solutions (0–1 μmol/L) were added to 384-well plates in combination with plasma and assay buffer. Control wells (mixture of DMSO, plasma, and assay buffer) were preincubated at room temperature for 5 minutes. The plate was then incubated at room temperature for 2 hours after adding the pNPP solution (2.5 μL/20 μL of total assay volume in a well). Each group was performed in triplicate; absorbance was measured at 405 nm.

For testing with monkey and human plasma, DS-1211 solutions (0–1 μmol/L) were added to 96-well plates in combination with plasma and assay buffer. Control wells were preincubated for 5 minutes at room temperature, after which 7.5 μL of the pNPP solution was added to each well (60 μL of total assay volume in a well) and the plate was incubated at 37°C for 1 hour for monkey plasma and 3 hours for human plasma. Each group was performed in triplicate; absorbance was measured at 405 nm.

The TNAP inhibition rates were calculated for each plasma and plotted on the *y*-axis against the log of DS-1211 concentrations on the *x*-axis. Inhibition rates were calculated using 100 – response rate, where response rate $= [(B - N) - (B_{pre} - N_{pre})]/[(B0 - N) - (B0_{pre} - N_{pre})] \times 100$; B, individual absorbance of test substance well after incubation; B_{pre}, individual value of test substance well before incubation; B0, mean absorbance of control well after incubation; $B0_{pre}$, mean value of control well before incubation; N, mean absorbance of well without enzymes after incubation; N_{pre}, mean value of well without enzymes before incubation.

Inhibition rate was plotted on the *y*-axis against the log of DS-1211 concentrations on the *x*-axis, and the IC_{50} values (DS-1211 concentration with a response value equivalent to the average of the estimated maximum [E_{max}] and minimum [E_{min}] inhibition rates) were estimated using nonlinear regression according to the following logistic model: $Y = Y_{min} + (Y_{max} - Y_{min})/(1 + 10^{[-\beta \times (X - ED0.5)]})$; β, slope (hill coefficient); ED0.5, common logarithm of IC_{50}; X, common logarithm of test substance concentration; Y, inhibition rate; Y_{max}, estimated maximal inhibition rate (E_{max}); Y_{min}, estimated minimal inhibition rate (E_{min}). Fixed values were not assumed for E_{min} and E_{max}.

In vitro off-target pharmacological actions

In vitro pharmacological actions of DS-1211 at 10 μmol/L on a total of 88 receptors, channels, transporters, and enzymes were evaluated in duplicate by enzyme and radioligand binding assays. DS-1211 was dissolved in DMSO and diluted 1000 times with assay buffer to make 10-μmol/L solution. Receptor binding and enzyme assays of reference substances were performed at five concentrations in duplicate to determine the IC_{50} and dissociation constant (Ki) values for receptor binding and the IC_{50} value for enzyme assays.

All assay procedures were performed following standard protocols. To validate the experiments, reference substances were tested concurrently with DS-1211 in each batch. Concurrent IC_{50} and Ki data were compared with historical data. Inhibitory effects of DS-1211 were expressed as percent inhibition relative to the control group. The threshold used to show affinities was when significant responses (≥50% inhibition) were noted.

In vivo ALP inhibition and PKs in mice

Thirty male mice were divided into 5 groups (n = 6/group; Supplemental Table S1) to receive a single oral dose of DS-1211 or vehicle. After group assignment, animals were housed socially, 3 animals per cage. Mice were fed a commercially available diet for rodents (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and received tap water *ad libitum*.

To prepare the dosing solution, the sodium salt of DS-1211 was reconstituted in its vehicle, 0.5% Methylcellulose 400 solution (0.5% MC, Wako Pure Chemical Industries), and 0.01, 0.03, 0.1, and 0.3 mg/mL DS-1211 solutions were prepared.

Mice were orally administered 10 mL/kg of their assigned dose, and blood was collected from the tail vein at 0 (predose), 1, 2, 4, 6, and 24 hours postdose time points. Plasma was separated from blood samples and used to determine plasma ALP activity and plasma DS-1211 concentrations.

A laboratory-developed ALP inhibition assay was used to determine the ALP activity in plasma. In a 384-well plate, plasma samples from each time point were added to assay buffer (1 mmol/L $MgCl_2$, 0.02 mmol/L $ZnCl_2$, purified water, and 50 mmol/L Tris–HCl [pH 7.5]). After allowing the plate to sit at room temperature for 5 minutes, 1 μL of the 4.6 mg/mL pNPP solution (pNPP reconstituted in purified water; final concentration, 0.58 mg/mL) was added to each well, and the plate was incubated at room temperature for 3 hours; absorbance was measured at 405 nm. Differential absorbance (ΔAbs) was calculated by subtracting the preincubation absorbance value from the absorbance after incubation with the test samples.

ALP activity in plasma after treatment was calculated at each time point as a percentage of the predose ALP activity ([ΔAbs after dosing/ΔAbs before dosing] × 100). Mean ALP activity at each time point was used to calculate mean ALP inhibition rate up to 6 hours after administration.

Plasma samples were analyzed for all groups at each time point for PK analysis. Plasma DS-1211 concentrations were determined using liquid chromatography tandem mass spectrometry (LC/MS/MS). The PK parameters calculated were maximum plasma concentration (C_{max}), time to reach the maximum plasma concentration (T_{max}), terminal elimination half-life ($T_{1/2}$), area under the plasma concentration-time curve up to the last quantifiable time (AUC_{last}), and area under the plasma concentration-time curve up to infinity (AUC_{inf}) were calculated.

PD biomarker analysis in mice

Changes in ALP activity, PPi, and PLP after single-dose DS-1211 administration were evaluated in blood samples from mice. Ninety male mice were assigned to receive a single dose of DS-1211 (1 or 10 mg/kg) or vehicle (0.5% MC). Mice were housed socially, 6 animals per cage. Animals received a rodent chow diet (FR-2, Funabashi Farm Co., Ltd., Funabashi, Japan) and were provided with tap water *ad libitum*.

The DS-1211 dosing solutions were prepared by reconstituting potassium salt of DS-1211 in 0.5% MC at two concentrations: 0.1 mg/mL and 1 mg/mL. The dosing solutions were prepared 2 days before administration and stored at −20°C protected from light until use.

Mice were orally administered 10 mL/kg of their assigned treatment dose and blood was collected from the tail vein or the abdominal vein at 0 (predose), 1, 2, 4, and 6 hours postdose from one animal per time point (Supplemental Table S1). Anticoagulants used for blood collection were heparin for ALP activity

and EDTA for PPi, PLP, and DS-1211 concentration measurements. To separate plasma from blood, collected blood was centrifuged for 5 minutes at 9100*g* and 4°C. Samples for the measurement of PPi were transferred to centrifugal filters and centrifuged again under the same conditions to obtain platelet-free plasma. Plasma samples were used to determine plasma ALP activity, plasma PPi concentration, plasma PLP concentration, and plasma DS-1211 concentrations.

A laboratory-developed ALP inhibition assay was used to determine the ALP activity in plasma, as described previously. Plasma was separated from abdominal vein blood to quantify plasma PPi, plasma PLP, and plasma DS-1211 concentrations. Platelet-free plasma samples were obtained for the measurement of PPi, as previously described. Plasma DS-1211 concentrations were determined using an internally validated LC/MS/MS method. To quantify plasma PPi, mouse plasma was mixed with either an assay mixture containing 2 mM $MgCl_2$, 1-M HEPES buffer, 684 μg/mL adenosine 5′-phosphosulfate sodium salt, ATP sulfurylase, and distilled water (+ATP_sul), or the same assay mixture without ATP sulfurylase (−ATP_sul). Samples were mixed, centrifuged, and then incubated for 30 minutes at 37°C, followed by 10 minutes at 90°C. After mixing, samples were centrifuged again at 9100 *g* for 20 minutes at 4°C and then added to a 96-well plate in combination with 5 × CTG reagent. Luminescence was measured after incubation at room temperature for 10 minutes; samples were performed in duplicate. To quantify plasma PLP, mouse plasma was treated using a protein precipitation method and analyzed using the LC/MS/MS method. Plasma samples were mixed and heated at 40°C for 30 minutes, after which they were centrifuged at 20,000*g* at 4°C for 5 minutes. The supernatant of each sample was transferred to a 96-well plate and analyzed in an autosampler as an LC/MS/MS injection sample.

In vivo ALP inhibition, pharmacokinetics, and biomarker analysis in monkeys

After single administration of DS-1211, ALP activity, plasma PPi concentration, and plasma DS-1211 concentrations were measured in 6 male cynomolgus monkeys (Shin Nippon Biomedical Laboratories, Ltd., Tokyo, Japan). Monkeys were assigned to receive a DS-1211 dose of 0.03, 0.1, 0.3, and 1.0 mg/kg or vehicle (0.5% MC). One monkey was housed per cage and animals were fed a laboratory diet (PS-A laboratory food, Oriental Yeast Co., Ltd., 90 g/d) with additional food (apples and oranges, about 50 g/d) and tap water *ad libitum*.

Each monkey received all DS-1211 dose levels, with a 7- to 14-day washout period between doses. The DS-1211 dosing solutions were prepared shortly before administration by reconstituting potassium salt of DS-1211 in 0.5% MC at the four different concentrations. On the day of testing, monkeys were orally administered 1 mL/kg of their assigned treatment dose under a fasted state (more than 12 hours from the last feeding). The laboratory diet and additional food were given to the monkeys 8 hours after dosing. Blood was collected at 0.5 hours predose (−0.5) and 0, 0.5, 1.0, 2.0, 4.0, 8.0, and 24 hours postdose (Supplemental Table S1). Plasma was obtained from blood for quantification of ALP activity, PPi concentration, and DS-1211 concentration. As previously described, samples for the measurement of PPi underwent an additional processing step to achieve platelet-free plasma.

A laboratory-developed ALP inhibition assay was used to determine the ALP activity in monkey plasma such as that in mouse plasma but modified in assay volume. Plasma samples were added to a 96-well plate in combination with an assay buffer (50 mM Tris–HCl [pH 7.5], 1 mM $MgCl_2$, and 0.02 mM $ZnCl_2$) and 4.6 mg/mL pNPP (final concentration 0.575 mg/mL; 7.5 μL per well in 60 μL of total assay volume) to measure ALP activity. Plates were incubated at room temperature for 1 hour protected from light. Each group was performed in one well; absorbance was measured at 405 nm. Differential absorbance (ΔAbs) was calculated for each time point. Plasma ALP activity after treatment was determined as ΔAbs at each time point as a percentage of the predose ALP activity ([ΔAbs after dosing/ ΔAbs before dosing] × 100). Mean ALP activity at each time point was used to calculate mean ALP inhibition ratio up to 8 hours after administration.

An ^{18}O-PPi solution (Niflumic acid [IS], 500 μg/mL in distilled water), 0.5-M EDTA solution, and methanol (MeOH) were mixed to prepare the IS/EDTA/MeOH solution. Dibutylamine (DBA) was diluted with dibutyl ammonium acetate (DBAA) to prepare the 0.01% DBA/DBAA solution. Plasma samples were combined with the IS/EDTA/MeOH solution, centrifuged, and the resulting supernatants were mixed with 0.01%DBA/DBAA solution. Control samples were directly combined with the IS/EDTA/MeOH solution and 0.01%DBA/DBAA solution. Sample treatment was performed on ice. PPi was quantified using internally validated LC/MS/MS methods.

Plasma protein binding and cytochrome P450 inhibitory potential of DS-1211

Plasma protein binding of DS-1211 in mice, rats, monkeys, and humans was investigated in vitro through ultracentrifugation. The concentration of DS-1211 in plasma and plasma supernatant were determined using internally validated LC/MS/MS methods. Plasma samples were mixed with DS-1211 and incubated at 37°C for 10 minutes. After incubation, samples were ultracentrifuged at 100,000 rpm (approximately 436,000*g*) at 4°C for 140 minutes. The total concentration of DS-1211 in plasma and the concentration of DS-1211 in plasma supernatant was measured and used to calculate protein binding (%). The percentage of plasma protein binding in mouse, rat, monkey, or human plasma ($n = 3$ per group) was calculated at three concentrations of 10, 100, and 1000 ng/mL DS-1211.

The inhibitory effects of DS-1211 on in vitro metabolism of substrates specific for human cytochrome P450 (CYP) isoforms (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A [testosterone], and CYP3A [midazolam]) were investigated using human liver microsomes with or without a 30-minute preincubation in the presence of DS-1211.

Oral bioavailability of DS-1211 in monkeys

DS-1211 was administered once orally at 0.1, 0.3, and 1 mg/kg in a dose-escalating manner, and intravenously at 0.3 mg/kg to 3 male cynomolgus monkeys to assess bioavailability (BA), distribution, and total body clearance (CL) of DS-1211. Plasma samples were collected and plasma concentrations of DS-1211 were measured using the LC/MS/MS method to calculate BA and PK parameters (CL and volume of distribution at steady state [Vd]). For oral dosing, blood samples were drawn predose and 0.25, 0.5, 1, 2, 4, 6, 8, 24, 32, and 48 hours postdose. For intravenous (IV) dosing, blood samples were drawn predose and 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 24, 32, and 48 hours postdose.

Data and statistical analysis

Data are presented as mean and standard deviation (SD), unless otherwise specified. Two-sided p values <0.05 were considered statistically significant for all analyses. Protein binding, enzymatic activity, enzyme inhibition rates, and IC_{50} values with their corresponding 95% confidence interval (CI) were calculated using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and EXSUS version 7.7 (CAC Croit).

For the study on pharmacological activity of 88 receptors, channels, transporters, and enzymes in response to DS-1211, inhibition percentages were analyzed using MathIQ (version 2.0.1.8; ID Business Solutions Ltd., Surrey, UK) and GraphPad Prism (GraphPad, La Jolla, CA, USA).

For the investigation of in vivo ALP inhibition and PKs in mice and monkeys, calculations of summary statistics, ALP activity, and ALP inhibition rates were performed using Microsoft Excel (Microsoft Corporation). The Dunnett multiple comparison procedure compared plasma ALP activity and mean ALP inhibition rates between the DS-1211 dosing groups and the vehicle group (SAS System Release 9.2, SAS Institute, Cary, NC, USA). To confirm dose dependency, a hypothesis test was performed using Spearman's rank correlation coefficient. Plasma concentration-time data were analyzed using noncompartmental methods and presented graphically. In the mice studies, Phoenix WinNonlin version 6.3 (Certara LP, Princeton, NJ, USA) was used for PK analysis. In the monkey experiment, biomarker concentration was analyzed through chromatogram analysis using Analyst (AB Sciex LLC, Framingham, MA, USA).

In the PD biomarker experiments in mice and monkeys, summary statistics, arithmetic mean (mean), standard error (SE) of plasma ALP, and plasma PLP concentration were calculated for each group at each time point. Mean plasma PPi concentration was calculated in the monkey experiment, while median plasma PPi concentration was calculated in the mouse experiment. All calculations were performed with Microsoft Excel. All statistical comparisons were performed using non-predefined post hoc tests (SAS System Release 9.2). In the mouse experiment, Dunnett's test was used to compare plasma ALP and plasma PLP activity at each time point in the treatment and control groups; steel test was used to compare plasma PPi concentration at each time point across groups. Bonferroni correction was applied to all comparisons, and two-sided p values < 0.0125 were considered statistically significant. In the monkey experiments, plasma ALP activity, mean ALP inhibition ratio up to 8 hours after DS-1211 administration, plasma PPi concentration, and area under the curve (AUC) of ΔPPi concentration up to 8 hours after administration were calculated for each time point and each group. Dunnett's tests were used to compare the mean ALP inhibition ratio and AUC of ΔPlasma PPi concentration across treatment groups. Two-sided p values < 0.05 were considered statistically

Table 1. V_{max} and Km Values at Tested Concentrations of DS-1211 Against TNAP Using pNPP as a Representative Substrate

DS-1211 concentration (nM)	V_{max} (nmol/min)	Km (mM)
0	1.07	0.25
5	0.81	0.18
10	0.72	0.17
20	0.50	0.10

Km = Michaelis–Menten constant; pNPP = *p*-nitrophenylphosphate; TNAP = tissue-nonspecific alkaline phosphatase; V_{max} = maximum velocity of the reaction.

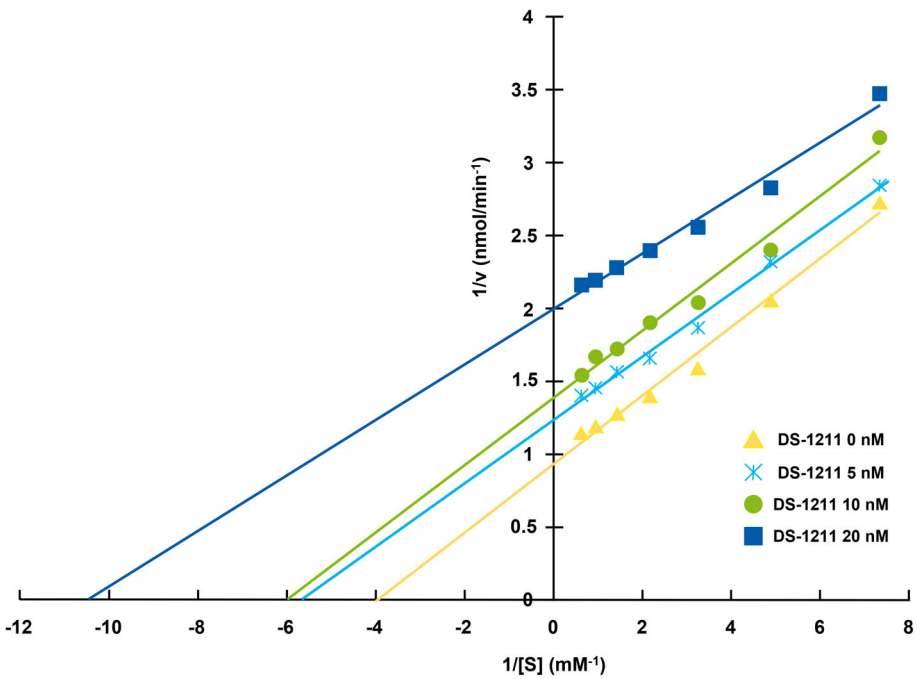

Fig. 2. Lineweaver-Burk plots showing uncompetitive inhibition of DS-1211 at different concentrations against TNAP using pNPP as a representative substrate. pNPP = *p*-nitrophenylphosphate; S = concentration of substrate; TNAP = tissue-nonspecific alkaline phosphatase; v = mean of enzymatic activity/30 min.

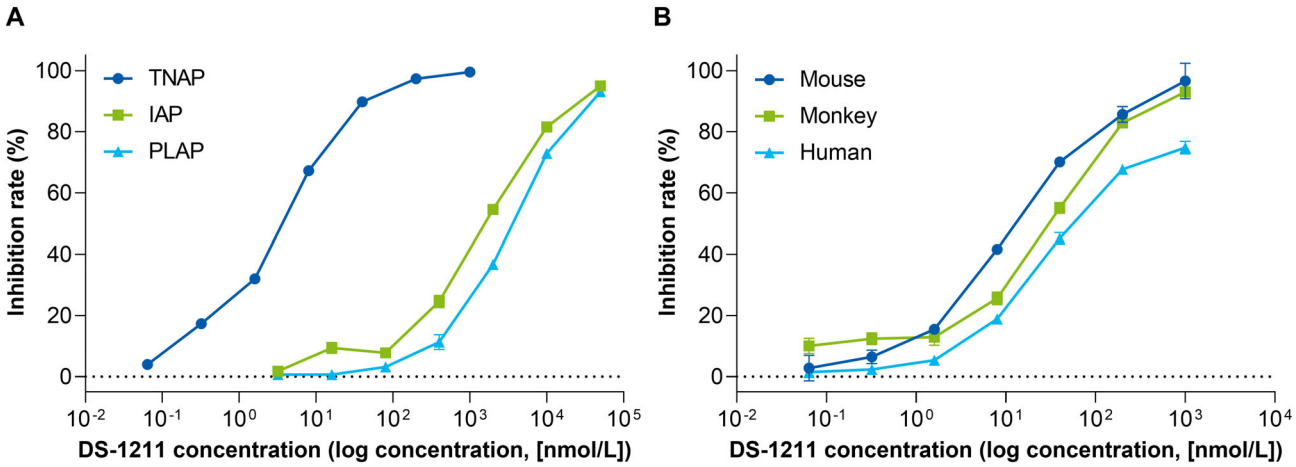

Fig. 3. Inhibitory effects of DS-1211 on (*A*) recombinant human TNAP, IAP, and PLAP, and (*B*) mouse, monkey, and human plasma using pNPP (1.15 mg/mL) as a representative ALP substrate. ALP = alkaline phosphatase; IAP = intestinal ALP; PLAP = placental ALP; pNPP = *p*-nitrophenylphosphate; TNAP = tissue-nonspecific ALP.

significant. Dunnett's test was also used to compare plasma ALP activity and plasma PPi concentration in the same groups at each time point, and Bonferroni correction was applied. Two-sided p values < 0.0083 were considered statistically significant.

Results

Inhibition mode of DS-1211 on the recombinant enzyme of human TNAP

Inhibition mode was evaluated by Lineweaver-Burk plots in the presence or absence of DS-1211 (Fig. 2). Lineweaver-Burk plots showed both V_{max} and Km values decreased as the concentration of DS-1211 increased (Table 1). These results suggest DS-1211 inhibits TNAP through an uncompetitive mode of action.

In vitro inhibitory effects of DS-1211

DS-1211 had a much greater inhibitory effect on the human TNAP enzyme compared with IAP or PLAP. A 3-order difference was observed in the IC_{50} for TNAP versus the IC_{50} for IAP or PLAP (Fig. 3*A*). The IC_{50} values (CI) for TNAP, IAP, and PLAP were 3.4 (3.0–3.7) nmol/L, 1560 (1380–1770) nmol/L, and 3510 (3340–3690) nmol/L, respectively.

DS-1211 was equally effective in inhibiting murine, simian/monkey, and human plasma ALP activity (Fig. 3*B*). The IC_{50} values (95% CI) for mouse, monkey, and human plasma were 13.0 (10.2–16.5) nmol/L, 36.3 (31.9–41.3) nmol/L, and 28.3 (25.4–31.6) nmol/L, respectively.

DS-1211 at 10 µmol/L showed no significant response (≥50% inhibition) on a total of 88 receptors, channels, transporters, and enzymes (Supplemental Table S2).

In vivo ALP inhibition, pharmacokinetics, and biomarker changes in mice

Plasma concentrations and PK parameters of DS-1211 are shown in Fig. 4*A* and Table 2. PK analysis showed C_{max}, AUC_{last}, and AUC_{inf} values increased with dose, whereas T_{max} and $T_{1/2}$ remained constant at each dose level. At DS-1211 dose levels of 0.3, 1.0, and 3.0 mg/kg, mean (SD) $T_{1/2}$ was 1.2 (0.3), 1.1 (0.1), and 1.4 (0.6), respectively. Plasma collected after a single dose of DS-1211 orally administered to mice showed DS-1211 inhibited ALP activity dose-dependently at dose concentrations of 0.01, 0.3, 1.0, and 3.0 mg/kg; inhibition continued for up to 6 hours after administration (Fig. 4*B*). DS-1211 inhibited significantly more ALP activity than the vehicle (Fig. 4*C*).

Further experiments showed DS-1211 significantly decreased plasma ALP activity with both dose levels (1 mg/kg or 10 mg/kg) and at all time points from 1 to 6 hours after administration of a single dose when compared with the control (Fig. 5). Additionally, a single dose of DS-1211 increased plasma PPi, plasma PLP, and plasma DS-1211 concentrations in mice compared with those receiving the vehicle over the first 6 hours after administration. Changes in biomarkers were generally dose-ordered.

In vivo ALP inhibition, pharmacokinetics, and biomarker changes in monkeys

DS-1211 and plasma PPi concentrations in monkeys increased with DS-1211 dose (Fig. 6*A*, *B*), whereas plasma ALP activity decreased with orally administered DS-1211 (Fig. 6*C*). After administration, DS-1211 significantly decreased plasma ALP activity with all DS-1211 doses up to 4 hours after administration and up to 8 hours after the 0.3- and 1.0-mg/kg dose groups compared with vehicle. DS-1211 significantly increased the mean ALP inhibition rate compared to vehicle up to 8 hours after administration in a dose-dependent manner (Fig. 6*D*). DS-1211 elicited a significant increase in plasma PPi concentration with 0.3- and 1-mg/kg doses up to 2 hours after administration; the 1-mg/kg dose elicited a significant increase in plasma PPi concentration at 0.5 hours after administration. The AUC of ΔPlasma PPi concentration increased with administration of DS-1211 up to 8 hours after administration in a dose-dependent manner for 0.03-, 0.1-, 0.3-, and 1-mg/kg doses; DS-1211 0.3 and 1 mg/kg significantly increased AUC of ΔPlasma PPi concentration compared with the vehicle ($p = 0.0083$ and $p < 0.0001$, respectively; Fig. 6*E*).

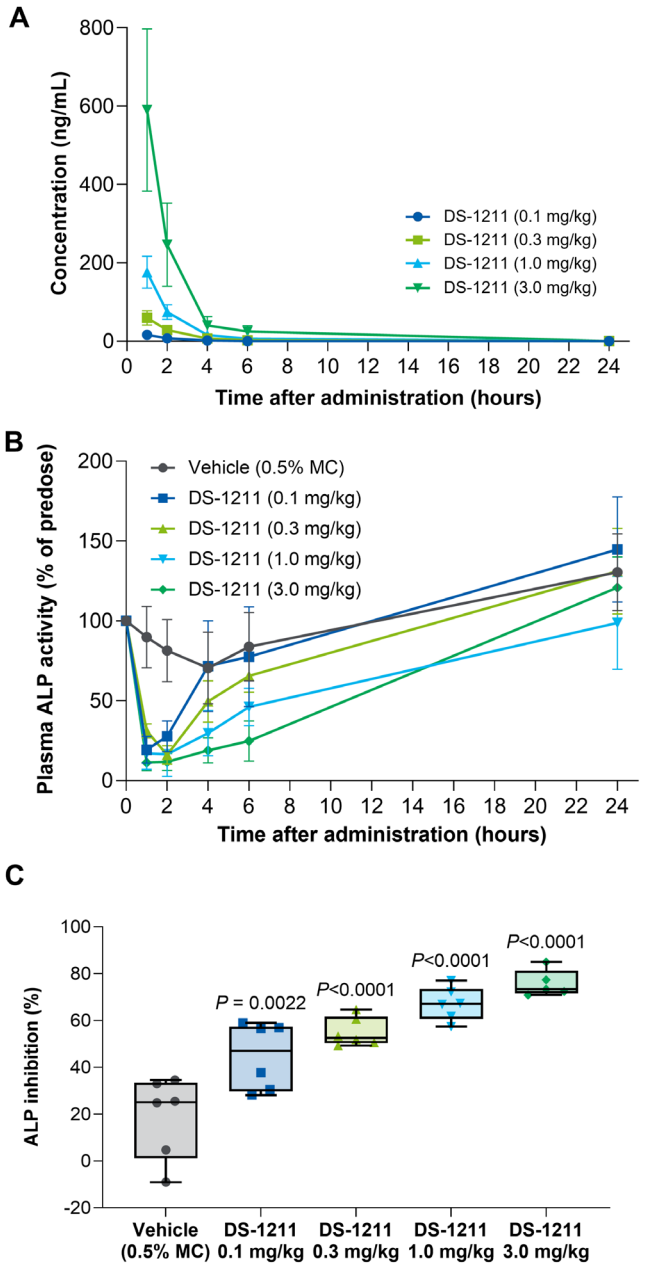

Fig. 4. Plasma (*A*) DS-1211 concentration and (*B*) ALP activity in mice after single oral administration of DS-1211 or vehicle (mean ± SD), and (*C*) ALP inhibition rate up to 6 hours after oral administration of DS-1211 or vehicle to mice. DS-1211 sodium salt was used in this experiment. ALP = alkaline phosphatase; MC = methylcellulose 400; SD = standard deviation.

Plasma protein binding and cytochrome P450 inhibitory potential of DS-1211

Plasma protein binding of DS-1211 at 10, 100, and 1000 ng/mL ranged from 71.7% to 73.0% in mice, 85.3% to 85.9% in rats, 86.3% to 87.2% in monkeys, and 79.3% to 80.4% in humans. There was no concentration dependence of protein binding in plasma in animals or humans.

In an investigation of direct and time-dependent inhibition of DS-1211 on metabolism of substrate specific for CYP isoforms in human liver microsomes, IC_{50} values of DS-1211 for CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A (testosterone), and 3A (midazolam) were all estimated to be >100 µmol/L.

Oral bioavailability of DS-1211 in monkeys

Single iv administration of DS-1211 in monkeys at a dose of 0.3 mg/kg yielded a mean (SD) Vd of 0.221 (0.020) L/kg and CL of 5.06 (0.70) mL/min/kg. Absolute oral BA was 73.9% (3.7%) as determined through single oral administration of the DS-1211 0.3 mg/kg dose in monkeys.

Discussion

Inhibition of TNAP is a potential therapeutic strategy to prevent ectopic soft tissue calcification by increasing the level of the potent endogenous anticalcification factor PPi.[11-14] Here, we report a comprehensive summary of studies establishing the biochemical and pharmacological profiles of the small molecule TNAP inhibitor, DS-1211. In vitro studies demonstrated that DS-1211 inhibited ALP through an uncompetitive mode, as both Km and V_{max} decreased as DS-1211 concentration increased. Furthermore, DS-1211 has a potent and selective inhibitory effect on TNAP. IC_{50} values for human TNAP were in the low nanomolar range, while IC_{50} values for human IAP and PLAP were orders of magnitude higher, indicating that DS-1211 is a highly selective inhibitor for human TNAP over IAP or PLAP. The inhibitory effect of DS-1211 on plasma ALP activity is similar across species with comparable IC_{50} values for mouse, monkey, and human plasma. Lastly, DS-1211 did not show significant response on 88 receptors, channels, transporters, and enzymes, suggesting that the risk of off-target adverse effects is low.

In vivo studies in mice and monkeys showed single oral administration of DS-1211 decreased ALP activity and increased plasma PPi, plasma PLP, and plasma DS-1211 concentrations in a dose-dependent manner. High oral bioavailability of DS-1211 was established. Lastly, no inhibitory effects of DS-1211 were observed on the activities of the CYP isoforms and no significant response on major other receptors, channels, transporters, and enzymes, suggesting there is a low risk of drug–drug interaction and off-target adverse effects. Results from the studies in this report suggest that DS-1211 is a highly potent and effective TNAP inhibitor in vivo.

This is the first thorough characterization of the inhibitory effects and pharmaceutical properties of the novel TNAP inhibitor, DS-1211. Results presented here show that the TNAP inhibition-PPi increase pathway is a common mechanism in humans and animal species in both the in vitro and in vivo setting. Species-related differences were observed in the relationship between PPi and TNAP inhibition, as the PPi increase with TNAP inhibition was clearly detected and more pronounced in monkeys and humans from the phase 1 study[20] than in mice. However, mice demonstrated a clear relationship between PLP and TNAP inhibition, indicating PLP may be a sensitive substrate affected by TNAP inhibition in mice. These data provide a foundation for future studies to further explore the DS-1211 mechanism of action.

Preclinical data is valuable to connect preclinical and clinical pharmacological profiles of novel therapeutics. Although several inhibitors of ALP isozymes, such as SBI-425, have been proposed, DS-1211 is the first selective TNAP inhibitor to advance t human

Table 2. Plasma PK Parameters in Mice Dosed Orally With DS-1211 Potassium Salt

	DS-1211 dose			
	0.1 mg/kg	0.3 mg/kg	1 mg/kg	3 mg/kg
C_{max} (ng/mL)	16.5 ± 1.0	59.7 ± 18.5	176 ± 41	590 ± 207
T_{max} (hours)	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
$T_{1/2}$ (hours)	NC	1.2 ± 0.3	1.1 ± 0.1	1.4 ± 0.6
AUC_{last} (ng·h/mL)	29.4 ± 7.2	119 ± 27	325 ± 73	1050 ± 350
AUC_{inf} (ng·h/mL)	NC	123 ± 27	335 ± 76	970 ± 96

Data shown as mean ± SD.

AUC_{inf} = area under the plasma concentration-time curve up to infinity; AUC_{last} = area under the plasma concentration-time curve up to the last quantifiable time; C_{max} = maximum plasma concentration; NC = not calculated; PK = pharmacokinetic; SD = standard deviation; T_{max} = time to reach the C_{max}; $T_{1/2}$ = terminal elimination half-life.

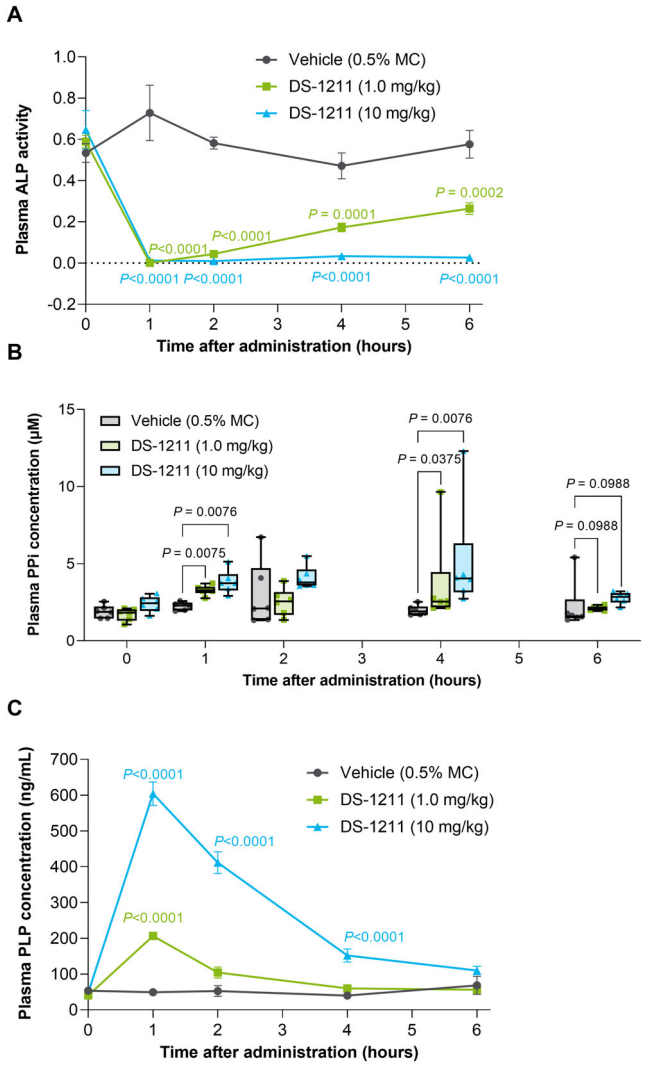

Fig. 5. Plasma concentration-time profiles of (*A*) ALP activity (ΔAbs at 405 nm, mean ± SE), (*B*) PPi concentration, and (*C*) PLP concentration (mean ± SE) after single oral administration of DS-1211 or vehicle to mice. DS-1211 potassium salt was used in this experiment. ΔAbs = differential absorbance; ALP = alkaline phosphatase; MC = methylcellulose 400; PLP = pyridoxal 5′-phosphate; PPi = pyrophosphate; SE = standard error.

testing.[1,15-17] Two first-in-human studies evaluated safety, tolerability, PK, and PD of single and multiple ascending doses of DS-1211, which appeared safe and well tolerated; PD assessments, as evaluated with PPi, PLP, and PEA concentration-time profiles, were consistent with exposure-dependent TNAP inhibition.[20] Results from the preclinical studies presented here corroborate the first-in-human clinical findings that oral administration of DS-1211 is associated with dose-ordered inhibition of ALP and dose-ordered elevation of the TNAP substrates, PPi, PLP, and PEA.[20] The current findings build upon those previously published by presenting a comprehensive pharmaceutical profile of DS-1211, including its mode of inhibition, its potent and selective inhibitory effect on TNAP, and its oral bioavailability.

There are few existing therapeutic drugs for the treatment of ectopic calcification and other bone mineralization diseases associated with reduced plasma PPi that have advanced to testing in humans. Like DS-1211, these therapies strive to elicit anticalcification effects; however, they have distinct pharmacological differences compared with DS-1211. For example, results from a phase 2 trial suggest that etidronate, the first generation of bisphosphonate, may reduce vascular calcification in patients with PXE.[27] Etidronate is a PPi mimetic and it elicits anticalcification efficacy without plasma PPi increase, which is different from DS-1211. Additionally, oral administration of PPi is ongoing for clinical trial preparation and may be efficient for ectopic calcification; it does not affect plasma PLP itself, a well-known biomarker for diagnosis of mineralization disorders of hypophosphatasia with levels correlated to clinical symptoms, in contrast with DS-1211. Notably, DS-1211 can be administered daily, whereas a cyclical regime (ie, treatment for 2 weeks every 12 weeks) is recommended for administration of etidronate.[27,28] Differences in the mechanism of action, toxicity profile, and dosing regimen of therapeutics may affect patient compliance and treatment efficacy.[29]

There are possible risks of TNAP inhibition, which should be considered. A continued and nearly complete or complete loss of TNAP activity is associated with hypophosphatasia (HPP), a rare inheritable mineralization disorder often leading to rickets, osteomalacia, or hypomineralization of teeth.[2] Excessive exogenous TNAP inhibition may lead to development of hypophosphatasemia,[30] a condition associated with clinical issues similar to those observed in HPP caused by accumulation of PPi in the extracellular matrix and disturbed PLP (vitamin B6) metabolism.[11] Appropriate clinical dosing of DS-1211 will be important to minimize possible side effects; potential clinical regimens will be investigated in more detail in future studies. Additionally, the DS-1211 half-life is relatively short; however, this does not take away from its potential to demonstrate anticalcification effects. The TNAP inhibitor SBI-425 also has a

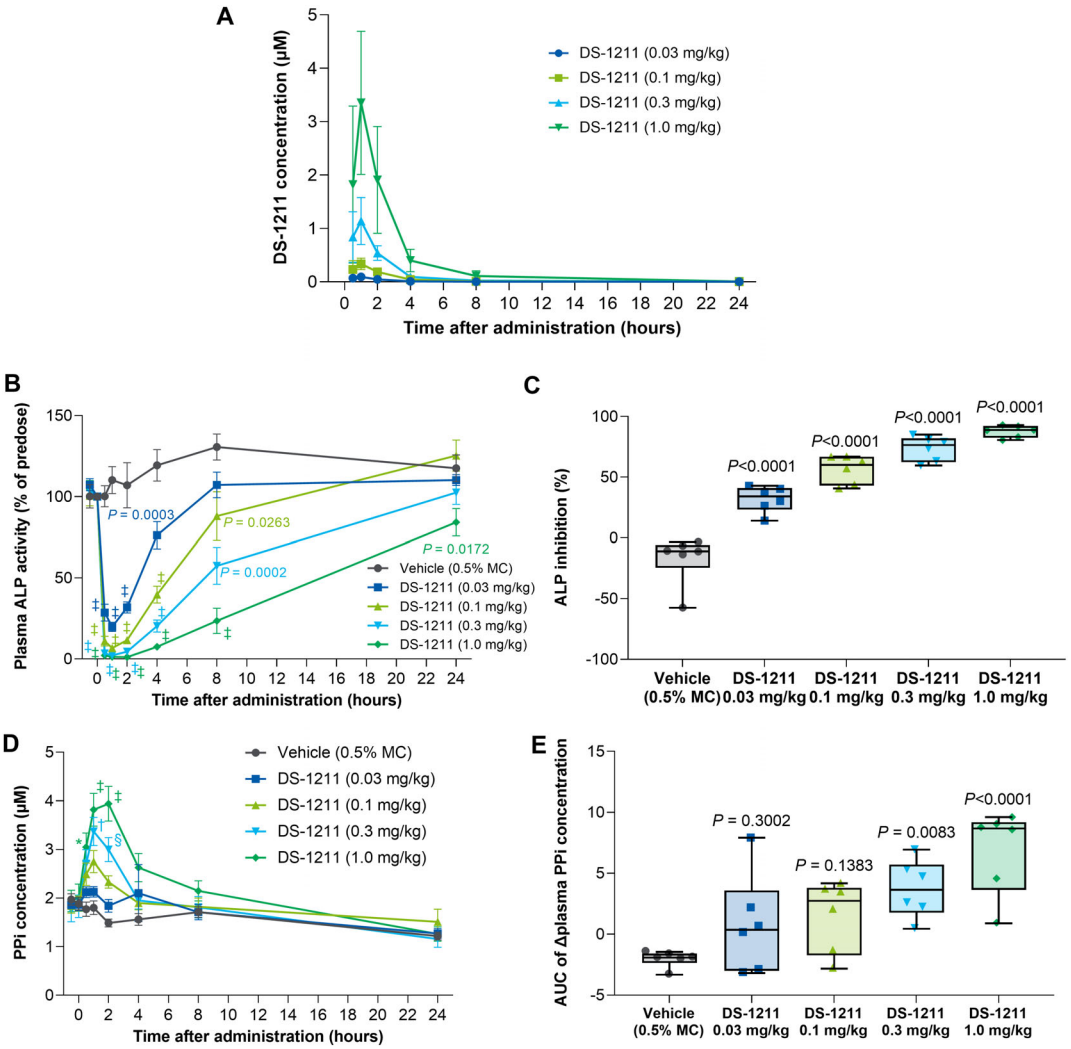

Fig. 6. Plasma (*A*) DS-1211 concentration (mean ± SD), (*B*) ALP activity (mean ± SE) after single oral administration of DS-1211 of vehicle to monkeys, (*C*) ALP inhibition rate up to 8 hours after administration of DS-1211 or vehicle in monkeys, (*D*) plasma PPi concentration (mean ± SE) after single oral administration of DS-1211 of vehicle to monkeys, and (*E*) AUC of change in plasma PPi concentration up to 8 hours after administration of DS-1211 or vehicle in monkeys. DS-1211 potassium salt was used in this experiment. $^{*}p = 0.0019$; $^{\dagger}p = 0.0004$; $^{\S}p = 0.0002$; $^{\ddagger}p < 0.0001$ versus vehicle. ALP = alkaline phosphatase; AUC = area under the curve; MC = methylcellulose 400; PPi = pyrophosphate; SD = standard deviation; SE = standard error.

relatively short half-life of 2.3 hours, and it has significant anticalcification effects in animal models of PXE.[1,7,19] Furthermore, transient increases in circulating PPi levels achieved by daily administration of exogenous PPi, either intraperitoneally or orally, lead to reduction in ectopic calcification in uremic rats and *ABCC6*-deficient mice.[31,32]

Interpretations of the results in this report are limited by the scope of the preclinical studies. Additional studies are warranted to further elucidate physiological activities considering the underlying mechanisms of DS-1211–mediated TNAP inhibition. Additionally, it is important to note that enzyme activity within the tissues may have a greater association with pathophysiologic conditions than circulating ALP activity. Although two phase 1 studies demonstrated the safety and tolerability of DS-1211 and PD data suggested that DS-1211 inhibits TNAP activity,[20] additional clinical studies are needed to further examine the safety, PK, and PD of DS-1211 in patients with ectopic calcification.

In conclusion, DS-1211 is a novel TNAP inhibitor that demonstrates a highly potent and selective inhibitory effect of TNAP in multiple animal species and in vitro models. The comprehensive findings in this report support further investigation of the efficacy and safety of DS-1211 in clinical studies with populations vulnerable to ectopic calcification.

Disclosures

All authors are employees of Daiichi Sankyo.

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Author Contributions

Kaori Soma: Data curation; formal analysis; investigation; project administration; supervision; writing – original draft; writing – review and editing. **Masanori Izumi:** Conceptualization; project administration; writing – review and editing. **Yuko Yamamoto:** Data curation; formal analysis; investigation; methodology; writing – review and editing. **Shojiro Miyazaki:** Methodology; writing – review and editing. **Kengo Watanabe:** Data curation; formal analysis; investigation; project administration; writing – original draft; writing – review and editing.

Data Availability Statement

Data that support the findings of this study are available from the corresponding author upon request. Some data may not be made available because of proprietary restrictions.

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