Pharmacokinetics and disposition of CS-0777, a sphingosine 1-phosphate receptor modulator, in rats and monkeys

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Abstract

1. Disposition and metabolism of CS-0777 (1-{5-[(3R)-3-amino-4-hydroxy-3-methylbutyl]-1-methyl-1H-pyrrol-2-yl}-4-(4-methylphenyl) butan-1-one), a selective sphingosine 1-phosphate receptor-1 modulator under development for autoimmune conditions was investigated following oral and/or i.v. bolus administration to rats and monkeys.

2. After oral administration of [14C]CS-0777, CS-0777 was well absorbed in rats and monkeys with total recoveries of over 90% of the dose, majorly in feces. CS-0777 and phosphorylated pharmacologically active metabolite of CS-0777 (M1) were highly bound to plasma proteins among rats, monkeys and humans (>93%).

3. The structures of 12 metabolites were identified and phosphorylation and two hydroxylation pathways were proposed as primary metabolism. In the blood of rats and monkeys, the major metabolite was M1 and a few phosphorylated metabolites were also detected. Meanwhile, in urine and feces of rats and monkeys, not phosphorylated, but oxidized CS-0777 metabolites and/or those various conjugated metabolites were observed. This suggests that CS-0777 and its oxidized metabolites would be phosphorylated in the body, but their phosphorylated metabolites would revert back to their dephosphorylated form again then be further metabolized and finally eliminated from the body.

4. Pharmacokinetic analysis using a reversible metabolism model revealed that the clearance of phosphorylation was larger than the clearance of dephosphorylation and elimination.

Keywords

CS-0777, dephosphorylation, distribution, pharmacokinetics, phosphorylation, reversible metabolism

History

Received 17 February 2015
Revised 6 April 2015
Accepted 6 April 2015
Published online 18 June 2015

Introduction

CS-0777 (1-{5-[(3R)-3-amino-4-hydroxy-3-methylbutyl]-1-methyl-1H-pyrrol-2-yl}-4-(4-methylphenyl)butan-1-one) is an amino alcohol prodrug and a sphingosine-1-phosphate (S1P) analogue after in vivo phosphorylation. Phosphorylated CS-0777 (M1) acts as an S1P receptor modulator expected to be efficient against various autoimmune diseases including multiple sclerosis (MS) (Nishi et al., 2011). The importance of S1P in the immune system has been demonstrated by another S1P receptor modulator, Gilenya (Fingolimod, FTY720) which has demonstrated efficacy in patients with remitting relapsing MS (Brinkmann et al., 2001; Cohen et al., 2010; Fujino et al., 2003; Kappos et al., 2010; O’Connor et al., 2009) and gained approval by the Food and Drug Administration in 2010. Activated fingolimod after phosphorylation by sphingosine kinase disrupts lymphocyte trafficking by decreasing lymphocyte egress from lymph nodes and the thymus (Chiba et al., 1998; Matloubian et al., 2004).

The S1P receptor is one of several G-protein coupled receptors and five mammalian S1P receptors (S1P1/Edg1, S1P2/Edg5, S1P3/Edg3, S1P4/Edg6 and S1P5/Edg8) that have been identified (Hla et al., 2001). Phosphorylated fingolimod binds to all S1P receptors except S1P2 (Brinkmann et al., 2002; Mandala et al., 2002). Meanwhile, M1 also exhibited binding to the S1P receptor, however, this was high selective binding to S1P1 compared to fingolimod (Nishi et al., 2011). It was suggested by the experiments using S1P1 knockout mice (Matloubian et al., 2004) that S1P1 is the primary S1P receptor for lymphocyte homing and trafficking to secondary lymphoid compartments. Therefore, a selective S1P1 modulator will be sufficient for the induction of lymphopenia and has the possibility to avoid potential side effects induced by the modulation of other S1P receptors apart from S1P1.
In healthy volunteers in a phase I single dose trial, the reduction of peripheral lymphocyte counts with increasing doses of CS-0777 was observed and there was a good correlation between lymphocyte reduction in circulation and M1 concentration in blood (Moberly et al., 2012a; Rohatagi et al., 2009). CS-0777 also resulted in a pronounced, dose-dependent decrease in lymphocyte and CD4 T cell subsets in MS patients in an open-label pilot study (Moberly et al., 2012b). Lymphocyte reduction in circulation was regarded to be an important process to represent suppression of an increased autoimmune effect. Therefore, phosphorylation of the parent compound in the body is considered to be an important process for the onset of pharmacological activity for CS-0777.

In this study, we investigated the pharmacokinetics, metabolism and excretion of CS-0777 in Sprague–Dawley rats and cynomolgus monkeys, which were used for the preclinical pharmacological and toxicological evaluation of this agent. Pharmacokinetics of M1 after administration of CS-0777 to rats and monkeys were also determined due to their importance from the pharmacological point of view. In addition, model analysis involving a reversible phosphorylation process between CS-0777 and M1 was conducted in order to obtain a deep understanding of their disposition.

Materials and methods

Materials

CS-0777, internal standards (ISs) (\(^{2}\)H\(_6\)-CS-0777 and \(^{2}\)H\(_6\)-M1), synthetic standards of metabolites [M1, the hydroxylated form of benzyl position of M1 (M2), the carboxylic acid form of M1 (M3), the hydroxy methyl form of CS-0777 (M4), the carboxylic acid form of CS-0777 (M5), the hydroxylated form of benzyl position of CS-0777 (M6) and the hydroxylated form of M5 (M7)] were synthesized by Daiichi Sankyo Co., Ltd (Tokyo, Japan). \(^{14}\)C-CS-0777 (1.80 MBq/mg) was synthesized at Sekisui Medical Co., Ltd. (Ibaraki, Japan) and its radiochemical purity was guaranteed to be more than 97% by high-performance liquid chromatography (HPLC) with radioactive flow detection. Sulfobutyl ether beta cyclodextrin sodium salt (SBE-\(\beta\)-CD) was purchased from CyDex, Inc. (Lenexa, KS). All other reagents and solvents used were commercially available and were of extra pure, guaranteed or liquid chromatography/mass spectrometry (LC/MS) grade.

Animal studies

All animal studies were conducted with approval in accordance with the guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd. and Mitsubishi Chemical Medience Corporation (Ibaraki, Japan). We used male Sprague–Dawley rats (182.6–232.4 g), which were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and male cynomolgus monkeys (2.6–4.6 kg), which were purchased from Nafovanny (Long Thanh, Vietnam). All animals were acclimated to the experimental conditions before use for at least 1 week for rats and 6 weeks for monkeys. Food and water were supplied ad libitum throughout the acclimatization and experimental period; however, before administration, the animals were fasted overnight and fed at approximately 6 h post-dose for rats and 8 h post-dose for monkeys.

Dosing solutions were prepared by dissolving each test compound (CS-0777 and \(^{14}\)C-CS-0777) in 4% (w/v) SBE-\(\beta\)-CD solution. The dosing volume was set at 2 ml/kg. The other conditions, including the dosage administered, are specified in each experiment.

Pharmacokinetic studies in rats

CS-0777 was administered intravenously via the tail vein or by oral gavage to rats (n = 4 for each group) at doses of 0.1, 0.3, 1 and 3 mg/kg for pharmacokinetic study. Approximately 150 µl of blood was collected from the jugular vein with a heparinized syringe at the following times under ethyl ether anesthesia: pre-dose and 0.083, 0.25, 0.5, 1, 3, 6, 8, 10, 24, 48 and 72 h post-dose for the intravenous dose; and pre-dose and 0.25, 0.5, 1, 3, 6, 8, 10, 24, 48 and 72 h post-dose for oral dose. Some of the blood sample (0.1 ml) was immediately mixed with 0.1 ml of methanol containing 0.02 N HCl. Plasma was obtained from a portion of the blood sample by centrifugation. Blood and plasma samples were stored at in a freezer (−20°C) until analysis.

Pharmacokinetic studies in monkeys

CS-0777 was administered intravenously via the saphenous vein or by oral gavage to monkeys (n = 4 for each group) at doses of 0.2, 0.5 and 1 mg/kg in a dose escalation manner with washout periods of over 4-week between doses. Approximately 1 ml of blood was collected from the cephalic vein with a heparinized syringe at pre-dose and 0.083, 0.25, 0.5, 1, 3, 6, 8, 10, 24, 32, 48, 72, 120, 168, 240 and 336 h post-dose for intravenous dosing and at pre-dose and 0.25, 0.5, 1, 3, 6, 8, 10, 24, 32, 48, 72, 120, 168, 240 and 336 h post-dose for oral dosing. Immediately after collection of the blood, a couple of blood samples (0.1 ml for each sample) were mixed with 0.1 ml of methanol containing 0.02 N HCl. Blood and plasma samples were stored in a freezer (−20°C) until analysis.

Mass balance study in rats

\(^{14}\)C-CS-0777 (0.5 mg/kg, 0.9 MBq/kg) was administered intravenously via the tail vein (n = 4) or by oral gavage (n = 4) to rats. After dosing, each rat was housed individually in a glass metabolic cage (Sugiyama-gen Co., Ltd., Tokyo, Japan), and urine and feces were collected for 336 h at 0–24, 24–48, 48–72, 72–120, 120–168, 168–216, 216–264 and 264–336 h post-dose.

Mass balance study in bile duct-cannulated rats

Rats were subjected to cannulation with a flexible polyethylene tube (PE-10; Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) into the common bile duct and fixed by placing a ligature around the tube to prevent dislocation under anesthesia with ethyl ether inhalation. After recovery from the anesthesia, \(^{14}\)C-CS-0777 (0.5 mg/kg, 0.9 MBq/kg) was administered intravenously via the tail vein (n = 4) or by
oral gavage (n = 3) to rats. Each animal was individually accommodated in a Bollman cage (Sugiyama-gen Co., Ltd.). The bile was collected at intervals of 0–1, 1–3, 3–6, 6–24, 24–30 and 30–48 h post-dose. The urine was collected at intervals of 0–24 and 24–48 h post-dose.

Mass balance study in monkeys

[^14]C CS-0777 (0.5 mg/kg, 0.9 MBq/kg) was administered intravenously via the saphenous vein (n = 4) or by oral gavage (n = 4) to monkeys. After dosing, each monkey was housed individually in a metabolic cage and urine and feces were collected for 504 h post-dose at 24 h intervals.

Tissue distribution of radioactivity in rats

After oral administration of[^14]C CS-0777 at a dose of 1 mg/kg (1.8 MBq/kg) to Sprague–Dawley rats (n = 1), blood was taken from the abdominal aorta under diethyl ether anesthesia at designated time points up to 12 week. Subsequently, tissues were collected from each carcass. A part of each blood sample was centrifuged to obtain plasma.

Sample collection for profiling and identification of metabolites in rats

For profiling and identification of metabolites in rats, blood was collected via the abdominal aorta at 10 h after oral administration of[^14]C CS-0777 (3 mg/kg, 3.6 MBq/kg) to rats (n = 8) under ethyl ether anesthesia. Two volumes of distilled water were added to the blood samples for hemolysis prior to the addition of six volumes of acetonitrile and then the supernatant after centrifugation was obtained as the extracted blood sample. Urine and bile was collected for a period of 0–24 h post-dose from the rats (n = 4) and the bile duct-cannulated rats (n = 4), respectively, administered orally[^14]C CS-0777 (3 mg/kg, 3.6 MBq/kg). All the samples were stored in a freezer (−20°C) until analysis.

Sample collection for profiling and identification of metabolites in monkeys

For profiling and identification of metabolites in monkeys, blood was collected from the carotid artery of monkeys (n = 2) euthanized by pentobarbital sodium injection at 10 h after oral administration of[^14]C CS-0777 (3 mg/kg, 3.6 MBq/kg). Simultaneously, urine samples were collected for a period of 0–10 h post-dose from the same monkeys. Bile was also collected from the gallbladder using an injection needle attached to a 10-ml syringe. In common with the treatment of rat blood samples, two volumes of distilled water were added to the monkey blood samples for hemolysis prior to the addition of six volumes of acetonitrile and then the supernatant after centrifugation was obtained as the extracted blood sample. All the samples were stored in a freezer (−20°C) until analysis.

In vitro plasma protein binding

The plasma protein bindings of CS-0777 and M1 were determined according to the ultracentrifugation method described previously (Nakai et al., 2004) with some modification. The rat, monkey or human plasma samples spiked with CS-0777 or M1 at the final concentrations of 100, 300 and 1000 ng/ml were incubated at 37°C for 5 min. CS-0777 and M1 were dissolved in methanol and methanol containing 0.0002 N NaOH, respectively, and added to the plasma to make the concentration of these solutions 1%. After the incubation, the samples were transferred to centrifuge tubes (polycarbonate, 0.5ml tube, Hitachi Koki Co., Ltd., Ibaraki, Japan). An aliquot of 0.1 ml plasma sample was collected for determination of the total concentration and the rest of the plasma samples were centrifuged at 5.340 000 x g for 140 min at 4°C using ultracentrifuges (CS120FX and CS150GXL, Hitachi Koki Co., Ltd.) equipped with rotors (S120AT3, Hitachi Koki Co., Ltd.). After centrifugation, the tube was cut off a few millimeters below the top using a tube slicer (CentriTube Slicer, Beckman Instruments, Inc., Brea, CA) to discard the uppermost part of the supernatant fraction containing chylomicrons and very low-density lipoproteins. Then 0.1 ml of the upper part of the middle layer was taken as a supernatant. Samples were analyzed using liquid chromatography (LC)–tandem mass spectrometry (MS/MS).

Phosphorylation and dephosphorylation in rat tissue homogenates

Rat tissue homogenates and erythrocyte lysate were prepared from male Sprague–Dawley rats. Brain, heart, lung, liver, kidney, spleen, thymus, testis, small intestine, muscle and bone marrow were homogenized with 50mM potassium phosphate buffer (PBS) by a Polytron homogenizer (PT1200E, Kinematica AG, Littau, Switzerland) to prepare a 12.5% (w/v) homogenate. The erythrocyte was collected after centrifugation of the blood at 2000 × g for 10 min at 4°C and washed by PBS. The erythrocyte was lysed by 10mM PBS with shaking for 10 min, then 12% (w/v) erythrocyte lysate in 50mM PBS was obtained by adding appropriate quantities of 80mM PBS. For preparation of the homogenates and erythrocyte lysate for dephosphorylation study, 10mM HEPES buffer was used instead of PBS.

For phosphorylation study, the sample in a final volume of 100 μl was incubated at 37°C for 5 min with 438 nM CS-0777 in 50mM PBS, containing 1mM dithiothreitol (DTT), 5mM MgCl2, 1mM ATP, 10mM creatine phosphate, 0.1mg/ml creatine phosphokinase and 10% tissue homogenate or erythrocyte lysate. For dephosphorylation study, the sample in a final volume of 100 μl was incubated at 37°C for 5 min with 438nM M1 in 10mM HEPES, containing 1mM DTT, 5mM EDTA and 10% tissue homogenate or erythrocyte lysate. The reaction was terminated by adding methanol containing 0.02 N HCl. Concerning the phosphorylation study in the blood, rat and monkey blood specimens were obtained from six male Sprague–Dawley rats and three male cynomolgus monkeys, respectively. Human blood was obtained from healthy volunteers (2 males and 2 females) according to a protocol approved by the Research Ethics Committee at Daiichi Sankyo Co. Ltd. Blood was collected using a heparinized syringe. The blood (100μl) was incubated at 37°C with 29.2nM CS-0777 for 5 min. The reaction was terminated by adding methanol containing 0.02 N HCl.
Sample analysis

Quantitative analysis of CS-0777 and M1 in blood, plasma, supernatant of plasma after ultracentrifugation and tissue homogenates

The concentrations of CS-0777 and M1 in blood, plasma, supernatant of plasma after ultracentrifugation and tissue homogenate were determined by a validated analytical method using solid-phase extraction of each analyte from the blood, plasma and supernatant of plasma after ultracentrifugation, followed by utilizing an LC-MS/MS system consisting of a Quattro Ultima Pt MS/MS (Micromass UK, Ltd, Manchester, UK) connected to an Alliance 2795 Separations Module (Waters Corp., Milford, MA). The plasma sample (100 µl) including study samples or control plasma (for standard and quality control) were mixed with 0.7 ml of 0.01 N HCl, 50 µl of internal standard (IS, 100 ng/ml C14-CS-0777 and 2H6-C14-M1 in methanol containing 0.0002 N NaOH) and 50 µl of methanol for study samples or standard or quality control samples in methanol containing 0.0002 N NaOH. The mixed samples were applied onto an Empore Disk Plate (UR, 3 M), which had been preconditioned by washing with methanol (0.5 ml x 2), followed by distilled water (0.5 ml x 2). After the Empore Disk Plate had been washed with distilled water (1 ml x 2), the analyte was eluted with 0.25 ml of 0.02 N HCl/methanol into a 96-well collection plate. 0.03 ml of distilled water was added to the eluted solution in the collection plate and injected onto the LC-MS/MS. The blood or tissue homogenate sample mixed with methanol containing 0.02 N HCl (200 µl) including study samples or control blood mixed with methanol containing 0.02 N HCl (for standard and quality control) were mixed with 1 ml of 0.01 N HCl, 50 µl of IS (100 ng/ml C14-CS-0777 and 2H6-C14-M1 in methanol containing 0.0002 N NaOH) and 50 µl of methanol for study samples or standard or quality control samples in methanol containing 0.0002 N NaOH. The mixed samples were centrifuged (CF15R, Hitachi Koki Co., Ltd.) at 15 000 rpm for 3 min (4°C). The supernatant was applied onto the Empore Disk plate and similar extraction operations to the plasma samples were conducted. Regarding the supernatant of plasma after ultracentrifugation, the same procedure as the plasma sample was applied using phosphate buffer saline as a control matrix. An XTerra RP18 column (5 µm, 3.0 mm x 150 mm, Waters Corp.) was used for separation of analytes at a column oven temperature of 35°C. The flow rate was 0.25 ml/min with a mobile phase composed of methanol, distilled water, formic acid and ammonium acetate (550/450/10.77, v/v/v). Ionization was conducted in the positive-ion mode at a source temperature of 150°C. CS-0777 and its IS were analyzed in the multiple reaction monitoring (MRM) mode using the mass transitions of m/z 331 → 148 and m/z 349 → 154, respectively. M1 and its IS were analyzed in MRM mode using the mass transition of m/z 423 → 308 and m/z 429 → 314, respectively. The calibration curves were generated using the analyte to IS peak area ratios by weighted (1/x) least-squares linear regression over the concentration ranges of 0.2–200 ng/ml for CS-0777 and 0.5–500 ng/ml for M1. This assay was well validated, since the precision and accuracy were less than 15% for the quality control samples prepared at low, medium and high concentrations of each analyte (less than 20% at the lower limit of quantification).

Radioactivity analysis

Aliquots of rat bile (20 µl) and urine (25–500 µl) were mixed with 0.5–1 ml of tissue solubilizer (NCS-II; GE Healthcare Japan Corporation, Tokyo, Japan) and 10 ml of scintillation cocktail (Hionic-Fluor; PerkinElmer Life and Analytical Sciences, Waltham, MA). Approximately 1 g of monkey urine and cage washing were mixed with 10 ml of scintillation cocktail (Clear-sol I; Nacalai Tesque, Kyoto, Japan). Radioactivity in the samples was counted using a liquid scintillation counter (Tri-Carb 2300TR; PerkinElmer Life and Analytical Sciences), where the counting was corrected by an external standard method.

For mass balance studies in rats, feces were soaked in distilled water (70–160 ml) and were then homogenized using Polytron (PT10/35; Kinematica AG). Aliquots of the fecal homogenate (0.4 ml) were mixed with 2 ml of NCS-II and 10 ml of Hionic-Fluor, and then were analyzed with a liquid scintillation analyzer. The carcasses after final sampling at 336 h post-dose were solubilized in 5 N NaOH by warming at 50°C for 24 h. Aliquots of the solubilized carcass (0.3 ml) were mixed with 10 ml of Hionic-Fluor and then were analyzed with a liquid scintillation analyzer.

For mass balance study in monkeys, feces were soaked with purified water of about 4-fold (v/v) volume of the feces, and were then homogenized using the Polytron. Then, approximately 0.5 g of the fecal homogenate was solubilized in 1 ml of tissue solubilizing agent by mechanical solubilization using a Biomerit instrument (Sekisui Medical Co. Ltd.) for about 30 min. The solubilized samples were mixed with 10 ml of Hionic-Fluor and then were analyzed with a liquid scintillation analyzer.

For tissue distribution study in rats, an aliquot 0.1 ml of the blood and plasma samples was solubilized with 2 ml of tissue solubilizer Soluene-350 (PerkinElmer, Inc.). Cerebrum, liver and kidney were weighed, dissected and homogenized by Polytron after the addition of about 2-fold volume (v/w) of saline. About 0.5 g of the homogenate was solubilized with 2 ml of Soluene-350. Except for cerebrum, liver and kidney, the tissues were collected entirely or cut to a size of about 0.2 g of their wet weight and solubilized with 2 ml of Soluene-350. In the case of blood, 0.4 ml of benzene was saturated with benzoyl peroxide for decolorization. After the addition of 10 ml of Hionic-fluor, all of the samples were subjected to radioactivity measurement using a 1900CA or 2500TR model liquid scintillation counter (PerkinElmer Life and Analytical Sciences), where counting was collected by an external standard method. The radioactivity concentration was expressed as an equivalent (eq.) value of [14C]CS-0777 per gram or milliliter. The lower limit of quantification (LLOQ) was defined to be twice the background radioactivity.

Sample preparation for qualitative metabolite profiling by RI-HPLC and structure analysis of metabolites

The extracted blood samples were pooled and the pooled sample was concentrated and lyophilized in vacuo. The residues were re-dissolved in a 20% acetonitrile aqueous solution and an aliquot was analyzed by radioactivity detection high-performance liquid chromatography (RI-HPLC), LC-MS and LC-MS/MS for structure analysis.
of the metabolites. The individual urine and bile samples collected were centrifuged and the supernatants were pooled for each matrix. An aliquot of the supernatant was analyzed by RI-HPLC, LC-MS and LC-MS/MS for structure analysis of the metabolites.

**Qualitative metabolite profiling by RI-HPLC and structure analysis of metabolites by LC-MS and LC-MS/MS**

The analysis of metabolite profiles in each matrix was conducted on a Hitachi low-pressure gradient elution HPLC system that consisted of an L-7100 pump, L-7300 column oven, D-7500 chromato-integrator and L-7420 UV–Vis detector or L-7400S UV detector (Hitachi High-Technologies Corp., Tokyo, Japan). The column used was a YMC-Pack Pro C18 (150×6.0 mm ID, S-5 μm, YMC Co., Ltd., Kyoto, Japan) run at 40°C and a flow rate of 1.0 ml/min. The mobile phases A and B were distilled water and acetonitrile, respectively, both containing 0.1% trifluoroacetic acid (v/v). One gradient program began at 20% B, and was increased linearly to 50% B in 30 min and to 80% B in 3 min for analysis of rat blood. The other gradient program began at 12% B, and was increased linearly to 40% B in 30 min and to 70% B in 10 min for analysis of rat and monkey excrement and monkey blood. The metabolites were detected by an ALOKA RLC 700 radio analyzer system (RLC-701 radioanalyzer, LFC-101 liquid flow controller, ALOKA Co., Ltd., Tokyo, Japan) and Clear-sol I was pumped (RLC-701 radioanalyzer, LFC-101 liquid flow controller, ALOKA Co., Ltd., Tokyo, Japan) run at 40°C/C14°C2 and elution flow from HPLC (1 ml/min) was split by a tee splitter to the HPLC system. The LC-MS was conducted using ESI spectrometer (Waters Corp.), which was directly coupled to the HPLC system. The LC-MS was conducted using ESI spectrometer through an ESI interface at a flow rate of 0.1 ml/min.

**Data analysis**

The pharmacokinetic parameters of CS-0777 and M1 in blood were calculated by non-compartmental analysis (NCA) using WinNonlin Professional, Version 4.0.1 or 5.2.1 (Pharsight Corp., Mountain View, CA). The maximum blood concentration (Cmax) and the time to Cmax (Tmax) were obtained based on the observation data. The half-life (t1/2) was calculated by the regression analysis of three or more log-transformed data points in the terminal phase. The area under the concentration versus time curve up to the last measurable time (AUC0-tz) was calculated by the trapezoidal method. AUC up to infinity (AUC0-inf) was determined using the equation:

\[
\text{AUC}_{0-\text{inf}} = \text{AUC}_{0-tz} + C_{tz}/\lambda
\]

where \(C_{tz}\) is the last measurable concentration and \(\lambda\) is the terminal elimination rate constant. The absolute oral bio-availability (F) of CS-0777 and M1 were calculated as a percentage of the ratio of AUC0-inf after oral administration to the mean AUC0-inf after intravenous administration of CS-0777.

Percentage of plasma protein binding of CS-0777 and M1 was calculated as follows: protein binding (\%) = (1 - \(C_t/C_p\))×100, where \(C_t\) is the concentration of test substance in a supernatant sample after ultracentrifugation of plasma, and \(C_p\) is the concentration of test substance in plasma samples.

The in vitro phosphorylation rate of CS-0777 or dephosphorylation rate of M1 in the tissue homogenates and erythrocyte lysate was determined from the concentration of M1 and CS-0777, respectively, at 5 min as the apparent rate (pmol/min/g tissue).

In vitro phosphorylation clearance in rat, monkey and human blood was calculated by dividing the phosphorylation rate at 10 min incubation by the substrate concentration (29.2 nM of CS-0777).

**Model analysis involving reversible metabolism kinetics**

The computer software WinNonlin Professional was used for the model based PK analysis of CS-0777 and M1 after administration of CS-0777 to rats and monkeys. The coefficient of variation (CV, %) was also calculated. The fitting of mean blood CS-0777 and M1 concentrations (\(C_p\) and \(C_{m,p}\), respectively) versus time \(t\) for the four doses (for rats) or three doses (for monkeys) of intravenous and oral administration (\(D_{va}\) and \(D_{po}\), respectively) was performed simultaneously by nonlinear regression.

**Rats**

A one-compartment linear model with reversible metabolism was constructed to describe the absorption and disposition of CS-0777 and M1 following oral and intravenous administration of CS-0777 to rats. The model assumes that the irreversible metabolism of CS-0777 followed by renal and biliary excretion was mainly responsible for the elimination process of the compounds (Figure 1A). The mass-balance equations for this model are described as follows:

(For intravenous administration)

\[
\frac{dC_p}{dt} = -(k_{pm} + k_e) \cdot C_p + \frac{k_{mp} \cdot V_m}{V_p} \cdot C_m
\]

(2)

(For oral administration)

\[
\frac{dC_p}{dt} = \frac{D_{po} \cdot BA \cdot k_a}{V_p} \cdot e^{-k_e \cdot t} - (k_{pm} + k_e) \cdot C_p + \frac{k_{mp} \cdot V_m}{V_p} \cdot C_m
\]

\[
\frac{dC_m}{dt} = \frac{k_{pm} \cdot V_p}{V_m} \cdot C_p - k_{mp} \cdot C_m
\]

where \(V_p\) and \(V_m\) (ml/kg) are the apparent distribution volume of CS-0777 and M1 respectively, \(k_{pm}\) and \(k_{mp}\) (h⁻¹) are the first-order conversion rate constants from CS-0777 to M1 and
from M1 to CS-0777, respectively, $k_e$ (h$^{-1}$) is the elimination rate constant of CS-0777, $k_a$ (h$^{-1}$) is the first-order absorption rate constant of CS-0777 and BA is oral bioavailability of CS-0777.

**Monkeys**

A two-compartment linear model with reversible metabolism was constructed to describe the absorption and disposition of CS-0777 and M1 following oral and intravenous administration of CS-0777 to monkeys. The mass-balance equations for this model, shown schematically in Figure 1(B), are described to as follows:

(For intravenous administration)

$$
\frac{dC_p}{dt} = -(k_{pm} + k_{px} + k_e) \cdot C_p + \frac{k_{mp} \cdot V_m}{V_p} \cdot C_m + \frac{k_{xp}}{V_p} \cdot A_{px}
$$

(5)

$$
\frac{dC_m}{dt} = \frac{k_{pm} \cdot V_p}{V_m} \cdot C_p - k_{mp} \cdot C_m
$$

(6)

$$
\frac{dA_{px}}{dt} = k_{px} \cdot V_p \cdot C_p - k_{xp} \cdot A_{px}
$$

(7)

(For oral administration)

$$
\frac{dC_p}{dt} = \frac{D_{po} \cdot BA \cdot k_a \cdot e^{-k_e \cdot t}}{V_p} - (k_{pm} + k_{px} + k_e) \cdot C_p + \frac{k_{mp} \cdot V_m}{V_p} \cdot C_m + \frac{k_{xp}}{V_p} \cdot A_{px}
$$

(8)

$$
\frac{dC_m}{dt} = \frac{k_{pm} \cdot V_p}{V_m} \cdot C_p - k_{mp} \cdot C_m
$$

(9)

$$
\frac{dA_{px}}{dt} = k_{px} \cdot V_p \cdot C_p - k_{xp} \cdot A_{px}
$$

(10)

where $A_{px}$ (nmol/l/kg) is the amount of CS-0777 in the peripheral compartment, $k_{px}$ and $k_{xp}$ (h$^{-1}$) are transfer rate constants between the central and peripheral compartment for CS-0777, and the other parameters are the same definitions as ones in the rat PK model. Similar to rats, the fitting of mean blood concentrations of CS-0777 and M1 versus time for three doses of intravenous and oral administration was performed simultaneously.

**Calculation of clearance**

The phosphorylation clearance for CS-0777, dephosphorylation clearance for M1 and elimination clearance for CS-0777 ($CL_{phos}$, $CL_{dephos}$ and $CL_{elim}$, respectively) are defined as follows:

$$
CL_{phos} = k_{pm} \times V_p
$$

$$
CL_{dephos} = k_{mp} \times V_m
$$

$$
CL_{elim} = k_e \times V_p
$$

**Results**

**Pharmacokinetics**

**Pharmacokinetic parameters by non-compartmental analysis**

Pharmacokinetic parameters by non-compartmental analysis (NCA) after intravenous and oral administration of CS-0777 at doses of 0.1, 0.3, 1 and 3 mg/kg to rats and at 0.2, 0.5 and 1 mg/kg to monkeys are summarized in Table 1. The AUC of CS-0777 increased with the dose in rats after intravenous or oral administration of CS-0777. For monkeys, the AUC of CS-0777 also increased with the dose after intravenous or oral administration of CS-0777. The mean terminal half-life ($t_{1/2}$) of CS-0777 was in the range between 5.40 and 12.0 h in rats and between 18.1 ($n=1$) and 38.1 h in monkeys. The pharmacologically active metabolite, M1, was observed in the blood from the first observation time point (5 or 15 min post-dose) in both animals after intravenous or oral administration of CS-0777. For rats, the $t_{max}$ of M1 was 4.5–7.5 h after intravenous administration and 8.0–9.5 h after oral administration, and then the blood concentrations of M1 decreased with $t_{1/2}$ of 10.7–12.9 h. The AUC and $C_{max}$ of M1 increased with the dose of CS-0777 after intravenous and oral administration. For monkeys, the $t_{max}$ of M1 was 1.0–2.5 h after intravenous administration and 5.75–8.0 h after oral administration, and then the blood concentrations of M1 decreased with $t_{1/2}$ of 59.9–76.9 h. The AUC and $C_{max}$ of M1...
Pharmacokinetics of CS-0777 in rats and monkey

The mean BA of CS-0777 was 64.2–88.7% in rats and 22.5–26.2% in monkeys. The BA of M1 calculated as the AUC ratio of M1 after oral and intravenous administration of CS-0777 was 67.8–90.8% in rats and 45.3–51.5% in monkeys.

The C_max and AUC of M1 after oral administration of CS-0777 were almost 10-fold or larger than those of CS-0777 in both species. (C_max: 8.0- to 14.0-fold, AUC_0-tz: 13.3- to 29.6-fold in rats and C_max: 27.5- to 36.8-fold, AUC_0-tz: 47.3- to 82.1-fold in monkeys).

### Model analysis involving reversible metabolism kinetics

#### Rats

The blood concentrations of CS-0777 and M1 after intravenous and oral administrations of CS-0777 at doses of 0.1, 0.3, 1 and 3 mg/kg to rats were simultaneously well-fitted to a one-compartment model involving reversible metabolism (Figure 2). The k_pm and k_mp were 0.917 and 0.268 h⁻¹, respectively, the k_d and k_d were 0.260 and 0.323 h⁻¹, respectively, the V_p and V_m were 7.99 and 2.44 l/kg, respectively, and the BA was 0.811. The coefficient of variation (CV, %) values were small (<12%) (Table 2).

The phosphorylation clearance of CS-0777 (CL_phos, 122 ml/min/kg) was 11.2-fold larger than dephosphorylation clearance of M1 (CL_dephos, 10.9 ml/min/kg). The elimination clearance of CS-0777 (CL_elim) was calculated as 34.6 ml/min/kg. The BA calculated by the model analysis was comparable to the value of CS-0777 (64.2–88.7%) estimated based on the NCA (Table 1).

#### Monkeys

The blood concentrations of CS-0777 and M1 after intravenous and oral administrations of CS-0777 at doses of 0.2, 0.5 and 1 mg/kg to monkeys were simultaneously well-fitted to a two-compartment model involving the reversible metabolism (Figure 3). The k_pm and k_mp were 3.06 and 0.327 h⁻¹, respectively; the k_d and k_d were 0.427 and 0.171 h⁻¹, respectively; the V_p and V_m were 4.32 and 2.44 l/kg, respectively, and the BA was 0.465, and CV values were between 6.3–22.2% (Table 2).

The CL_phos and CL_dephos were 220 and 13.3 ml/min/kg, respectively, indicating that CL_phos in monkeys was 16.5-fold larger than CL_dephos. The CL_elim for CS-0777 was calculated as 30.7 ml/min/kg. The BA calculated by the model analysis was 6.3- to 22.2% (Table 2).

Regarding CS-0777, monkeys exhibited a 1.8-fold larger value than rats.

### In vitro plasma protein binding

The mean plasma protein binding of CS-0777 was 97.6–97.9% in rats, 97.7% in monkeys, and 97.1–97.3% in humans at 100–1000 ng/ml. Regarding M1, the mean plasma protein binding was 95.3–95.7% in rats, 93.3–93.8% in monkeys, and 95.3–95.7% in humans at 100–1000 ng/ml.

### Urinary, fecal and biliary excretion of radioactivity

It took 72 and 168 h to reach 90% or more recovery of total radioactivity after oral administration to rats and monkeys.

### Table 1. Pharmacokinetic parameters and bioavailability of CS-0777 and M1 after intravenous administration of CS-0777 to rats and monkeys

| Animal | Dose (mg/kg) | Route (po, iv) | C_max (ng/ml) | t_max (h) | AUC_0–inf (ng·h/ml) | t_1/2 (h) | BA (%) |
|---------|--------------|----------------|---------------|-----------|---------------------|----------|--------|
| Rat | 0.1 | iv | 5.2 ± 0.2 | 12 ± 2.2 | 41 ± 0.2 | 106 ± 0.2 | 4.8 ± 0.3 | 96.4 ± 0.6 |
| | 0.3 | po | 1.89 ± 0.2 | 26.4 ± 3.4 | 4.75 ± 3.0 | 3.02 | 22.1 ± 1.6 | 0.96 ± 0.1 |
| | 1 | po | 5.64 ± 0.6 | 64.3 ± 9.3 | 4.25 ± 2.0 | 9.7 | 8.5 ± 0.9 | 1.02 ± 0.1 |
| | 3 | po | 18.2 ± 2.0 | 124 ± 19 | 3.25 ± 1.5 | 9.8 | 5.7 ± 0.9 | 1.02 ± 0.1 |
| Monkey | 0.1 | iv | 7.99 ± 0.1 | 11.9 ± 1.5 | 4.72 ± 3.0 | 3.02 | 22.1 ± 1.6 | 0.96 ± 0.1 |
| | 0.3 | po | 5.90 ± 0.1 | 61.9 ± 9.5 | 4.25 ± 2.0 | 9.7 | 8.5 ± 0.9 | 1.02 ± 0.1 |
| | 1 | po | 18.3 ± 2.0 | 125 ± 19 | 3.25 ± 1.5 | 9.8 | 5.7 ± 0.9 | 1.02 ± 0.1 |

Each value is the mean ± standard deviation (SD) of four animals in rat study and three animals in monkey study. The BA-value (%) of M1 was calculated by dividing the AUC of M1 after oral administration of CS-0777 by that after intravenous administration of CS-0777 at the same dosage.

iv, intravenous; po, oral; n.a., not applicable; n.c., not calculated.
respectively (Table 3). After oral administration to rats and monkeys, the mean recovery of total radioactivity in excreta 336 and 504 h post-dose accounted for approximately 96 and 98%, respectively. The urinary and fecal excretions of radioactivity up to 336 h post-dose in rats were 13.67 and 82.37%, respectively. The urinary and fecal excretions of radioactivity up to 504 h post-dose in monkeys were 25.45 and 66.28%, respectively. Fecal excretion was the major route of elimination of drug-related radioactivity in both animals. After intravenous administration to rats and monkeys, fecal excretion was also the major route of the elimination, while the recovery of the radioactivity in feces exhibited a slight decrease compared to those after oral administration.

After oral and intravenous administration of [14C]CS-0777 at a dose of 0.5 mg/kg to bile duct-cannulated rats, biliary excretion of the radioactivity was 52.54 and 47.62% of the dose, respectively, up to 48 h post-dose (Table 3).

### Tissue distribution

After oral administration of [14C]CS-0777 at a dose of 1 mg/kg to Sprague–Dawley rats (Table 4), the radioactivity concentrations in most of the tissues reached their maximum at 8 h post-dose. The radioactivity concentrations in most of the tissues were higher than that in the plasma. The tissue to plasma concentration ratio in heart, lung, liver, kidney, adrenal gland, spleen, pancreas and bone marrow were above 10 at 8 h post-dose. The radioactivity concentrations in many of the tissues were not detected at 1 week post-dose and exhibited a similar elimination profile to that in the plasma. In cerebellum and liver, the radioactivity was detected by 1 week, post-dose. In testis, radioactivity was detected by 2 weeks, post-dose.

### In vivo metabolite profiles

Representative radiochromatograms for metabolites of [14C]CS-0777 in rat and monkey blood, bile and urine are
Figure 3. Blood concentration versus time profiles of CS-0777 and M1 after intravenous or oral administration of CS-0777 at a dose of 0.2, 0.5 and 1 mg/kg to monkeys. Blood concentrations are expressed as the mean ± SD of four animals. The symbols represent the observed CS-0777 or M1 concentrations. The solid lines and dashed lines represent the fittings of CS-0777 and M1, respectively, based on the reversible metabolism model analysis indicated in Figure 1(B).

![Graph showing blood concentration versus time for CS-0777 and M1 after intravenous and oral administration to monkeys.](image)

Table 3. Cumulative excretion of radioactivity in bile, urine and feces after administration of [14C]CS-0777 to rats and monkeys.

| Species                  | Route          | Dose (mg/kg) | Period | Bile        | Urine       | Feces       | Total       | Remaining in carcass (%) of dose |
|--------------------------|----------------|--------------|--------|-------------|-------------|-------------|-------------|-------------------------------|
| Bile-duct cannulated rat | Intravenous    | 0.5          | 0–48 h | 52.54 ± 12.95 | NA          | NA          | NA          | NA                            |
|                          | Oral           | 0.5          | 0–48 h | 47.62 ± 4.47  | NA          | NA          | NA          | NA                            |
| Rat                      | Intravenous    | 0.5          | 0–48 h | NA          | 14.83 ± 0.94 | 74.83 ± 1.73 | 92.60 ± 0.73 | 1.42 ± 0.71                     |
|                          | Oral           | 0.5          | 0–48 h | NA          | 15.93 ± 0.95 | 73.87 ± 1.73 | 89.81 ± 1.18 | NA                            |
|                          | Oral           | 0–72 h       | NA     | 15.93 ± 0.95 | 73.87 ± 1.73 | 89.81 ± 1.18 | NA          | NA                            |
|                          | Oral           | 0–336 h      | NA     | 15.93 ± 0.95 | 73.87 ± 1.73 | 89.81 ± 1.18 | NA          | NA                            |
| Monkey                   | Intravenous    | 0.5          | 0–48 h | NA          | 17.43 ± 2.77 | 19.49 ± 13.15| 41.32 ± 10.53| 1.42 ± 0.71                     |
|                          | Oral           | 0–48 h       | NA     | 17.43 ± 2.77 | 19.49 ± 13.15| 41.32 ± 10.53| NA          | NA                            |
|                          | Oral           | 0–168 h      | NA     | 24.85 ± 3.39 | 53.81 ± 3.31 | 84.90 ± 4.40 | NA          | NA                            |
|                          | Oral           | 0–504 h      | NA     | 24.85 ± 3.39 | 53.81 ± 3.31 | 84.90 ± 4.40 | NA          | NA                            |

NA, not applicable. Total excretion in monkey was sum of the urine, feces excretion and cage washing recovery.
shown in Figures 4 and 5, respectively. Radiochromatographic analysis and further LC/MS revealed 12 metabolite peaks in rat and monkey blood, urine and bile. Major components were elucidated as follows: M1 in rat blood, M5 in rat urine, M5 and M8 in rat bile, M1 in monkey blood, M5 in monkey urine and M5 and M10 in monkey bile. The major metabolite in rat and monkey blood, M1 was not detected in rat and monkey urine and bile.

### Identification of metabolites

The structures of the 12 metabolites were elucidated by LC-MS/MS. The elucidated structures of eight metabolites were further identified by comparing their mass spectra and retention times on HPLC with those of synthetic standards. The product ions and their assignments for all of the metabolites are summarized in Table 5.

### Mass spectral fragmentation of CS-0777

The LC/MS spectrum of CS-0777 in positive ion mode showed a protonated molecule [M + H]+ at m/z 343. The LC-MS/MS spectrum of CS-0777, which was obtained by collision-induced dissociation of the ion [M + H]+ at m/z 343, and the proposed fragmentation scheme are shown in the ‘‘Supplementary material’’. The product ion spectrum of m/z 343 produced major fragment ions at m/z 254, 161 and 148. M1: M1 was detected as major metabolite only in blood. In positive ion mode, M1 gave a protonated molecule, [M + H]+ at m/z 423, 80 Da higher than parent CS-0777. The product ion spectrum of m/z 423 and proposed fragmentation are indicated in Table 5 and Supplemental material. The mass fragmentation of M1 was consistent with that of synthesized M1. Therefore, M1 was identified as phosphorylated form of CS-0777.

M2: M2 was also detected in rat and monkey blood. M2 gave a characteristic ([M + H]−H2O)+ ion at m/z 421. Therefore, the molecular weight of M2 was proposed to be 438. The major fragment ions produced from the product ion spectrum of m/z 421 is shown in Table 5 (See M2). Furthermore, the LC-MS/MS spectrum and HPLC retention time of M2 were identical to those of the synthetic standard. Based on these results, M2 was identified as a hydroxylated form of benzyl position of M1.

M3: M3 was only detected in monkey blood. M3 gave a protonated molecule, [M + H]+ at m/z 453, 30 Da higher than M1. The product ion spectrum of m/z 453 gave fragments at m/z 438, 298 and 191 (Table 5). Furthermore, the LC-MS/MS spectrum and HPLC retention time of M3 were identical to those of the synthetic standard. As a result, M3 was identified as the carboxylic acid form of M1.

M4 and M6: M4 was detected in rat urine and M6 was detected in rat and monkey urine. M4 and M6 produced protonated molecule, [M + H]+ at m/z 359, corresponding to the addition of 16 Da relative to CS-0777. The characteristic fragment ions which were assigned to be the ions produced from each [M + H]+ at m/z 359 were indicated in Table 5. Finally, M4 and M6 was determined to be the hydroxy methyl form of CS-0777 and hydroxylated form of the benzyl position of CS-0777, respectively, by comparing mass fragmentation and HPLC retention times with the synthetic standards.

M5: M5 was detected as a major metabolite in rat and monkey except in rat blood. M5 gave a protonated molecule, [M + H]+ at m/z 373, 30 Da higher than that of parent compound and proposed fragmentation are shown in Table 5 and Supplemental material. The LC-MS/MS spectrum and HPLC retention time of M5 were identical to those of the synthetic standard. Therefore, M5 was confirmed as the carboxylic acid form of CS-0777.

M7 and M8: Both M7 and M8 were observed in urine and bile. The protonated molecule, [M + H]+ of M7 was detected at m/z 389, corresponding to an addition of 16 Da to M5. M8 gave a protonated molecule, [M + H]+, at m/z 387, 2 Da lower than that of M7. The fragment ions of M7 and M8 were illustrated in Table 5. Finally, M7 and M8 were estimated to be the hydroxylated form and keto form of M5, respectively.
M9, M10 and M11: M9 was observed in urine and bile of both rats and monkeys. M10 was detected in rat bile and monkey urine and bile. M11 was detected only in monkey bile. Mass spectral analysis of M9 (at m/z 549 in positive mode) indicated loss of glucuronic acid, giving rise to a fragment ion at m/z 373, corresponding to M5. The characteristic fragment ion patterns estimated to be produced from m/z 373 corresponded to the pattern from M5. Also, the fragment ion at m/z 460, corresponding to elimination of the amino-methylpropanol part from m/z 549, indicated that the conjugation site of M9 was at the carboxyl group. M10 and M11 ([M + H]+ at m/z 480 and m/z 430, respectively) exhibited 107 and 57 Da at a higher molecular weight than that of M5, respectively, suggesting that M10 and M11 were a taurine conjugate and glycine conjugate of M5, respectively. In the product ion spectrum of M10 and M11, similar characteristic fragment ions were detected. That is, M9, M10 and M11 were identified as an acyl glucuronide, a taurine conjugate and a glycine conjugate of M5, respectively.

M12: M12 was detected only in monkey urine. The product ion spectral analysis of M12 (at m/z 535 in positive ion mode) indicated loss of glucuronic acid, giving rise to a fragment ion at m/z 341, corresponding to M4 and M6. The fragment ions at m/z 341 exhibited similar fragmentation patterns to the ions of M6. Finally, M12 was confirmed to be an O-glucuronide of M6.

Figure 4. Radiochromatographic profiles of CS-0777 and metabolites in blood, urine and bile of rats after a 3 mg/kg oral administration of [14C]CS-0777. The HPLC condition in analysis of blood was different from that of the other matrices.
Phosphorylation and dephosphorylation in rat tissue homogenates

The phosphorylation activity of CS-0777 was not observed in the brain, heart and lung among the rat tissue homogenates examined in this study (Figure 6). Among the tissues found to have phosphorylation activity, the liver exhibited the highest phosphorylation rate (72.8 pmol/min/g tissue) and next was erythrocyte (26.8 pmol/min/g tissue). Moderate phosphorylation was observed in thymus (21.2 pmol/min/g tissue) and bone marrow (18.6 pmol/min/g tissue). The testis, kidney, small intestine, spleen and muscle had weak phosphorylation activity (<10 pmol/min/g tissue).

Dephosphorylation activity of M1 was detected in all tissues examined in this experiment (Figure 6). The dephosphorylation rates were very high in the brain and kidney (280 and 154 pmol/min/g tissue, respectively). The lung, liver, spleen, testis and bone marrow exhibited a moderate dephosphorylation rate (40.7–76.1 pmol/min/g tissue) and the phosphorylation rate was low (16.2–23.6 pmol/min/g tissue) in muscle, small intestine, thymus and heart. The lowest rate was observed in the erythrocyte (8.47 pmol/min/g tissue).

The intrinsic phosphorylation clearance (CL\textsubscript{phos}) of CS-0777 in blood was nearly the same between rat and monkey (0.0377 and 0.0363 ml/min/ml blood, respectively; Table 6). In human blood, the intrinsic CL\textsubscript{phos} (0.0209 ml/min/ml

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Figure 5. Radiochromatographic profiles of CS-0777 and metabolites in blood, urine and bile of monkeys after a 3 mg/kg oral administration of [14C]CS-0777.
Table 5. Summary of mass spectral data for CS-0777 and its metabolites in rats and monkeys.

| Parent or Metabolite | Chemical Structure and Proposed Fragmentation Scheme | Molecular Ion [M+H]+ | Characteristic Product ions |
|----------------------|--------------------------------------------------|---------------------|----------------------------|
| **Parent**           | ![Chemical Structure](image)                      | 343                 | 326, 308, 254, 161, 148, 143, 131, 117, 105 |
| **M1**               | ![Chemical Structure](image)                      | 423                 | 405, 325, 308, 268, 254, 232, 161, 148, 143 |
| **M2**               | ![Chemical Structure](image)                      | 421                 | 323, 306, 288, 266, 252, 232, 159, 148, 131 |
| **M2’**              | ![Chemical Structure](image) (as [(M+H)-H2O]+)    | 421                 | 323, 306, 288, 266, 252, 232, 159, 148, 131 |
| **M3**               | ![Chemical Structure](image)                      | 453                 | 355, 338, 320, 298, 284, 232, 191, 173, 148 |
| **M4**               | ![Chemical Structure](image)                      | 359                 | 341, 324, 306, 288, 270, 252, 175, 164, 161, 159, 148, 147, 146, 131, 129, 117, 105, 94 |
| **M5**               | ![Chemical Structure](image)                      | 373                 | 356, 338, 320, 284, 191, 173, 148, 136 |
| Parent or Metabolite | Chemical Structure and Proposed Fragmentation Scheme | Molecular Ion [M+H]^+ | Characteristic Product ions |
|----------------------|------------------------------------------------------|-----------------------|----------------------------|
| M6                   | ![Chemical Structure of M6](Image)                  | 359                   | 341, 324, 306, 288, 252, 209, 192, 174, 159, 148, 131 |
| M7                   | ![Chemical Structure of M7](Image)                  | 389                   | 371, 354, 336, 318, 300, 282, 209, 192, 189, 174, 161, 148 |
| M8                   | ![Chemical Structure of M8](Image)                  | 387                   | 370, 352, 334, 298, 205, 177, 149, 148 |
| M9                   | ![Chemical Structure of M9](Image)                  | 549                   | 531, 514, 496, 460, 373, 356, 338, 320, 284, 191, 148 |
| M10                  | ![Chemical Structure of M10](Image)                 | 480                   | 462, 445, 427, 391, 355, 338, 320, 298, 268, 175, 173, 148, 147, 146 |
| M11                  | ![Chemical Structure of M11](Image)                 | 430                   | 413, 395, 341, 338, 320, 268, 175, 173, 148, 147, 146 |
| M12                  | ![Chemical Structure of M12](Image)                 | 535                   | 341, 324, 306, 288, 252, 148 |
blood) of CS-0777 was about 55% of it in rat and monkey. By correction of the clearance with the blood volume per kg body weight, the CL_phos was 2.03, 2.65 and 1.54 ml/min/kg in rats, monkeys and humans, respectively.

**Discussion**

After the administration of CS-0777 within the dose range examined, its pharmacologically active metabolite, M1, exhibited higher AUC than CS-0777 and almost linear pharmacokinetics in rats and monkeys (Table 1). The oral bioavailabilities of CS-0777 and M1 after the administration of CS-0777 were high (>60%) in rats, while those of CS-0777 (<26.2%) and M1 (<51.5%) were moderate in monkeys. The plasma protein binding of CS-0777 and M-1 were high (>90%) and had minimal species difference among rat, monkey and human.

The radioactivity derived from [14C]CS-0777 after oral dosing was mainly excreted into feces in rats (82.37%) and monkeys (66.28%). The amount of radioactivity excreted in bile (52.54%) at 48 h after intravenous administration was 77.5% of the amount excreted in feces (67.77%) in rats (Table 3). Therefore, the radioactivity was considered to be excreted in feces mainly via bile, and partly via gastrointestinal secretion in rats. CS-0777 was hardly detected in the bile and urine after administration of [14C]CS-0777 to rats and monkeys (Figures 4 and 5), indicating that the metabolism would be predominant factor for disposition of CS-0777. The majority of the radioactivity was excreted in a few days in rats while it required one week or more to excrete 90% of the radioactivity in monkeys. This difference might reflect the difference of the $t_{1/2}$ of CS-0777 and M1 between rats (9.43–11.6 h) and monkeys (38.1–62.2 h).

A proposed scheme of metabolic pathways of CS-0777 in rats and monkeys is presented in Figure 7. Metabolite profiling and identification of metabolites indicated that the primary metabolic route of CS-0777 consists of three biotransformation pathways: (1) formation of active metabolite (M1) via phosphorylation of aminoalcohol moiety; (2) hydroxylation at the methyl group of the benzene ring (M4); and (3) hydroxylation at the benzyl position (M6).

Almost linear pharmacokinetics of M1 was confirmed as well as CS-0777 and the elimination phase of M1 was in parallel with CS-0777 (Figures 2 and 3). Therefore, the phosphorylation of CS-0777 was considered to be reversible. In fact, fructosamine 3-kinase (FN3K) and the fructosamine 3-kinase-related protein (FN3K-RP) (Yonesu et al., 2011), and alkaline phosphatase (ALP) (Sakurai et al., 2013) were identified as the responsible enzymes for the phosphorylation of CS-0777 and the dephosphorylation of M1, respectively. Fingolimod, another S1P agonist, had been reported to also have a reversible phosphorylation process in its disposition (Zollinger et al., 2011) as well as an endogenous substance, sphingosine (Liu et al., 2012). Sphingosine kinase type 2 and partially by sphingosine kinase type 1 (Paugh et al., 2003) and lipid phosphate phosphatase (LPP) 1a and 3 (Mechtcheriakova et al., 2007; Yamanaka et al., 2008) for phosphorylation and dephosphorylation, respectively, were involved with the disposition of fingolimod as well as sphingosine. It is very interesting to note that the enzymes responsible for the reversible phosphorylation of CS-0777, FN3K, FN3K-RP and ALP have no relevance for the disposition of sphingosine in spite of the fact that CS-0777 has similar activation process as fingolimod.

Further metabolites of M1, a hydroxylated form of M1 (M2) and carboxylic acid form of M1 (M3, only in monkeys), was detected in the blood, but not in the urine and bile (Figures 4 and 5). In addition, it was observed that M1 barely underwent oxidation in liver microsomes compared to the oxidation of CS-0777 (data not shown). Therefore, M2 and M3 were estimated to be produced via reversible phosphorylation from M5 and M6 (described below), respectively, as well as M1.

M4 underwent further oxidation, forming the carboxylic acid metabolite, M5. M5 was phosphorylated to M5 or metabolized successively via hydroxylation at the benzyl position to M7 and formed its dehydrogenated metabolite, M8. Moreover, M5 was conjugated with glucuronic acid (M9), taurine (M10) and glycine (M11). As another metabolic pathway, M6 underwent further oxidation at the methyl group of the benzene ring, forming M7 or phosphorylation, forming M2. Therefore, the formation of M7 will be involved with two metabolic pathways from M5 and M6.

Although M3 was detected only in monkey blood and more conjugated metabolites were detected in monkey bile compared to rat bile (Figures 4 and 5), minimal difference in metabolism exists qualitatively between rats and monkeys.

The pharmacokinetic model involving the reversible metabolism and unidirectional elimination of CS-0777 through oxidative metabolism (Figure 1) were well described through the blood concentrations of CS-0777 and M1 in both animals after administration of CS-0777. The CL_phos in rats (122 ml/min/kg) and monkeys (220 ml/min/kg) surpassed the
hepatic blood flow of rats (55.2 ml/min/kg) and monkeys (43.6 mL/kg) (Davis & Morris, 1993), indicating the contribution of extra hepatic tissue to the phosphorylation of CS-0777. In fact, phosphorylation activity was observed in various tissue homogenates in rats (Figure 6) and in rat, monkey and human blood (Table 6). However, the phosphorylation clearance of CS-0777 in rat blood (2.03 ml/min/kg) was a much lower value compared to the estimated in vivo $CL_{phos}$. The in vivo $CL_{phos}$ of monkeys was 1.8-fold high compared to that of rats. This difference seems to resemble the difference of $CL_{phos, body}$ evaluated from in vitro blood phosphorylation activity (Table 6) between rats and monkeys with differential of 1.3-fold. In vivo $CL_{elim}$ was similar between rats (34.6 ml/min/kg) and monkeys (30.7 ml/min/kg). In contrast, $t_{1/2}$ of CS-0777 in monkeys was approximately 1.5- to 5-fold longer than that of rats, indicating that in vivo $CL_{elim}$ did not directly affect the elimination of CS-0777. The $CL_{dephos}$ of M1 was the smallest one among the other CLs in both animals and was considered to be a rate-limiting process of the disposition of CS-0777. Then, the 1.5-fold large $CL_{phos}/CL_{dephos}$ value in monkeys (16.5) compared to that in rats (11.2) indicates more phosphorylation-biased equilibrium in

Figure 7. Chemical structure of [14C]CS-0777 and proposed metabolic pathways of CS-0777 in rats and monkeys. *Denotes position of 14C label.

Table 6. Phosphorylation clearance of CS-0777 in rat, monkey and human blood.

|                        | Rat                  | Monkey              | Human               |
|------------------------|----------------------|---------------------|---------------------|
| Phosphorylation clearance (ml/min/ml blood) | 0.0376 ± 0.0039 | 0.0361 ± 0.0058 | 0.00208 ± 0.0030 |
| Blood volume$^a$ (ml/kg) | 54                   | 73.4                | 74.3                |
| Phosphorylation clearance$^b$ (ml/min/kg) | 2.03 ± 0.21 | 2.65 ± 0.42 | 1.54 ± 0.23        |

$^a$Davis & Morris (1993).

$^b$CL$_{phos, body}$ (ml/min/kg) = CL$_{phos, blood}$ (ml/min/ml blood) $\times$ blood volume (ml/kg).
monkeys than in rats. In addition, contrary to rats, the blood concentrations of CS-0777 and M1 in monkeys were well fitted to two-compartment distribution. This may indicate that tissue distribution of CS-0777 is different between rats and monkeys. Since terminal \( t_{1/2} \) was represented as a function of the hybrid of the rate constant among the compartments, the longer \( t_{1/2} \) of CS-0777 in monkeys than in rats could be observed.

The estimated \( V_p \) and \( V_m \) were high (>2.44 l/kg in both animals; Table 2). In fact, the tissue concentrations of radioactivity derived from \( {}^{14} \)C-CS-0777 exhibited 10-fold or more higher compared to that in the plasma at 24 h after oral administration to rats except for brain, skin, testis, eyeball and thymus (Table 4). Among them, the \( V_m \) was smaller than that \( V_p \) in both animals (Table 2). This may be caused by the physicochemical property of M1, that would be higher water solubility and lower membrane permeability compared to CS-0777 due to the highly polar phosphate group in its structure.

It is already known that many compounds showed reversible metabolism pharmacokinetics (Cheng & Jusko, 1993). As described above, sphingosine and fingolimod have a reversible metabolism process via phosphorylation. Among them, CS-0777 is the first example of the model analysis involving reversible metabolism via phosphorylation. In addition, it was revealed that fingolimod phosphate did not exceed fingolimod itself in exposure in clinical (Kovarik et al., 2009). On the other hand, the exposure of M1 was much larger than that of CS-0777 in clinical (Moberly et al., 2012a) and the superiority of phosphorylation is maintained in humans as well as rats and monkeys.

The phosphorylation of CS-0777 was observed in the liver, kidney, spleen, thymus, testis, small intestine, muscle, bone marrow and erythrocyte in rats (Figure 6), and also in the brain, lung and heart at higher concentration of CS-0777 (data not shown). The observed tissues’ FN3K and FN3K-RP activities in mice and rats (liver, lung, heart, kidney, brain, spleen, muscle, testis, thymus and erythrocyte) (Delplanque et al., 2004) were consistent with the tissues observed phosphorylation of CS-0777. The dephosphorylation of M1 in rats was observed in all tissues examined and thought to be also consistent with the distribution of the ALP that is well known to exist widely in the body (Van Hoof & De Broe, 1994). The blood (erythrocyte) and liver were estimated to be major responsible tissues for the phosphorylation of CS-0777 from superiority in rate at the same substrate concentration (Figure 6). In vitro phosphorylation clearance in rat blood (2.03 ml/min/kg) was inconsistent with the model fitted value of 122 ml/min/kg in rat. Unfortunately, phosphorylation clearance in the liver, which was estimated in a similar manner to the blood, was an approximately similar value to that in the blood (data not shown). Therefore, we could not explain the inconsistency between in vivo and in vitro situations only by taking account of the contribution of the tissues other than the blood. Since CS-0777 is a highly protein binding compound, we guess we should have estimated the in vitro phosphorylation clearance with the correction of an unbound fraction of CS-0777 in blood and tissue homogenates (Poulin et al., 2012). In addition, there remains a difficulty of acquiring in vitro–in vivo extrapolation (IVIVE) of metabolism via non-CYP enzymes such as aldehyde oxidase (Akabane et al., 2012) and N-glucuronidation (Kilford et al., 2009). Currently, the optimal condition for testing the activities of FN3K and FN3K-RP is still unclear besides the stability of these enzymes during the examination. Therefore, the experimental condition in this study may not be suitable for the confirmation of IVIVE of phosphorylation of CS-0777. We are focused on elucidating tissues involved in the phosphorylation of CS-0777 and dephosphorylation of M1 and we have largely succeeded in the aims in this study.

The phosphorylation activity of CS-0777 in the blood maintained regardless of species (Table 6), indicating that whole blood (erythrocyte) would be one of the important organs for the phosphorylation of CS-0777.

Conclusions

In this study, orally administered CS-0777 was absorbed well, distributed widely in tissues and slowly excreted into urine and bile in rats and monkeys. The pharmacologically active form, M1 via reversible phosphorylation, was detected in the blood as the main metabolite in rats and monkeys. The main elimination process of CS-0777 was oxidative biotransformation followed by the conjugation.

Declaration of interest

The authors report no declarations of interest.

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Supplementary material available online.
Supplementary Figures S1 and S2