Alpha-Amylase Inhibitor, CS-1036 Binds to Serum Amylase in a Concentration-Dependent and Saturable Manner

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ABSTRACT

(2R,3R,4R)-4-hydroxy-2-(hydroxymethyl)pyrrolidin-3-yl 4-O-(6-deoxy-β-D-glucopyranosyl)-α-D-glucopyranoside (CS-1036), which is an α-amylase inhibitor, exhibited biphasic and sustained elimination with a long t1/2 (18.4–30.0 hours) in rats and monkeys, but exhibited a short t1/2 (3.7–7.9 hours) in humans. To clarify the species differences in the t1/2, the plasma protein binding of CS-1036 was evaluated by ultrafiltration. A concentration-dependent and saturable plasma protein binding of CS-1036 was observed in rats and monkeys with the dissociation rate constant (Kd) of 8.95 and 27.2 nM, and maximal binding capacity (Bmax) of 52.8 and 22.1 nM, respectively. By the assessments of the recombinant amylase and immunoprecipitation, the major binding protein of CS-1036 in rats was identified as salivary amylase (Kd = 5.64 nM). CS-1036 also showed concentration-dependent and saturable binding to human salivary and pancreatic amylase, with similar binding affinity in rats. However, the protein binding of CS-1036 was constant in human plasma (≤10.2%) due to the lower serum amylase level compared with rats and monkeys. From the calculation of the unbound fraction (fu) in plasma based on in vitro Kd and Bmax, the dose-dependent increase in fu after oral administration is speculated to lead to a dose-dependent increase in total body clearance and a high area under the curve/dose at lower doses, such as 0.3 mg/kg in rats.

Abbreviations: AGP, α1-acid glycoprotein; AUC, area under the plasma concentration versus time curve; AUClast, area under the plasma concentration versus time curve up to the last quantifiable time; Bmax, maximal binding capacity; Cb, bound concentration; CL, total body clearance; CLr, renal clearance; Cmax, maximum plasma concentration; CS-1036, (2R,3R,4R)-4-hydroxy-2-(hydroxymethyl)pyrrolidin-3-yl 4-O-(6-deoxy-β-D-glucopyranosyl)-α-D-glucopyranoside; Ct, total concentration; Cu, unbound concentration; CL, unbound concentration; Fe, urinary excretion ratio; fu, unbound fraction; Foral, oral bioavailability; GFR, glomerular filtration rate; IP, immunoprecipitation; Kd, dissociation rate constant; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PBS, phosphate-buffered saline; PK, pharmacokinetics; rAmy1, recombinant rat salivary amylase; tmax, time to reach maximum plasma concentration; VSS, volume of distribution at steady state.

Introduction

(2R,3R,4R)-4-hydroxy-2-(hydroxymethyl)pyrrolidin-3-yl 4-O-(6-deoxy-β-D-glucopyranosyl)-α-D-glucopyranoside (CS-1036), shown in Fig. 1, inhibits both salivary and pancreatic α-amylase in the gastrointestinal tract and therefore blocks starch digestion, and as a consequence depresses glucose absorption (Honda et al., 2013). α-Amylase is responsible for starch digestion in digestive tracts. Salivary and pancreatic amylases, which exhibit high homologies in humans (>95%, α-amylase-1: locus NP_004029; α-amylase 2A: locus NP_000690; and α-amylase 2B: locus NP_066188), are known as subtypes for α-amylase (Gumucio et al., 1988; Mashige et al., 1989). Serum amylase is known as a biomarker for the pancreatic function (Ranson, 1997), and both amylases are almost equally secreted into human plasma. The docking study using a pancreatic amylase X-ray crystal structure inferred that the pyrrolidine ring of CS-1036 interacted with the active center, and the disaccharide on the other side of CS-1036 bound to the starch binding site of pancreatic amylase (data not shown).

CS-1036 was mainly excreted into urine from systemic circulation, and exhibited biphasic elimination profiles with relatively long t1/2 (18.4–30.0 hours at ≥1 mg/kg for intravenous administration) in rats and monkeys (Honda et al., 2013). The total body clearance (CL) of CS-1036 in rats and monkeys (2.25–3.44 ml/min/kg) was comparable to the glomerular filtration rate (GFR) (Davies and Morris, 1993; Honda et al., 2013). In rats and monkeys, CS-1036 exhibited a small volume of distribution at steady state (ca. 0.2 l/kg) comparable to the extracellular fluid volume (Davies and Morris, 1993), and low oral bioavailability (Foral) of 1% to 2% (Honda et al., 2013). In general, the renal clearance (CLr) is determined by GFR, the unbound fraction (fu), renal secretion, and renal reabsorption, indicating that plasma protein binding plays an important role in the CLr (Rowland and Tozer, 2011). Serum albumin, which is a major target of the plasma protein binding of drugs, is generally more abundant (520–750 μM, molecular weight 67,000) than drug concentration, and therefore, the protein binding of most of the drugs that bind to albumin is constant in the therapeutic concentration range (Wright et al., 1996; Rowland and Tozer, 2011). At high drug concentrations in plasma or in the presence of competitors, a saturation of plasma protein binding to serum albumin occurs, and two binding sites with different binding affinities to albumin are occasionally attributed to the concentration-dependent binding (Urjen et al., 1981; Stoeckel and Koup, 1984; Roberts et al., 2011). The other well-known drug-binding plasma protein is α1-acid glycoprotein.
glycoprotein (AGP), and some drugs show nonlinear plasma protein binding due to a limited binding capacity to AGP (9–24 μM, molecular weight 42,000) (Wright et al., 1996; Fuse et al., 1999; Fuse et al., 2000).

To clarify the causes for long t1/2 values, the plasma protein binding of CS-1036 in rats and monkeys was evaluated by ultrafiltration, and the pharmacokinetics (PK) and plasma protein binding of CS-1036 in humans were also investigated to evaluate species differences in the elimination phase. Furthermore, the target binding protein of CS-1036 in plasma was identified, and the relationship between plasma protein binding and in vivo elimination of CS-1036 was investigated.

### Materials and Methods

CS-1036 and its internal standard substance, R-187454 (R-H form of CS-1036), were synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Amylase antibody (MAK < H-S-AMY > M-Tuf66C7-IgG) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The control antibody (IgG from murine serum), albumin from rat serum, albumin from human serum, amylase from human saliva, and amylase from human pancreas were purchased from Sigma-Aldrich Co., (St. Louis, MO). Other reagents and solvents used were commercially available and either of guaranteed, high-performance liquid chromatography or liquid chromatography–mass spectrometry grade.

**Animals.** All animal experimental procedures were performed in accordance with the institutional animal care guidelines of Daiichi Sankyo Co., Ltd. Male F344/DuCrjHrl (F344) rats at 7 weeks of age and male Sprague-Dawley (Crl:CD(SD)) rats at 8 weeks of age were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Kanagawa, Japan) and then used after an acclimatization period of 5–8 days. Male cynomolgus monkeys at 3–5 years of age were purchased from CLEA Japan, Inc. (Tokyo, Japan) after quarantine and reared for over 3 years in a controlled animal area. The monkeys used in this study were 6–8 years of age and had not been administered any drugs for at least one week before the experiment. Diets were freely accessible for rats and supplied once daily for monkeys. Water was given ad libitum throughout the experiments.

**Construction of Recombinant Rat Salivary Amylase.** After the isolation of the rat parotid gland from Sprague-Dawley rats under diethyl ether in-solution, rat parotid gland was reared for over 3 years in a controlled animal area. The monkeys used in this study were 6–8 years of age and had not been administered any drugs for at least one week before the experiment. Diets were freely accessible for rats and supplied once daily for monkeys. Water was given ad libitum throughout the experiments.

**Protein Binding Assay of CS-1036 by Ultrafiltration.** For in vitro plasma protein binding, blood was taken from the abdominal aorta of F344 rats, from the femoral vein of monkeys, and from the antecubital median vein of healthy male volunteers (30–41 years old), the latter with confirmed, informed consent. The plasma was obtained by centrifugation. Aliquots of 10 μl of each CS-1036 solution were mixed with 990 μl of rat, monkey, and human plasma (final concentrations 22.7, 113, 227, 1,130, and 11,300 nM; n = 4), PBS (n = 2, for the evaluation of adsorption to the equipment for ultrafiltration), rat albumin solution (40 mg/ml, n = 2), control or amylase IP-plasma of rats (n = 2), control or amylase IP-plasma of human (final concentrations 11.3, 22.7, and 113 nM; n = 2), rat salivary amylase solution (final concentrations 11.3, 22.7, 113, 227, 1,130, and 11,300 nM; n = 2), human albumin (50 mg/ml, final concentrations of CS-1036: 22.7, 113, 227, and 11,300 nM; n = 2), human salivary or pancreatic amylase solution (10 U/ml, final concentrations of CS-1036: 22.7, 113, 227, and 1130 nM; n = 2). After the samples were incubated at 37°C for 10 minutes, 50 μl of the sample was used for the determination of the total concentration (Ct) of CS-1036 by liquid chromatography–tandem mass spectrometry (LC-MS/MS) measurement, and the rest of the sample was applied to ultrafiltration by Centrifree (YM-30, Millipore Corp., Billerica, MA). After centrifugation, 50 μl of filtered sample was used for the determination of the unbound concentration (Cu) of CS-1036. The adsorption of CS-1036 to the ultrafiltration equipment was confirmed to be negligible (data not shown).

**PK in Healthy Male Subjects.** The study was approved by the ethics committee/institutional review board, and was conducted in compliance with ethical principles that originated in the Declaration of Helsinki and in accordance with the International Conference on Harmonization Guideline E6 for good clinical practice and other applicable local regulations. After being advised of the nature, purpose, and the possible risks and benefits associated with the study, all subjects were required to give informed and written consent prior to participation in the study. All subjects were in good health as determined by their medical history, physical examination, and clinical laboratory tests conducted prior to the study based on inclusion and exclusion criteria. Sixty subjects were healthy men aged 21–43 years with a body mass index between 19.2 and 25.0 kg/m² and without a known medical reason to prevent participation. This was a randomized, double-blind, placebo-controlled, sequential, dose-escalating study. Subjects received a single dose of CS-1036 (5, 10, 20, 40, 80, or 160 mg, n = 8 each) or placebo (n = 12) as an oral solution under fasting conditions, and completed breakfast within approximately 15 minutes after administration. CS-1036 from the bottle was dissolved in a solution containing the same ingredients as the placebo. Serial blood samples were drawn prior to dosing and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36, and 48 hours after the dose. Then, blood samples were centrifuged to yield plasma. Urine samples were collected continuously up to 48 hours after the dose. The total volume of urine at each period was recorded. The plasma and urine samples were stored at −20°C.

**Measurement of Amylase Activity.** The amylase activities in rat plasma, recombinant amylase, and corresponding IP samples diluted with PBS were measured using an amylase activity measurement kit (Sekisui Medical Co.,...
Ltd., Tokyo, Japan). The absorbance of the sample at 660 nm was normalized by that at 405 nm, and the amylase activity was calculated from the mean value of the duplicate (VERSAmax, Molecular Devices, LLC, Sunnyvale, CA). The enzyme calibrator plus (Sysmex Corp., Kobe, Hyogo, Japan) was used as the reference standard to adjust the amylase activity of recombinant rat salivary amylase. The specific activity of recombinant rat salivary amylase (U/mg protein) was determined and the amylase concentration in rat plasma (mg or µg protein/ml) was subsequently calculated.

**PK Analysis.** The PK parameters of CS-1036 in plasma were calculated using WinNonlin Professional (Version 4.0.1 or 5.2; Pharsight Corp., Mountain View, CA) based on a noncompartmental method in the same way as previously reported (Honda et al., 2013). In humans, the urinary excretion ratio (Fe) was determined by multiplying the urinary concentration, urine volume and by 100, and then normalized by the dose. The CLR was determined by dividing the amount of drug excreted in urine up to 48 hours after the dose by the plasma concentration versus time curve (AUC) up to 48 hours.

**Data Analysis.** The fu and the protein binding (%) were calculated by the following Eqs. 1 and 2, respectively:

\[
fu = \frac{Cu}{Ct} \quad (1)
\]

\[
\text{Protein binding(%) } = \frac{Ct - Cu}{Ct} \times 100 \quad (2)
\]

To estimate the dissociation rate constant (KD) and maximal binding capacity (Bmax) of CS-1036, the Cu and fu of CS-1036 were fit to the following model (Eq. 4, which was converted from the Langmuir model; Eq. 3) for saturable binding equilibriums (Wright et al., 1996), using the computer software WinNonlin Professional (Version 4.0.1. or 5.2.1, Pharsight Corp.).

\[
Cb = \frac{B_{\text{max}} \cdot Cu}{K_D + Cu} \quad (3)
\]

\[
fu = \frac{Cu}{Cu + Ch} = \frac{K_D + Cu}{K_D + Cu + B_{\text{max}}} \quad (4)
\]

where Cb is the bound concentration and Bmax is defined by multiplying the number of CS-1036 binding sites in the protein by the binding protein concentration.

Using the estimated KD and Bmax, the fu of CS-1036 in rat and monkey PK studies (Honda et al., 2013) was calculated by the following Eq. 5, which was converted from Eq. 4.

\[
fu = \frac{Ct - K_D - B_{\text{max}} + \sqrt{(Ct - K_D - B_{\text{max}})^2 + 4 \cdot K_D \cdot Ct}}{2 \cdot Ct} \quad (5)
\]

**Statistical Analysis.** The reported AUC up to the last quantifiable time (AUClast) of CS-1036 in rats and monkeys (Honda et al., 2013) was normalized by the dose. AUC/dose was compared among the dose levels by a Siegel-Tukey test. P values of less than 0.05 were considered to be statistically significant.

**Results**

**In Vitro Plasma Protein Binding of CS-1036 in Rats, Monkeys, and Humans.** Plasma protein binding in rats, monkeys, and humans was evaluated by ultrafiltration. The fu of CS-1036 in rat and monkey plasma in the nominal concentration range of 2.7–11,300 nM increased in a concentration-dependent manner (Fig. 2, A and B), but over the same concentration range the fu in humans (0.898 ± 0.032) showed no concentration dependency (Fig. 2C). The plasma protein binding of CS-1036 in rats and monkeys was well explained by the Langmuir model. As shown in Table 1, the KD of CS-1036 in monkey plasma (27.2 nM) was approximately 3-fold higher than that in rat plasma (8.95 nM). The Bmax of CS-1036 in monkey plasma (22.1 nM) was approximately half of that in rat plasma (52.8 nM).

**CS-1036 Binding to Rat Albumin, Rat Salivary Amylase, and Human Salivary and Pancreatic Amylase.** As shown in Fig. 3A, CS-1036 does not bind to either rat or human albumin. The Bmax of CS-1036 in rat plasma (52.8 nM) was much lower than the plasma albumin and the AGP level in rats (ca 350 µM and 7 µM, respectively) (Kawase et al., 2013). Therefore, the binding proteins of
CS-1036 in plasma were considered to be minor proteins. The serum amylase, which is potentially the same as the pharmacological target protein of CS-1036 and tested clinically for serum chemistry (Ranson, 1997), was considered as a possible candidate for the binding protein of CS-1036. For the evaluation of the target binding protein of CS-1036 in plasma, the recombinant rat salivary amylase was constructed and used with an adjustment of the amylase activity almost the same as that in rat plasma (1,220 U/l), since rats showed a remarkable concentration-dependency in the plasma protein binding. Then, CS-1036 showed a concentration-dependent and saturable binding to rat salivary amylase as observed in rat plasma, and the $K_D$ and $B_{\text{max}}$ of CS-1036 to rat salivary amylase were estimated to be 5.64 and 95.4 nM, respectively (Fig. 3B and Table 1). CS-1036 also showed a concentration-dependent binding to human salivary and pancreatic amylase, with $K_D$ values of 4.92 and 8.74 nM and $B_{\text{max}}$ values of 52.1 and 81.6 nM, respectively (Fig. 3C and D, respectively). The amylase activities of human salivary and pancreatic amylase in this evaluation were set much higher ($\geq$10-fold) than those of in vivo serum amylase (Takasaka et al., 2003).

**Effects of IP on CS-1036 Binding.** After the IP of amylase in rat plasma by anti-human salivary amylase antibody, the crossreactivity of the amylase antibody was confirmed by the decrease of amylase activities in rat IP-plasma (<20% of the control) (Fig. 4C). In a similar manner, the plasma protein binding of CS-1036 in rat amylase IP-plasma decreased to 22.2%–36.3% of rat control IP-plasma (Fig. 4A). The enzyme activity of rat amylase IP-amylase was almost completely depressed to 2.9% of rat control IP-amylase (Fig. 4C). The protein binding of CS-1036 in rat amylase IP-amylase decreased to 6.4%–10.4% of rat control IP-amylase (Fig. 4B). Thus, the enzyme activity in rat amylase IP- and control IP-plasma showed a correlation with the fu of CS-1036 in rat amylase IP and control IP salivary amylase.

**PK of CS-1036 in Healthy Male Subjects.** The plasma concentration-time profiles and PK parameters of CS-1036 in healthy male subjects after oral administration are shown in Fig. 5 and Table 2, respectively. The time to reach maximum plasma concentration ($t_{\text{max}}$) was observed at 1.25–1.75 hours after administration. The maximum plasma concentration ($C_{\text{max}}$) and AUC up to infinity (AUC$_{0-\infty}$) of CS-1036 increased in an almost dose-proportional manner over the dose range of 5–160 mg. The Fe was $<1\%$, which was similar to the $F_{\text{oral}}$ in monkeys (Honda et al., 2013). The $t_{1/2}$ (3.7–7.9 hours) was shorter than those in intravenous administration ($\geq3$ mg/kg (23.6–30.0 hours) and in oral administration at 10 mg/kg (12.6–18.3 hours) in rats and monkeys (Honda et al., 2013), which were determined with sufficient time points at the elimination phases. Less than 1% of the dose was excreted into urine up to 48 hours after oral administration, and the CL$_R$ of CS-1036 was relatively constant (82.3–119.1 ml/min), regardless of the dose.

**Estimation of the fu in Rat and Monkey PK Studies.** The fu for the plasma concentrations of CS-1036 in rats and monkeys, which was previously reported (Honda et al., 2013), was calculated by Eq. 5 using the in vitro $K_D$ and $B_{\text{max}}$ of CS-1036 in plasma (Table 1). After intravenous administration of CS-1036, the fu for CS-1036 was calculated to be $\geq0.88$ up to 2 hours after the dose for rats (Fig. 6A) and up to 4 hours after the dose for monkeys for all doses (Fig. 6B). It decreased with time and became almost constant near the end of the elimination phase (ca. 0.15 for rats and 0.56 for monkeys). After oral administration of CS-1036, the maximum values of the fu for CS-1036 were estimated to be $\geq0.88$ up to 2 hours after the dose for rats (Fig. 6A) and up to 4 hours after the dose for monkeys for all doses (Fig. 6B). It decreased with time and became almost constant near the end of the elimination phase (ca. 0.15 for rats and 0.56 for monkeys).

### TABLE 1

$K_D$ and $B_{\text{max}}$ of CS-1036 in rat and monkey plasma, and rat and human amylase

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>$K_D$</th>
<th>CV (%)</th>
<th>$B_{\text{max}}$</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Plasma</td>
<td></td>
<td>8.95</td>
<td>26.2</td>
<td>52.8</td>
<td>8.6</td>
</tr>
<tr>
<td>Salivary amylase</td>
<td></td>
<td>5.64</td>
<td>19.9</td>
<td>95.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Monkey Plasma</td>
<td></td>
<td>27.2</td>
<td>38.6</td>
<td>22.1</td>
<td>20.9</td>
</tr>
<tr>
<td>Human Salivary amylase</td>
<td></td>
<td>4.92</td>
<td>50.3</td>
<td>52.1</td>
<td>11.8</td>
</tr>
<tr>
<td>Pancreatic amylase</td>
<td></td>
<td>8.74</td>
<td>44.3</td>
<td>81.6</td>
<td>11.0</td>
</tr>
</tbody>
</table>
was observed at the $t_{\text{max}}$ of CS-1036, which exhibited a remarkable dose-dependency especially in rats (0.2 to 0.9 from 0.3 to 10 mg/kg) compared with that in monkeys (0.6 to 0.9 from 0.3 to 10 mg/kg). Then, the fu was decreased with time, and finally became almost constant near the end of the elimination phase (ca. 0.15 for rats and 0.56 for monkeys), being similar values to those after intravenous administration. In addition, the higher $K_D$ and lower $B_{\text{max}}$ in monkeys compared with rats were considered to result in smaller changes in the fu of CS-1036 in monkeys (0.6–0.9) compared with corresponding values in rats (0.2–0.9). At 0.3 mg/kg after oral administration to rats, the plasma concentration of CS-1036 was lower than the $B_{\text{max}}$ in the overall observed period, and then the fu was almost constant (ca. 0.2).

**Discussion**

The reasons for species differences in the $t_{1/2}$ of CS-1036 were investigated from the viewpoint of plasma protein binding. A concentration-dependent and saturable plasma protein binding of CS-1036 was observed in rats and monkeys, but not in humans (where fu was consistently $\approx 0.9$). In rat plasma, CS-1036 exhibited a lower $K_D$ (8.95 nM) and higher $B_{\text{max}}$ (52.8 nM) than in monkeys ($K_D$ 27.2 nM and $B_{\text{max}}$ 22.1 nM). On the other hand, CS-1036 did not bind to rat albumin, and it showed a lower $B_{\text{max}}$ than the plasma concentrations of albumin (ca. 350 μM) and AGP (ca. 7 μM) in rats (Kawase et al., 2013). As a candidate for CS-1036 binding protein, serum amylase, which is known as a biomarker for pancreatic function (Ranson, 1997) and is potentially the same as the pharmacological target protein of CS-1036, was evaluated. Rats exhibit salivary (Amy1) and pancreatic (Amy2-1 and Amy2-2) amylase (Sugino, 2007), and the former was reported as a major subtype in rat plasma (Takeuchi et al., 1975; Tomasik et al., 1995). Then, the concentration-dependent binding to recombinant rat salivary amylase was observed with a similar $K_D$ (5.64 nM) to values obtained from rat plasma (8.95 nM). In the IP of rat amylase, anti-human salivary amylase antibody showed suppressive effects on the plasma amylase activity and protein binding of CS-1036 in rat plasma and rat salivary amylase. From these results, CS-1036 binding protein in rat plasma was identified as salivary amylase, which is a novel finding to show that drugs bind to serum amylase. The $\alpha$-glycosidase inhibitor acarbose, which also exhibits $\alpha$-amylase inhibitory effects (Hiele et al., 1992), showed relatively long $t_{1/2}$ in rats, dogs, and humans (23–24 hours) with a concentration-dependent plasma protein binding in rats and dogs (Ahr et al., 1989), suggesting that serum amylase may contribute to the plasma protein binding of acarbose.

Human salivary and pancreatic amylases exhibit relatively high homologies with rat amylases (>80%), and both amylases are almost
equally secreted into human plasma (Gumucio et al., 1988; Mashige et al., 1989). Indeed, CS-1036 also showed concentration-dependent and saturable plasma protein binding to human pancreatic and salivary amylase with $K_D$ values of 8.74 nM and 4.92 nM, respectively, which were similar to values seen in rat plasma and rat salivary amylase. However, the concentration-dependent and saturable plasma protein binding of CS-1036 was not observed in human plasma. Amylase activity in human plasma was reported to be 40–162 U/l (Takasaka et al., 2003), and that in rats was 10–30-fold higher (1,220 U/l) in the present study, indicating that the $B_{\text{max}}$ is much higher in rats than in humans. Thus, in rats and monkeys, since the plasma protein binding of CS-1036 was concentration-dependent and saturable, the $t_{1/2}$ became longer (18.4–30.0 hours for $\geq$1 mg/kg i.v.) (Honda et al., 2013). In contrast, in humans, the plasma protein binding was effectively saturated and constant, and the $t_{1/2}$ became shorter (3.7–7.9 hours). In the case of the dipeptidyl peptidase-4 inhibitor linagliptin, which also binds to the pharmacologic target protein and exhibits concentration-dependent and saturable plasma protein binding, the model-based simulation suggests that the higher $B_{\text{max}}$ leads to a longer $t_{1/2}$ of the total concentration (Retlich et al., 2009). CS-1036 is a small and highly hydrophilic molecule with a trisaccharide structure (molecular weight 441.43) and is excreted into urine (Honda et al., 2013). As anticipated, given that the plasma protein binding appears to be saturated in humans at the doses studied, the CLR of CS-1036 in

### TABLE 2

PK parameters of CS-1036 after single oral administration of CS-1036 to male healthy subjects

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>AUC$_{last}$ (ng·h/ml)</th>
<th>AUC$_{0-inf}$ (ng·h/ml)</th>
<th>$t_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>$t_{1/2}$ (h)</th>
<th>CL/F (ml/min)</th>
<th>$V_d/F$, apparent volume of distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5.047 (37.8)</td>
<td>9.080 (69.1)</td>
<td>1.250 (1.00, 2.00)</td>
<td>1.456 (25.3)</td>
<td>3.686 (106.9)</td>
<td>9173 (69.2)</td>
<td>2928.9 (33.1)</td>
</tr>
<tr>
<td>10</td>
<td>11.461 (27.2)</td>
<td>15.167 (22.7)</td>
<td>1.500 (1.00, 3.00)</td>
<td>2.128 (28.6)</td>
<td>3.696 (11.3)</td>
<td>10987 (22.7)</td>
<td>3515.6 (23.8)</td>
</tr>
<tr>
<td>20</td>
<td>27.098 (51.9)</td>
<td>34.008 (52.6)</td>
<td>1.750 (1.00, 2.50)</td>
<td>4.568 (56.1)</td>
<td>4.501 (39.1)</td>
<td>9802 (52.6)</td>
<td>3818.6 (57.4)</td>
</tr>
<tr>
<td>40</td>
<td>43.111 (43.2)</td>
<td>54.744 (42.5)</td>
<td>1.750 (1.00, 2.00)</td>
<td>6.352 (31.8)</td>
<td>5.131 (46.5)</td>
<td>12177 (42.5)</td>
<td>5407.3 (38.1)</td>
</tr>
<tr>
<td>80</td>
<td>112.165 (20.3)</td>
<td>128.230 (18.4)</td>
<td>1.750 (1.00, 4.00)</td>
<td>17.059 (22.2)</td>
<td>6.261 (45.8)</td>
<td>10400 (18.4)</td>
<td>5636.6 (45.0)</td>
</tr>
<tr>
<td>160</td>
<td>233.675 (29.4)</td>
<td>246.375 (30.1)</td>
<td>1.250 (0.25, 12.00)</td>
<td>25.855 (39.3)</td>
<td>7.871 (47.7)</td>
<td>10824 (30.1)</td>
<td>7375.6 (35.8)</td>
</tr>
</tbody>
</table>

$V_d/F$, apparent volume of distribution.
humans was almost constant and was comparable to values reported in an earlier study (Davies and Morris, 1993), indicating that CS-1036 is eliminated mainly by glomerular filtration. In rats and monkeys, CS-1036 is also thought to be eliminated mainly by glomerular filtration. The plasma protein binding is one of the key determinant factors for the CLR, and the concentration-dependent plasma protein binding should alter the CL in a dose-dependent manner, although the CL in rats and monkeys was almost constant at doses of 0.3–10 mg/kg for intravenous administration (Honda et al., 2013).

To explain why the dose-dependent CL was not observed, given the concentration-dependent and saturable plasma protein binding seen in rats and monkeys, the time-dependent changes of the fu were simulated for rats and monkeys. For intravenous administration, the fu was close to 1 hour and reached up to 2 hours for rats and 4 hours for monkeys (Fig. 6), and AUC_{0-2h} for rats and AUC_{0-4h} for monkeys corresponded to >85% of total AUC for rats and 90% for monkeys, respectively. Therefore, the CL in both animals was thought to be constant in this dose range. On the other hand, for oral administration, the fu changed dramatically with the doses (Fig. 7), because a low

**Fig. 7.** Time profiles of the calculated fu of CS-1036 after oral administration of CS-1036 to rats (A) and monkeys (B). The fu was calculated from the mean plasma concentrations, which were presented in an earlier report (Honda et al., 2013).

**Fig. 8.** Relationships between dose-normalized AUC and dose after intravenous (A) and oral administration (B) to rats and monkeys. The reported AUC_{last} (Honda et al., 2013) was normalized by the dose. The data are expressed as the mean ± S.E. (n = 3 or 4). *p < 0.05.
$F_{\text{oral}}$ of CS-1036 in rats and monkeys (1% to 2%) leads to the much lower plasma concentrations ($C_{\text{max}}$ 36.7–646 nM for rats and 11.8–344 nM for monkeys at 0.3–10 mg/kg) than those after intravenous administration (Honda et al., 2013). Therefore, for oral administration, the CL is considered to increase with the dose in proportion with the increase of the fu at higher doses. Indeed, the AUC/dose, which is inversely correlated with the CL, was higher in rats at low doses, such as 0.3 mg/kg for oral administration, than at higher doses (Fig. 8). In a similar case, the concentration-dependent and saturable plasma protein binding of linagliptin led to nonlinear pharmacokinetics after both oral and intravenous administration with less than a dose-proportional increase in exposure in the clinical trials (Heise et al., 2009; Retlich et al., 2010; Graefe-Mody et al., 2012). In addition, for the case of insulin-like growth factor-1, which also exhibited concentration-dependent and saturable plasma protein binding, the CL for total insulin-like growth factor-1 increased with the dose, whereas the AUC/dose was decreased with the dose (Mizuno et al., 2001). On the other hand, for oral administration in monkeys, the higher AUC/dose at the lowest dose was not observed, but the AUC/ dose of CS-1036 increased with the dose. This might be due to low exposures of CS-1036 close to the lower limit of quantification at lower doses in the PK study for oral administration, and there were fewer time points at which concentrations were determined at the lower doses compared with higher doses in monkeys.

In conclusion, CS-1036 showed concentration-dependent and saturable plasma protein binding in rats and monkeys, but apparently not in humans. $\alpha$-Amylase in plasma was identified as the target plasma binding protein, and species differences in serum amylase levels in humans.

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