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

Tomohiro Honda, Yoko Kaneno-Urasaki, Takashi Ito, Takako Kimura, Nobuko Matsushima, Hiromi Okabe, Atsushi Yamasaki and Takashi Izumi

Drug Metabolism & Pharmacokinetics Research Laboratories, R&D Division, Daiichi Sankyo Co., Ltd., Tokyo, Japan (T.H., Y.K., T. Ito, T. Izumi)
Medicinal Chemistry Research Laboratories, R&D Division, Daiichi Sankyo Co., Ltd., Tokyo, Japan (T.K.)
Translational Medicine & Clinical Pharmacology Department, R&D Division, Daiichi Sankyo Co., Ltd., Tokyo, Japan (N.M., H.O.)
Global Project Management Department, R&D Division, Daiichi Sankyo Co., Ltd., Tokyo, Japan (A.Y.)
Running Title:

CS-1036 Binds to Serum Amylase

Address correspondence to:

Tomohiro Honda,
Drug Metabolism & Pharmacokinetics Research Laboratories, R&D Division,
Daiichi Sankyo Co., Ltd., 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan
Tel: +81-3-3492-3131
Fax: +81-3-5436-8567
E-mail: honda.tomohiro.us@daiichisankyo.co.jp

Number of:

Text Pages: 29
Tables: 2
Figures: 8
References: 24
Abstract: 205 words
Introduction: 503 words
Discussion: 1092 words
Abbreviations:

AGP, \( \alpha_1 \)-acid glycoprotein

AUC, area under the plasma concentration versus time curve

AUC\text{last}, area under the plasma concentration versus time curve up to the last quantifiable time

Cb, bound concentration

CL, total body clearance

CL\text{R}, renal clearance

\( C_{\text{max}} \), maximum plasma concentration

Ct, total concentration

Cu, unbound concentration

\( F_e \), urinary excretion ratio

fu, unbound fraction

\( F_{\text{oral}} \), oral bioavailability

GFR, glomerular filtration rate

IP, immunoprecipitation

\( K_D \), dissociation rate constant

LC-MS/MS, liquid chromatography-tandem mass spectrometry

PBS, phosphate buffered saline

PK, pharmacokinetics

\( t_{\text{max}} \), time to reach maximum plasma concentration

\( V_{\text{ss}} \), volume of distribution at steady state
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 a biphasic and sustained elimination with a long $t_{1/2}$ (18.4–30.0 hours)
in rats and monkeys, but exhibited a short $t_{1/2}$ (3.7–7.9 hours) in humans. To clarify the
species differences in the $t_{1/2}$, 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 ($K_D$) of
8.95 and 27.2 nM and the $B_{\text{max}}$ of 52.8 and 22.1 nM, respectively. By the assessments of
the recombinant amylase and the immunoprecipitation, the major binding protein of
CS-1036 in rats was identified as salivary amylase ($K_D$; 5.64 nM). CS-1036 also showed
a concentration-dependent and saturable binding to human salivary and pancreatic
amylase with similar binding affinity to rats. However, the protein binding of CS-1036
was constant in human plasma ($\leq 10.2\%$) due to the lower serum amylase level compared
to animals. From the calculation of the $fu$ in the plasma based on in vitro $K_D$ and $B_{\text{max}},$
the dose-dependent increase in the $fu$ after oral administration is speculated to lead a
dose-dependent increase in the CL, and a high AUC/dose at the lower doses such as 0.3
mg/kg in rats.
Introduction

CS-1036 ((2R,3R,4R)-4-hydroxy-2-(hydroxymethyl)pyrrolidin-3-yl 4-O-(6-deoxy-β-D-glucopyranosyl)-α-D-glucopyranoside, 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). Alpha-amylase is responsible for the starch digestion in digestive tracts. Salivary and pancreatic amylases, which exhibits high homologies in humans (>95%, AMY1: NP_004029, AMY2A: NP_000690 and AMY2B: 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 the biphasic elimination profiles with relatively long $t_{1/2}$ (18.4–30.0 hours at ≥1 mg/kg for intravenous administration) in rats and monkeys (Honda et al., 2013). The 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 ($V_{ss};$ ca 0.2 l/kg) comparable to the extracellular fluid volume (Davies and Morris, 1993), and low oral bioavailability ($F_{oral},$ 1%–2%) (Honda et al., 2013). In general, the renal clearance ($CL_R$) is determined by GFR, the unbound fraction (fu), renal secretion and renal reabsorption, indicating that the
plasma protein binding plays an important role in the $\text{CL}_R$ (Rowland and Tozer, 2011). Serum albumin, which is a major target of the plasma protein binding of drugs, is generally more abundant ($520–750 \mu\text{M}$, molecular weight: 67,000) than drug concentration, and therefore, the protein binding of most of the drugs which bind to albumin is constant in the therapeutic concentration range (Wright et al., 1996; Rowland and Tozer, 2011). At high drug concentrations in the plasma or in the presence of competitors, a saturation of plasma protein binding to serum albumin occurs, and two binding sites with different binding affinity in albumin are occasionally attributed to the concentration-dependent binding (Urien et al., 1981; Stoeckel and Koup, 1984; Roberts et al., 2011). The other well-known drug binding plasma protein is $\alpha_1$-acid glycoprotein (AGP), and some drugs show a nonlinear plasma protein binding due to the limited binding capacity to AGP ($9–24 \mu\text{M}$, molecular weight: 42,000) (Wright et al., 1996; Fuse et al., 1999; Fuse et al., 2000).

To clarify the causes for long $t_{1/2}$, 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 the species difference in the elimination phase. Furthermore, the target binding protein of CS-1036 in plasma was identified, and the relationship between the plasma protein binding and the in vivo elimination of CS-1036 was investigated.
Materials and Methods

Materials.

CS-1036 and its internal standard substance, R-187454 (\(^2\)H\(_5\) form of CS-1036), were synthesized at Daiichi Sankyo Co., Ltd. Amylase antibody (MAK <H-S-AMY>M-Tu66C7-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, USA). 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/DuCrCrlj (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 at 6–8 years of age that had not been administered any drugs for at least one week before the experiment, were used in this study. 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 (rAmy1).

After the isolation of the rat parotid gland from SD rats under diethyl ether inhalation, rat parotid RNA was extracted by TRIzol reagent (Life Technologies Corp., Carlsbad, CA, USA) and rAmy1 cDNA was obtained by a reverse transcriptional-polymerase chain reaction (Invitrogen SuperScriptIII One Step RT-PCR system, Life Technologies Corp.) using the forward primer 5’-ggg gac aag ttt gta caa aaa agcagg ctt cac cAT GAG AGA ATA CTG CCA ACA TCA TAG C-3’, and the reverse primer 5’-ggg gac cac ttt gta caa gaa agc tgg gtc GAT TTT TGA CTC TAC ATG GAT TGC AAT G-3’. Amplified rAmy1 was purified by an Invitrogen PureLink PCR Purification kit (Life Technologies Corp.) and ligated to pDONR221 by a Gateway BP reaction prior to transformation into Escherichia coli strain TOP10 competent cells (Life Technologies Corp.). The consistency of rAmy1 in the vector with reported rAmy1 (NM_001010970) was confirmed by the DNA sequence analysis. A Gateway LR reaction was performed using the rAmy1 gene/pDNOR211 and pcDNA_DEST40 expression vector (Life Technologies Corp.). The rAmy1/pcDNA_DEST40 was amplified and purified by an Invitrogen PureLink HiPure Plasmid Maxiprep Kit (Life Technologies Corp.). After transfection to the FreeStyle 293 expression system, culture media was purified by His-tag affinity purification using Ni-NTA agarose to obtain the recombinant rat salivary amylase (0.71 mg protein/ml, 6 × histidine and V5 epitope fused to the C-terminus of rat salivary amylase).

Immunoprecipitation (IP).

The rat salivary amylase solution (10.7 µg protein/ml in phosphate buffered saline
(PBS, pH7.4)) and rat plasma (pooled, n = 15) were mixed with either control or amylase antibody solutions (1 mg protein/ml), using a ratio of 5/1 (v/v) and incubated at 4°C overnight. Then, protein G plus agarose (SantaCruz Technologies, Inc., Santa Cruz, CA, USA) was added to the sample, using a ratio of 3/4 (v/v, protein G agarose/plasma or rat salivary amylase solution) and incubated at 4°C for more than 1 hour. The sample was centrifuged at approximately 160 g for 1 minute at 4°C and the supernatant was used as IP-plasma or IP-amylase for amylase activity measurement and ultrafiltration.

**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) 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, 1130, 2270 and 11300 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-amylase (final concentrations: 11.3, 22.7 and 113 nM; n = 2), rat salivary amylase solution (final concentrations: 11.3, 22.7, 113, 227, 1130 and 2270 nM, n = 2), human albumin (50 mg/ml, final concentrations of CS-1036: 22.7, 113, 227 and 1130 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, USA). 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 (ICH) 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 to 43 with a body mass index between 19.2 and 25.0 kg/m², and were not found to have any 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 in 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.

**Determination of CS-1036 Concentration by LC-MS/MS.**

CS-1036 concentrations in the plasma, in vitro samples, their filtered samples and tissue samples were determined in accordance with a reported quantification method using LC-MS/MS with minor modifications (Honda et al., 2013).

**Measurement of Amylase Activity.**

The amylase activities in rat plasma, recombinant amylase and their 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, USA). 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 the plasma were calculated using WinNonlin Professional (Version 4.0.1 or 5.2; Pharsight Corp., Mountain View, CA, USA) based on a non-compartmental method in the same way as previously reported (Honda et al., 2013). In humans, the urinary excretion ratio ($F_e$) was determined by multiplying the urinary concentration, urine volume and by 100, and then normalized by the dose. The $CL_R$ 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 (%) was calculated by the following equations 1 and 2, respectively.

$$fu = \frac{Cu}{Ct} \quad \text{(1)}$$

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

To estimate the dissociation rate constant ($K_D$) and $B_{\text{max}}$ of CS-1036, the $Cu$ and $fu$ of CS-1036 were fit to the following model (equation 4, which was converted from the Langmuir model; equation 3) for saturable binding equilibriums (Wright et al., 1996), using the computer software WinNonlin Professional (ver. 4.0.1 or 5.2.1, Pharsight Corp., Mountain View, CA, USA).
Cb = \frac{B_{\text{max}} \cdot Cu}{K_D + Cu} \quad (3)

\text{fu} = \frac{Cu}{Cu + Cb}

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

where Cb is the bound concentration and \( B_{\text{max}} \) is defined by multiplying the number of CS-1036 binding sites in the protein by the binding protein concentration.

Using the estimated \( K_D \) and \( B_{\text{max}} \), the fu of CS-1036 in rat and monkey PK studies (Honda et al., 2013) was calculated by the following equation 5, which was converted from equation 4.

\text{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 area under the AUC up to the last quantifiable time (AUC_{last}) 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.

The plasma protein binding in rats, monkey and humans was evaluated by ultrafiltration. The fu of CS-1036 in rat and monkey plasma in the nominal concentration range of 22.7–11300 nM increased in a concentration-dependent manner (Figs. 2A 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 $K_D$ of CS-1036 in monkey plasma (27.2 nM) was approximately 3-fold higher than that in rat plasma (8.95 nM). The $B_{max}$ 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 binds to neither rat nor human albumin. The $B_{max}$ of CS-1036 in rat plasma (52.8 nM) was much lower than the plasma albumin and 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 the 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.
(1220 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 the $K_D$ of 4.92 and 8.74 nM and the $B_{\text{max}}$ of 52.1 and 81.6 nM, respectively (Figs. 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 in 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 cross reactivity of the amylase antibody was confirmed by the decrease of the 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$_{0-\text{inf}}$) of CS-1036 increased in an almost dose-proportional manner over the dose range of 5–160 mg. The $F_e$ 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 ≥3 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 CLR 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 study.

The fu for the plasma concentrations of CS-1036 in rats and monkeys, which was previously reported (Honda et al., 2013), was calculated by equation 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 ≥0.88 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 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 to that in monkeys (0.6 to 0.9 from 0.3 to 10 mg/kg), then 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 (Fig. 7). The $B_{\text{max}}$ in rats and monkeys was between the $C_{\text{max}}$ at 0.3 and 1 mg/kg for both animals, and then the fu at the $t_{\text{max}}$ resulted in wide ranges for oral administration. In addition, the higher $K_D$ and the lower $B_{\text{max}}$ in monkeys compared to those in rats were considered to result in smaller changes in the fu of CS-1036 in monkeys (0.6–0.9) compared to those 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 $\geq 0.9$). In rat plasma, CS-1036 exhibited a lower $K_D$ (8.95 nM) and higher $B_{\text{max}}$ (52.8 nM) than those 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 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 potentially the same as the pharmacological target protein of CS-1036, was evaluated. Rats exhibit salivary ($Amy1$) and pancreatic amylase ($Amy2-1$ and $Amy2-2$) (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 those 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. Alpha-glycosidase inhibitor, acarbose, which also exhibits $\alpha$-amylase inhibitory effects (Hiele et al., 1992), showed relatively long $t_{1/2}$ in rats and 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 a concentration-dependent and saturable plasma protein binding to human pancreatic and salivary amylase with the $K_D$ of 8.74 and 4.92 nM, respectively, which were similar to that 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. The 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 (1220 U/L) in the present study, indicating that the $B_{\text{max}}$ is much higher in rats than that 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). While, in humans, the plasma protein binding was effectively saturated and constant, the $t_{1/2}$ became shorter (3.7–7.9 hours). In the case of dipeptidyl peptidase-4 (DPP-4) inhibitor, linagliptin, which also binds to the pharmacological target protein and exhibits a concentration-dependent and saturable plasma protein binding, the model-based simulation suggests that the higher $B_{\text{max}}$ lead to a longer $t_{1/2}$ of the Ct (Retlich et al., 2009).

CS-1036 is a small and highly hydrophilic molecule with a trisaccharide structure (molecular weight; 441.43), and was 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 $\text{CLR}$ of CS-1036 in humans was almost constant and comparable to GFR (Davies and Morris, 1993), indicating that CS-1036 is eliminated mainly by...
glomerular filtration. In rats and monkeys, CS-1036 is also supposed 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 CLR and then 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 clarify the discrepancy why the dose-dependent CL was not observed even with the concentration-dependent and saturable plasma protein binding 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 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 corresponding 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 F_{oral} of CS-1036 in rats and monkeys (1%–2%) leads to the much lower plasma concentrations (C_{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, at low doses such as 0.3 mg/kg for oral administration in rats was higher than that in higher doses (Fig. 8). In a similar case, a concentration-dependent and saturable plasma protein binding of linagliptin lead 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 (IGF-1), which also exhibited a concentration-dependent and saturable plasma protein binding, the CL for total IGF-1 increased with the dose, and then 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 less time points of which concentrations were determined at the lower doses compared to those at higher doses in monkeys.

In conclusion, CS-1036 showed a concentration-dependent and saturable plasma protein binding in animals, but apparently not in humans. Alpha-amylase in the plasma was identified as the target plasma binding protein, and the species difference of the serum amylase level ($B_{max}$) is suggested to influence the elimination rate of CS-1036.
Acknowledgements

We would like to acknowledge Keiichi Fusegawa for assistance with the animal experiments, Dr. Toshimasa Ohnishi for assistance with data analysis, and Dr. Takuo Washio and Tsunenori Nakazawa for the clinical data.
Authorship Contributions

Participated in research design: Honda, Ito, Matsushima, Yamasaki and Izumi.

Conducted experiments: Honda, Matsushima, Yamasaki and Kaneno-Urasaki.

Contributed new reagents or analytic tools: Honda and Kaneno-Urasaki.

Performed data analysis: Honda, Kimura, Matsushima and Okabe.

Wrote or contributed to the writing of the manuscript: Honda, Ito and Izumi.
References


Retlich S, Duval V, Ring A, Staab A, Hüttner S, Jungnik A, Jaehde U, Dugi KA, and Graefe-Mody U (2010) Pharmacokinetics and pharmacodynamics of single rising intravenous doses (0.5 mg–10 mg) and determination of absolute bioavailability of the dipeptidyl peptidase-4 inhibitor linagliptin (BI 1356) in


Figure Legends

Fig. 1
Chemical structure of CS-1036.

Fig. 2
Relationship between the Ct and fu of CS-1036 in rat (A), monkey (B) and human plasma (C). The curve indicates the fitting results to the Langmuir model for the estimation of $K_D$ and $B_{max}$.

Fig. 3
Relationship between the Ct and fu of CS-1036 in rat and human albumin (A), rat salivary amylase (B), human salivary amylase (C) and pancreatic amylase (D). The curve indicates the fitting results to the Langmuir model for the estimation of $K_D$ and $B_{max}$.

Fig. 4
Protein binding (A and B) and amylase activity (C) in rat IP-plasma (A) and rat IP-amylase (B) after treatment of control and amylase antibody. The data are expressed as the mean of duplicate. Protein binding of rat control IP-salivary amylase at 11.3 nM of the Ct was not determined due to the quantification limit of the Cu.
Fig. 5
Plasma concentration-time profiles of CS-1036 after single oral administration of
CS-1036 to healthy male subjects. The data are expressed as the mean ± S.D. (n = 7 or
8).

Fig. 6
Time profiles of the calculated fu of CS-1036 after intravenous administration of
CS-1036 to rats (A) and monkeys (B). The fu was calculated from the mean plasma
concentrations, which was cited from the previous report (Honda et al., 2013).

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 was cited from the previous 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
Tables

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>$K_D$ (nM)</th>
<th>CV (%)</th>
<th>$B_{\text{max}}$ (nM)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Plasma</td>
<td>8.95</td>
<td>26.2</td>
<td>52.8</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>Salivary amylase</td>
<td>5.64</td>
<td>19.9</td>
<td>95.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Monkey</td>
<td>Plasma</td>
<td>27.2</td>
<td>38.6</td>
<td>22.1</td>
<td>20.9</td>
</tr>
<tr>
<td>Human</td>
<td>Salivary amylase</td>
<td>4.92</td>
<td>50.3</td>
<td>52.1</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>Pancreatic amylase</td>
<td>8.74</td>
<td>44.3</td>
<td>81.6</td>
<td>11.0</td>
</tr>
</tbody>
</table>
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>( \text{AUC}_{\text{last}} ) (ng·h/ml)</th>
<th>( \text{AUC}_{0-\text{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>( Vd/F ) (l)</th>
<th>Fe (%)</th>
<th>CL_R (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5.047</td>
<td>9.080</td>
<td>1.250</td>
<td>1.456</td>
<td>3.686</td>
<td>9173</td>
<td>2928.9</td>
<td>0.85</td>
<td>119.099</td>
</tr>
<tr>
<td></td>
<td>(37.8)</td>
<td>(69.1)</td>
<td>(1.00, 2.00)</td>
<td>(25.3)</td>
<td>(106.9)</td>
<td>(69.2)</td>
<td>(33.1)</td>
<td>(29.4)</td>
<td>(29.2)</td>
</tr>
<tr>
<td>10</td>
<td>11.461</td>
<td>15.167</td>
<td>1.500</td>
<td>2.128</td>
<td>3.696</td>
<td>10987</td>
<td>3515.6</td>
<td>0.74</td>
<td>94.660</td>
</tr>
<tr>
<td></td>
<td>(27.2)</td>
<td>(22.7)</td>
<td>(1.00, 3.00)</td>
<td>(28.6)</td>
<td>(11.3)</td>
<td>(22.7)</td>
<td>(23.8)</td>
<td>(27.5)</td>
<td>(21.9)</td>
</tr>
<tr>
<td>20</td>
<td>27.098</td>
<td>34.008</td>
<td>1.750</td>
<td>4.568</td>
<td>4.501</td>
<td>9802</td>
<td>3818.6</td>
<td>0.75</td>
<td>82.280</td>
</tr>
<tr>
<td></td>
<td>(51.9)</td>
<td>(52.6)</td>
<td>(1.00, 2.50)</td>
<td>(56.1)</td>
<td>(39.1)</td>
<td>(52.6)</td>
<td>(57.4)</td>
<td>(55.2)</td>
<td>(10.3)</td>
</tr>
<tr>
<td>40</td>
<td>43.111</td>
<td>54.744</td>
<td>1.750</td>
<td>6.352</td>
<td>5.131</td>
<td>12177</td>
<td>5407.3</td>
<td>0.71</td>
<td>92.078</td>
</tr>
<tr>
<td></td>
<td>(43.2)</td>
<td>(42.5)</td>
<td>(1.00, 2.00)</td>
<td>(31.8)</td>
<td>(46.5)</td>
<td>(42.5)</td>
<td>(38.1)</td>
<td>(21.0)</td>
<td>(19.0)</td>
</tr>
<tr>
<td>80</td>
<td>112.165</td>
<td>128.230</td>
<td>1.750</td>
<td>17.059</td>
<td>6.261</td>
<td>10400</td>
<td>5636.6</td>
<td>0.85</td>
<td>93.379</td>
</tr>
<tr>
<td></td>
<td>(20.3)</td>
<td>(18.4)</td>
<td>(1.00, 4.00)</td>
<td>(22.2)</td>
<td>(45.8)</td>
<td>(18.4)</td>
<td>(45.0)</td>
<td>(23.2)</td>
<td>(12.4)</td>
</tr>
<tr>
<td>160</td>
<td>233.675</td>
<td>246.375</td>
<td>1.250</td>
<td>25.855</td>
<td>7.871</td>
<td>10824</td>
<td>7375.6</td>
<td>0.93</td>
<td>96.335</td>
</tr>
<tr>
<td></td>
<td>(29.4)</td>
<td>(30.1)</td>
<td>(0.25, 39.3)</td>
<td>(47.7)</td>
<td>(30.1)</td>
<td>(35.8)</td>
<td>(31.3)</td>
<td>(9.3)</td>
<td></td>
</tr>
</tbody>
</table>

Values for \( t_{\text{max}} \) are shown as the median (minimum, maximum); other values are shown as the geometric mean (geometric coefficient of variation, %). \((n = 7 \text{ or } 8)\) CL/F, apparent total body clearance; \( Vd/F \), apparent volume of distribution.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8