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Marked Strain Differences in the Pharmacokinetics of a VLA-4 Antagonist, 4-[1-[3-Chloro-4-[N'-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidine-2-yl]methoxybenzoic acid, D01-4582, in Sprague-Dawley Rats are Associated with Albumin Genetic Polymorphism

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Running title

PK strain differences associated with albumin polymorphism

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Discussion: 1500 words

Abbreviations

EHBR: Eisai hyperbilirubinemic rat, E217βG: estradiol 17β-glucuronide, E-sul: estrone 3-sulfate, LUI: liver uptake index, AUC: area under the concentration-time curve, CL: total plasma clearance, V_{dss}: distribution volume at steady-state, E_h: hepatic extraction ratio, K_d: dissociation constant, nPt: concentrations of binding site, f_u: unbound fraction in plasma

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Abstract

Strain differences in pharmacokinetics of a VLA-4 antagonist, D01-4582, in Sprague-Dawley rat strains (SD rat and CD rat) and their mechanism were investigated. Total plasma clearances of D01-4582 were 31.5 and 5.23 mL/min/kg in SD and CD rats, respectively. From *in vivo* studies, hepatic uptake process was thought to be involved in the strain differences. Differences in the uptake of D01-4582 by isolated hepatocytes prepared from the both strains were not observed when hepatocytes were incubated with simple buffer, but marked differences were observed when hepatocytes were incubated with plasma. When the dissociation constants (K_d) for the plasma protein binding of D01-4582 were examined in six rat strains, each strain was classified into two groups: a high-K_d group which included SD rats, Brown Norway rats, and Wistar rats, and a low-K_d group which included CD rats, Lewis rats and EHBR. Since all rat strains in the low-K_d group showed higher AUC for D01-4582 than rats in the high-K_d group, it was considered that the strain differences in the pharmacokinetics of D01-4582 were due to differences in the binding affinity. Purified albumin also showed strain differences in K_d. The cDNA sequence of the albumin was analyzed and eleven substitutions were observed. Val238→Leu (G784→C, T786→C) and Thr293→Ile (C950→T) were found only in the high-K_d group, suggesting that these amino acid changes reduced the binding affinity of albumin for D01-4582. In conclusion, the strain differences in D01-4582 pharmacokinetics were suggested to be caused by an alteration in K_d, associated

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with albumin genetic polymorphism.

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Introduction

In the process of drug discovery and development, the properties of drug candidates are assessed and screened for their pharmacological, toxicological, and pharmacokinetic aspects. One important issue in the pharmacokinetic evaluation is the prediction of inter-individual variabilities in plasma concentrations in humans. For this purpose, the metabolic pathway, elimination route, and their relevant proteins are characterized, because metabolism and membrane transport, in most cases, govern the plasma concentration profiles of a drug. Once the relevant protein, *e.g.* CYP3A4, OATP1B1 etc., has been identified, information on the frequency of the variants with reduced activity helps us to estimate the risk of the occurrence of inter-individual variabilities (Sadee 1998, Pirmohamed et al. 2001, Tribut et al 2002, Evans et al 2003). Animal models, such as knockout mice and naturally occurring deficient animals, are useful for demonstrating the importance of the protein *in vivo*. If pharmacokinetics differ in model animals compared with that in normal animals, the protein of interest would play an important role *in vivo*. In other words, pharmacokinetic differences in model animals would imply the possibility that inter-individual variabilities in humans might be observed due to reduced expression or reduced activity of the corresponding determinant protein.

Recently, strain differences in the pharmacokinetics of several drugs have been reported in “normal” rats. Wistar-Imamichi rats have higher concentrations of acetohexamide than Sprague-Dawley rats owing to the reduced activity of carbonyl reductase in liver microsomes

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(Imamura et al. 2005). Metabolic clearances of diazepam differ among Sprague-Dawley rats, Brown Norway rats, and Wistar rats, in which differences in both the rate of elimination and the metabolic pathways of diazepam are due to polymorphic expression of the enzymes involved in diazepam metabolism (Saito et al. 2004, Sakai et al. 2005). These results are useful for predicting the variability in human pharmacokinetics if the mechanisms of such differences are completely understood. It would be worthwhile to fully characterize animals used in pharmacokinetic studies from the point of view of the genetic expression of transporters and metabolic enzymes, even if these animals are regarded as “normal”.

D01-4582, 4-[1-[3-Chloro-4-[N'-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidine-2-yl]methoxybenzoic acid (MW = 540.0; Figure 1a), is a drug candidate synthesized in our institute. In preclinical pharmacokinetic studies, the possibility was pointed out that the pharmacokinetics of D01-4582 might be different for two strains of Sprague-Dawley rats that were bred by two different breeders. Both rat strains are regarded as “normal” and have been used indiscriminately in pharmacological and pharmacokinetic studies without any problems. If the causative mechanisms of the strain differences are shared with humans, they might give rise to inter-individual pharmacokinetic variability in humans. Since it is important to understand all possible risks of drug candidate before the drugs are administered to humans, we carried out a detailed investigation of the causative mechanism.

In the present study, strain differences in the pharmacokinetics of D01-4582 were

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demonstrated first, and then the mechanism was investigated. The main focus was on the association of albumin genetic polymorphism with protein binding and the uptake of D01-4582 by the liver. Finally, the impact of the strain differences in the pharmacokinetics of D01-4582 on evaluation of possible risks in humans was discussed.

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Materials and methods

Chemicals

D01-4582 and 4-[(4*S*)-Fluoro-1-phenylacetyl-(2*S*)-pyrrolidinyl]methoxybenzoic acid, D11-1430, were synthesized by the Medicinal Chemistry Research Laboratory, Daiichi Pharmaceuticals (Tokyo, Japan). Radiolabeled [^{14}C]-D01-4582 was synthesized by Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan), with a radiochemical purity >98 % and an initial specific activity of 984 kBq/mg. The radiolabel was introduced into the aromatic ring of D01-4582, which was metabolically stable and was not cleaved. D82-7319, an internal standard for the analysis of D01-4582, was synthesized by the Medicinal Chemistry Research Laboratory, Daiichi Pharmaceuticals (Tokyo, Japan). ^{3}H-inulin (405 μCi/mg, Lot No. 3467110), ^{14}C-inulin (2.4 μCi/mg, Lot No. 3346025), ^{3}H-estradiol 17β-glucuronide (40.5 Ci/mmol, Lot No. 3467-060), and ^{3}H-estrone sulfate (43.1 Ci/mmol, Lot No. 3363-726) were obtained from PerkinElmer (Wellesley, MA). Estradiol 17β-glucuronide sodium salt and estrone 3-sulfate sodium salt were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

Animals

Male Sprague-Dawley rats, aged 7-9 weeks, were supplied by two distinct breeders, Charles River Japan (Yokohama, Japan) and Japan SLC Inc. (Shizuoka, Japan). The former

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are designated as CD rats and the latter as SD rats. According to breeder's information, SD rats were introduced from Charles River, USA in 1968 and bred since then. CD rats were developed by the International Genetic Standard System in 1992 and introduced to Charles River Japan in 1994. Both rats are closed-colony animals. Male Wistar rats and EHBR were obtained from Japan SLC Inc, and male Lewis rats and Brown Norway rats were obtained from Charles River Japan. Rats of comparable age were enrolled in each study. Feed and water were provided *ad libitum*, with the exception that rats were fasted for 16 h before the studies. The rats were housed in an animal room where the temperature and relative humidity were maintained at 23 ± 2°C and 55 ± 15%, respectively. Lighting was controlled to provide a 12 h light/dark cycle. All studies were approved by the Animal Ethics Committee of Daiichi Pharmaceuticals.

Animal studies

For the pharmacokinetics study, D01-4582 was dissolved in saline with 3 eq. mol NaOH for intravenous dosing or suspended in a 0.5 % methylcellulose aqueous solution for oral dosing. In the intravenous administration study, D01-4582 (10 mg/5 mL/kg) was administered to SD, CD, Wistar, Lewis, Brown Norway rats, and EHBR via the right jugular vein under light ether anesthesia. In the oral administration study, D01-4582 was administered orally into the stomach. Blood samples were collected directly from the left

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jugular vein with heparinized syringes at every designated time point under light ether anesthesia. Rats were conscious during the study except for the blood sampling operation. Plasma was prepared by centrifugation of the blood at 1500 × *g* for 15 min at 4°C. Rats were euthanized under ether anesthesia.

For the biliary excretion study, the bile duct was cannulated with PE10 tubing under ether anesthesia. Each rat was held in a Bollman cage and allowed to recover from the anesthesia before proceeding with the experiments. SD rats and CD rats were given ^{14}C-D01-4582 intravenously at 2 mg/kg. Bile and urine samples were collected at intervals for up to 8 h. Rats were euthanized under ether anesthesia.

For the integration plot analysis, D01-4582 was administered via the jugular vein under ether anesthesia. Blood samples were collected with a heparinized syringe from the abdominal aorta at 1, 2, 3, 4, and 5 min after administration, and approximately 200-mg liver was also obtained, simultaneously. Plasma was prepared by centrifugation of the blood at 1500 × *g* for 15 min at 4°C. Rats were euthanized under ether anesthesia.

For the liver uptake index (LUI) study, ^{3}H-estrone 3-sulfate (E-sul), ^{3}H-estradiol 17β-glucuronide (E217βG), and ^{14}C-D01-4582 were dissolved in rat plasma prepared beforehand from SD rats and CD rats. ^{14}C-inulin or ^{3}H-inulin was also added, as appropriate, to the plasma as a vascular marker. Under ether anesthesia, the plasma containing a radiolabeled test compound and its respective vascular marker was injected rapidly into the

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portal vein immediately after ligation of the hepatic artery. Eighteen seconds after bolus administration, the portal vein was cut, and the whole liver was excised and then minced. Approximately 100-mg liver was transferred to a scintillation vial. Radioactivity in the injected plasma was also counted to determine the total activity administered.

Isolated rat hepatocyte study

Isolated rat hepatocytes were prepared from an SD rat and a CD rat aged 9 weeks by the collagenase perfusion method (Yamazaki et al., 1993). Isolated hepatocytes (viability >90%) were suspended in Krebs-Henseleit buffer, adjusted to 5.0 x 10^6 viable cells/mL, and stored on ice until used. After preincubation at 37°C for 3 min, hepatocytes were incubated with three different matrices as described below at a final concentration of 1.0 x 10^6 viable cells/mL. D01-4582 and E217βG, as probe substrates, were dissolved separately in these matrices and the matrices were pre-warmed before being added to the hepatocyte suspension. Under the first condition, hepatocytes were incubated with the buffer. Under the second condition, hepatocytes were incubated with rat plasma prepared from the corresponding rat strain. Under the third condition, hepatocytes were incubated under the "replaced condition," where hepatocytes were incubated with rat plasma prepared from the other strain. In other words, SD rat hepatocytes were incubated with CD rat plasma and *vice versa* to investigate the effect of the plasma origin on the substrate uptake. Substrate concentrations were 3.5 and

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100 μM under the first condition and 45 μM under the other conditions. These concentrations were chosen in consideration of the detection limit and solubility of the compounds. At 40 s, 1, 2, and 5 min after starting the incubation, a 200 μL sample of the incubation mixture was collected and placed in a centrifuge tube (250 μL) containing 50 μL 2 N NaOH under a layer of 100 μL oil (density, 1.015, a mixture of silicone oil and mineral oil, Sigma-Aldrich, St. Louis, MO). Subsequently, the sample tube was centrifuged at 6500 × *g* for 30 s at 4°C. During this process, the hepatocytes pass through the oil layer into the alkaline solution. After an overnight incubation at room temperature to dissolve the cells in the alkali, the centrifuge tube was cut and each compartment was transferred to a scintillation vial. The compartment containing dissolved cells was neutralized with 50 μL 2 N HCl, mixed with scintillation cocktail, and the radioactivity was determined in a liquid scintillation counter.

Protein binding study

Blood was obtained from Wistar rats, EHBR, Lewis rats, and Brown Norway rats as well as SD rats and CD rats with 0.1% w/v EDTA as an anti-coagulant under light ether anesthesia. Plasma was prepared by centrifugation of the blood at 1500 × *g* for 15 min at 4°C.

After preincubation for 3 min at 37°C, 10 μL of the D01-4582 standard solution was added to 490 μL plasma and incubated for 30 min at 37°C. The final concentrations of D01-4582 were 1.85, 9.26, 18.5, 37.0, 55.6, 92.6, 138, 185, 278, 370 μM. At the end of the

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incubation period, 210 μL of each sample was centrifuged in a 0.23 PC tube (Hitachi Koki Co., Ltd.) at 200000 × *g* for 16 h at 4°C. The supernatant (50 μL) was collected and subjected to LC-MS/MS analysis (TSQ-700, Thermo Electron, Waltham, MA).

Albumin was purified from SD and CD rat plasma by affinity column chromatography (Watanabe et al. 2001). Plasma was dialyzed for 48 h at 4°C against 100 volumes of distilled water, followed by a further 24 h against 100 volumes of 200 mM sodium acetate buffer at pH 5.5. Then the solution was loaded on to a Blue Sepharose CL-6B column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). The column was washed with about 5 bed volumes of 200 mM sodium acetate buffer at pH 5.5, and then the albumin was eluted with 3 M sodium chloride. The eluted albumin was freeze-dried, and stored at -20°C until used. The purity of the albumin fraction was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue. Albumins were solubilized with Sörensen buffer at a concentration of 1 μM and incubated with D01-4582 at final concentrations of 0.1, 0.2, 0.5, 0.7, 0.8, and 1 μM. The binding assay procedure was the same as previously described.

Determination of cDNA sequences of rat albumins

The cDNA sequences of albumins were determined by the direct sequencing method for the six strains of rats employed in the pharmacokinetic study. Total RNA was extracted from

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the liver using RNeasy (QIAGEN, Hilden, Germany) followed by purification of poly A^+ RNA by Oligotex-dT30 (Takara, Japan). First-strand cDNA was synthesized by reverse transcription using an LA PCR Kit (AMV) Ver. 1.1 (Takara, Japan) under the conditions of 30°C for 10 min, 50°C for 30 min, 99°C for 5 min, and 5°C for 5 min. To analyze the base sequence of albumin cDNA, polymerase chain reaction (PCR) was performed with specific primer sets. The following primer set was designed according to the reference sequence registered in GenBank (Accession No. NM 134326): 5'-AGAAGCACACAAGAGTGA-3' (nt 72 to 89) and 5'-CTGAGATGGTTGTGATGTG-3' (nt 1847 to 1829). The PCR reaction included 0.2 mM dNTP mixture, 1xPCR buffer, 1 mM $MgSO_4$, 0.3 μM forward primer, 0.3 μM reverse primer, 20 μL cDNA sample, and 2 U KOD-Plus (Toyobo, Osaka, Japan). Distilled water was added to give a total volume of 100 μL. PCR conditions were: denaturation at 94 °C for 15 s (first cycle, 2 min), annealing at 46°C for 30 s, and extension at 68°C for 2 min over a total of 25 cycles. PCR products were subjected to electrophoresis on 1.5% agarose gel and stained with ethidium bromide for fluorescence band detection. DNA bands were purified using a GFX PCR DNA Gel Band Purification Kit (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). After the residual primer and dNTP were digested with the PCR Product Pre-Sequencing Reagent Pack (Toyobo, Osaka, Japan), a sequencing reaction for purified PCR product was performed using a CEQ DTCS Quick Start Kit (Beckman Coulter, Inc., Fullerton, CA). The DNA sequence was determined with a CEQ8000

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(Beckman Coulter, Inc., Fullerton, CA). Several additional primers were designed and used as sequence reaction primers. The presence of mutations was determined by comparison with the reference sequence.

Analytical procedures

Thin Layer Chromatography (TLC)

Bile samples collected in the biliary excretion study were subjected to TLC analysis. Each bile sample (10 μL) was loaded onto a TLC plate (Silicagel 60 F_{254}, Merck KGaA, Darmstadt, Germany), and the plate was placed in the solvent chamber. The composition of the organic solvent was butanol : acetic acid : water = 4 : 1: 2. After the TLC plate was allowed to dry, it was covered in plastic wrap and placed in contact with a BAS-SR2025 plate (Fuji Photo Film, Tokyo, Japan). Results were analyzed with a FLA-3000 (Fuji Photo Film, Tokyo, Japan)

Sample preparation for LC-MS/MS analysis

Samples with high concentrations were diluted appropriately with blank matrix. Methanol (150 μL) containing the internal standard was added to the plasma and bile samples (50 μL) followed by vigorous mixing and centrifugation at 10000 × *g* for 15 min at 4°C. Supernatant (20 μL) was introduced into the LC-MS/MS. The liver samples were weighed

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and homogenized with 2-mL acetonitrile containing the internal standard. After centrifugation at 1500 × *g* for 15 min at 4°C, supernatant (20 µL) was introduced into the LC-MS/MS. Calibration lines were freshly prepared by adding D01-4582 to blank matrix at several concentrations.

LC-MS/MS analysis

Analyses were performed using a Waters HPLC system (Aliance 2695, Waters, Milford, MA) coupled to a TSQ-700 (Thermo Electron, Waltham, MA) via an electrospray ionization interface for mass analysis and detection. Chromatographic separation was achieved on a Symmetry Shield RP8 column (50 x 2.1 mm i.d.; Waters, Milford, MA) with gradient elution using an ammonium formate buffer (0.01 M) and methanol as the mobile phase at a flow rate of 0.2 mL/min. The column temperature was 40°C and the total run time was 12 min for each injection. The mass spectrometer was operated in positive ion mode. The MS−MS transition selected to monitor D01-4582 was from m/z 540.2 to a product ion at m/z 401.6. Internal standard was monitored using the transition from m/z 488.2 to m/z 350.2. Each transition was alternately monitored at a dwell time of 0.3 s.

Liquid scintillation counter analysis

Plasma, bile, and urine samples were mixed with 10 mL scintillation cocktail (Aquasol

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II, Packard, USA) and counted directly for radioactivity. Liver samples were dissolved in a solubilizing agent (Soluene-350; PerkinElmer Life and Analytical Sciences, Boston, MA) and incubated at 55°C overnight, followed by the addition of liquid scintillation cocktail (Hionic-Fluor; PerkinElmer Life and Analytical Sciences, Boston, MA). The radioactivities were determined in a liquid scintillation counter (LS6000SE, Beckman Coulter, Fullerton , CA).

Data analysis

Pharmacokinetic parameters

The area under the concentration-time curve (AUC), total plasma clearance (CL), and volume of distribution at steady-state (V_{dss}) were calculated by the non-compartmental method without extrapolation using a validated program developed in-house. Oral bioavailability was calculated from the AUC ratio after oral and intravenous administration.

Integration plot analysis

Since the efflux or elimination of radioactivity from a tissue is negligible when the tissue uptake is measured within a short period, the tissue uptake of D01-4582 could be evaluated by using the following formula (Kim et al., 1988; Liu et al., 1992).

$$C_{liver}/C_p = CL_{uptake,plasma} \bullet AUC_{(0\text{-}t)}/C_p \qquad (1)$$

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where C_{liver} and C_p represent concentrations in the liver and plasma, respectively, and $AUC_{(0\text{-}t)}$ represents the area under the C_p-time curve from time 0 to t. Uptake clearance ($CL_{uptake,plasma}$) was obtained from the slope of the plot. Uptake clearances per body were calculated by multiplying $CL_{uptake,plasma}$ by liver weight (40 g liver/kg)

LUI study

The hepatic extraction ratio (E_h) was estimated from the data based on the following formula, with the assumption that the ratio of test compound radioactivity in the vascular space to injected radioactivity is the same as the rate of vascular marker radioactivity in the liver to injected radioactivity (Liu et al., 1992). :

$$E_{app} = (R_{s,liver} + R_{s,vascular}) / R_{s,inject},$$

$$E_h = E_{app} - R_{s,vascular} / R_{s,inject},$$

$$= E_{app} - R_{p,vascular} / R_{p,inject}: \quad (2)$$

where E_{app} represents the apparent hepatic extraction ratio; $R_{s,liver}$, $R_{s,vascular}$, and $R_{s,inject}$ represent the radioactivity of the substrate taken up in the liver, radioactivity of substrate remaining in the vascular space, and radioactivity of substrate injected, respectively; and subscripts s and p represent substrate and vascular marker, respectively.

Binding parameters

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The binding characteristics were examined initially by Scatchard plot to determine the availability of only one principle class of binding site. The dissociation constant (K_d) and concentration of binding site (nPt) were estimated by fitting the results to the following equation using a nonlinear least-square program (*WinNonlin* from Pharsight, Mountain View, CA, USA),

$$C_b = nPt \cdot C_f / (K_d + C_f), \qquad (3)$$

where C_b and C_f represent the molar concentrations of bound and free D01-4582, respectively.

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Results

Pharmacokinetic study

The plasma concentration profiles after intravenous and oral administration are shown in Figures 2a and 2b, respectively, in which the concentrations of D01-4582 were always higher in CD rats than in SD rats regardless of the route of administration. The AUC was approximately six times higher in CD rats than in SD rats after intravenous administration. The AUC after oral administration was approximately nineteen times higher in CD rats than in SD rats (Table 1). The bioavailability was 3.3 fold higher in CD rats than in SD rats (Table 1).

The plasma concentration profiles were also examined in an additional four rat strains, *i.e.*, Lewis, Wistar, Brown Norway rats and EHBR (Figure 3). Wistar and Brown Norway rats showed relatively low plasma concentrations like SD rats, whereas Lewis rats and EHBR showed relatively high plasma concentrations like CD rats. Strictly speaking, differences in pharmacokinetics between SD rats and CD rats are not strain differences but breeder differences. However, for the convenience of explanation, we will call it a strain difference from now on.

Biliary excretion study

After intravenous administration of ^{14}C-D01-4582, almost all the radioactivity was

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recovered in the bile (Table 2); however, the cumulative excretion of radioactivity in urine up to 8 h was less than 1% of the dose in both rat strains.

Integration plot analysis study and LUI study

The distribution of D01-4582 to the liver was investigated by integration plot analysis and LUI study. Uptake clearances estimated by integration plot analysis exhibited a five-fold difference (0.672 and 0.137 mL/min/g liver for SD and CD rats, respectively) (Figure 4), which corresponded to the extent of the *in vivo* difference in CL of D01-4582. The uptake clearances per body weight were estimated to be 26.9 and 5.48 mL/min/kg for SD and CD rats, respectively, which were comparable with the total CL in each rat strain.

The results of the LUI study are shown in Figure 5. The hepatic extraction ratios corrected by vascular marker were 0.60 and 0.11 for SD and CD rats, respectively. Probe substrates for hepatic organic anion transporting polypeptide (Oatps), E-sul and E217βG, did not show any strain differences for the hepatic extraction ratio in the LUI study.

Isolated hepatocyte study

The uptake of D01-4582 by hepatocytes was investigated under three conditions. Under the first condition, where hepatocytes were incubated with the buffer, D01-4582 was taken up more efficiently at a low concentration than at a high concentration by both SD and CD rat

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hepatocytes (Figure 6a). On the other hand, both SD and CD rat hepatocytes took up D01-4582 to a similar extent.

Under the second condition, hepatocytes were incubated with plasma prepared from the corresponding rat strain to examine the effect of plasma protein binding. In this case, SD rat hepatocytes took up D01-4582 more efficiently than CD rat hepatocytes (Figure 7a). We observed no strain differences in the uptake of E217βG by SD and CD rat hepatocytes under the first (Figure 6b) and second conditions (Figure 7c).

Under the third condition, the effect of plasma on the uptake of D01-4582 was examined by incubating rat hepatocytes with plasma prepared from the other rat strain. SD rat hepatocytes incubated with CD rat plasma took up D01-4582 to a lesser extent than when they were incubated with SD rat plasma. In contrast, CD rat hepatocytes took up D01-4582 more efficiently when they were incubated with SD rat plasma than did the SD rat hepatocytes under this replaced condition (Figure 7b).

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Protein binding study

The results of protein binding study in six rat strains are shown in Figure 8a and Table 3. These six rat strains were classified into two groups, a low-K_d group and a high-K_d group (Table 3). The former group included CD rats, EHBR, and Lewis rats, and the latter group included SD rats, Wistar rats, and Brown Norway rats. Estimated nPts (ca. 350 ~ 580 μM)

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were around the concentration of albumin in plasma.

The binding of D01-4582 to albumin was investigated using purified albumin from SD and CD rat plasma (Figure 8b). Similar to the results of binding study with whole plasma, CD rat albumin showed a higher binding affinity for D01-4582 than SD rat albumin. The K_ds for SD rat albumin and CD rat albumin were 3.86 ± 2.76 and 0.276 ± 0.034 μM (Mean ± SE), respectively.

Determination of cDNA sequences of rat albumins

The cDNA sequences of albumin genes were determined (Table 4) and eleven mutations were found. When we examined the albumin sequence of a single CD rat, G1291→A was determined as heterozygous. Three additional CD rats were found to be G/G. The reason for these conflicting results was unclear. Among the observed mutations, four were associated with amino acid changes. G784→C and T786→C compose the identical codon, resulting in an alteration of Val238 to Leu. C950→T and G1291→A led to alterations of Thr293 to Ile and Val407 to Ile, respectively. The Val238→Leu mutation has been already registered in Swiss-Prot (http://ca.expasy.org/cgi-bin/niceprot.pl?P02770). However, the Thr293Ile and Val407Ile mutations in the present study were new.

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Discussion

Since both SD and CD rats are Sprague-Dawley rats, D01-4582 would have been expected to show comparable plasma concentration profiles. However, marked strain differences were observed (Figure 2) and this was also the case for Lewis, Wistar, Brown Norway rats and EHBR (Figure 3), demonstrating the existence of two phenotype of D01-4582 pharmacokinetics. At first, metabolism and/or elimination were supposed to be possible causes of the strain differences. However, considering the fact that the plasma concentration showed marked differences at five minutes after intravenous administration (Figure 2a), processes other than metabolism and elimination were considered to be possible causes.

The liver was considered to be the main elimination organ for ^{14}C-D01-4582 and would be associated with the strain differences from the results of the biliary excretion study (Table 2). According to the results of the integration plot analysis and the LUI study (Figures 4, 5), the uptake clearance of D01-4582 was different between SD and CD rats. It is known that anion transporters (Oatps and Oats) are expressed on the sinusoidal membrane of rat hepatocytes (Meijer et al. 1999). Based on results of the LUI study and the isolated hepatocyte study, the overall activities of the transporters involved in the uptake of E217βG and E-sul, typical substrates of Oatps, were suggested to be at similar levels in both SD and CD rats (Figures 5, 6b). Although active transport was suggested to be involved in the uptake

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of D01-4582 (Figure 6a), transporters did not seem to be the cause of the strain differences. The strain difference in the hepatic uptake of D01-4582 was only reproduced in the presence of plasma in the isolated hepatocyte study (Figure 7a) and the uptake was altered under the replaced condition (Figure 7b), suggesting that the strain difference in the hepatic uptake was due to interaction of D01-4582 with plasma proteins.

In the protein binding study, strain differences were found in K_d for the six rat strains tested (Table 3) and they were classified into two groups, high- K_d group and low-K_d group. Interestingly, this classification agreed with the classification based on the pharmacokinetic phenotype (Figures 2, 3). Therefore, the differences in binding affinity of D01-4582 were considered to cause the strain differences in pharmacokinetics. This consideration was strongly supported by the analysis in which the unbound concentrations of D01-4582 were compared (Figure 9), which showed that the plasma concentration profiles for SD and CD rats were almost superimposable.

Purified albumin also showed a strain difference in K_d (Figure 8b), suggesting that albumin could be the main binding protein of D01-4582 and the strain difference in plasma protein binding could be caused by differences in the binding affinity of albumin. Analysis of the cDNA sequence of rat albumin gene revealed eleven mutations, and four of those led to three amino acid changes (Table 4). While Val238→Leu and Thr293→Ile were only found in the high-K_d group, Val407→Ile was found in EHBR (low-K_d group) albumin as well as in the

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high-K_d group. Therefore, Val407→Ile might have a limited effect on K_d, and mutations of Val238→Leu and/or Thr293→Ile were considered to have a critical effect on the binding affinity of D01-4582.

Albumins are highly homologous among species (Peters 1996) so that the three-dimensional structure of rat albumin was predicted to be similar to that of human albumin by SWISS-MODEL (Schwede et al. 2003, Guex et al. 1997, Peitsch et al. 1995). In human albumin, Leu238 and Val293 are located on the third and the sixth alpha-helix in domain IIA, respectively. These two amino acid residues are close to the major binding regions, site I (Sudlow et al. 1975) known as the warfarin site. Leu238 forms the binding cavity of site I (Curry et al. 1998, Matsushita et al. 1998, Ghuman et al. 2005); in contrast, Val293 of human albumin has not been reported to be involved directly in ligand binding. Leu407 of human albumin is located on the second alpha-helix in domain IIIA. Although this amino acid residue is close to the binding region, site II (Sudlow et al. 1975) known as the diazepam site, direct involvement in ligand binding has not been reported. Val238 and Thr293 of rat albumin would be located in domain IIA and might be close to binding site I. Since mutations of Val238→Leu or Thr293→Ile in rat albumin were associated with an alteration in the binding affinity of D01-4582, D01-4582 was thought to bind to site I of rat albumin. However, the binding characteristics vary considerably among species (Kosa et al. 1997), even although albumins are highly homologous. Therefore, further studies are needed to

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show direct involvement of Val238 and Thr293 in ligand binding in rat albumin.

There are a few reports of an effect of albumin polymorphism on ligand binding affinity. Kragh-Hansen et al. reported altered binding characteristics of salicylate, warfarin, diazepam (Kragh-Hansen et al. 1990a) and endogenous substances (Kragh-Hansen et al. 1990b, Kragh-Hansen et al. 1996) for human albumin. Familial dysalbuminemic hyperthyroxinemia is associated with Arg218Pro and Arg218His. These substitutions increase the binding affinity of thyroxine (Petersen et al. 1994, Wada et al. 1997) and reduce the affinity for warfarin (Petersen et al. 2000). Association of a monkey albumin mutation with altered binding affinity of bilirubin has been reported (Watkins et al. 1993). The present study is the first report of the effects of genetic polymorphisms of rat albumin on the binding affinity and pharmacokinetics of a ligand.

Whereas over 60 variants of human serum albumin are reported , the prevalence of each variant is very small so that the cumulative frequency of all variants has been reported to be about 0.1% in humans (Peters 1996). In contrast, multiple alleles of albumin are reported for rabbits (Ferrand et al. 1992), cattle (Panepucci et al. 1991), and monkeys (Watkins et al. 1993). If albumin polymorphism affects the pharmacokinetics and pharmacological effects of a drug candidate, care should be taken in the selection of strains or individual experimental animals.

So far, strain differences in K_d are observed only for D01-4582 and its chemical

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derivatives. According to our preliminary study, the pharmacokinetics of D11-1430, a part of D01-4582 (Figure 1b), did not show strain differences, which implied no strain difference in K_d for D11-1430. Besides, warfarin and diazepam showed only a minor, if any, strain difference in their K_d (data not shown). By using D01-4582 and its derivatives as probe compounds, the binding characteristics of rat albumin could be investigated with regard to structure-affinity relationships. Besides, subsites of site I have been proposed for human albumin with *n*-butyl *p*-aminobenzoate and azapropazone as binding probes (Yamasaki et al. 1996, Fehske et al. 1982) and, so, D01-4582 and its derivatives would be good probes for identifying the binding site, differentiating sub-sites of site I of rat albumin.

The strain difference in the plasma concentration was extremely marked after oral administration. This would be caused by elevated E_h in SD rats, which resulted in reduced bioavailability as well as increased CL. This suggests that the bioavailability could vary among individuals if inter-individual variability in protein binding was observed in humans. However, inter-individual variability in pharmacological effects or adverse events would be limited for the following two reasons. Firstly, the prevalence of a variant of human albumin is so low that it would be rare to observe elevated plasma concentrations that were caused by human albumin polymorphisms. Since risks associated with polymorphisms are relative issues, risks associated with albumin polymorphism might be less significant compared with other common polymorphisms, such as, polymorphisms in metabolic enzymes. Secondly,

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even if plasma concentrations were elevated owing to albumin polymorphism, the exposure to pharmacologically and toxicologically active components, in other words, the exposure to the unbound fraction of D01-4582, would not be affected by protein binding when D01-4582 is administered orally (Benet et al. 2002). Assuming that D01-4582 is eliminated mainly by the liver in humans, as is the case for rats, the exposure to the unbound fraction ($AUC_{oral,\ free}$) can be expressed as follows:

$$AUC_{oral,\ free} = Dose/CL_{int}, \qquad (4)$$

where CL_{int} represents hepatic intrinsic clearance. Exposure to unbound D01-4582 after oral administration is independent of f_u. The steady-state unbound concentration would be comparable among subjects regardless of the albumin polymorphism, even although the total (*i.e.* bound plus unbound) plasma concentrations showed marked differences. In this case, alteration of f_u corrects for inter-individual variability in exposure to the active component. Therefore, dose adjustment will not be required. Collectively, risks owing to albumin polymorphism are considered to be minor in humans. Inter-strain differences in D01-4582 pharmacokinetics in rats might not imply inter-individual pharmacological and toxicological variability in humans.

In conclusion, it was demonstrated that strain differences in D01-4582 pharmacokinetics in rats were caused by an alteration in K_d. Albumin genetic polymorphism could be associated with this difference. With this preclinical information, we would expect limited

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clinical risks that might arise owing to albumin polymorphism with regard to pharmacological and toxicological aspects. These considerations will help us in future drug development programs.

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Legends for Figures

Figure 1. Structure of D01-4582 (a) and D11-1430 (b) in relation to the radio-labeled site.

Figure 2. Plasma concentration profiles of D01-4582 after intravenous (a) and oral (b) administration at a dose of 10 mg/kg. Closed circles represent SD rats, and open circles represent CD rats (n = 3 for intravenous administration, n = 4 for oral administration). Each point represents the mean and SD.

Figure 3. Plasma concentration profiles of D01-4582 in four strains of rats after intravenous administration at a dose of 10 mg/kg. Open triangles and squares represent Lewis rats and EHBR, respectively. Closed triangles and squares represent Wistar and Brown Norway rats, respectively. Each point represents the mean and SD (n=3).

Figure 4. Integration plot analysis for D01-4582 in SD and CD rats. D01-4582 was administered intravenously to SD and CD rats at a dose of 0.5 mg/kg, and the rats were killed at each time point (n = 3 / time point). Closed circles represent SD rats, and open circles represent CD rats. Broken lines are regression lines for each rat strain.

Figure 5. Hepatic extraction ratios of D01-4582 and probe compounds in SD and CD rats obtained by the liver uptake index method. Each bar and vertical bar represents the mean and SE (n = 3). E-sul: estrone 3-sulphate; E217βG: estradiol 17β-glucuronide

Figure 6. Uptake of D01-4582 (a) and estradiol 17β-glucuronide (b) by SD and CD rat hepatocytes in buffer. Closed circles represent SD rat hepatocytes, and open circles represent

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CD rat hepatocytes. Hepatocytes were incubated with D01-4582 at 3.5 μM (solid lines) and 100 μM (broken lines) or with estradiol 17β-glucuronide (1 μM). Each point represents the mean and SE (n = 3).

Figure 7. Uptake of D01-4582 (45 μM) (a, b) and estradiol 17β-glucuronide (1 μM) (c) by SD and CD rat hepatocytes in the presence of rat plasma. Closed circles represent SD rat hepatocytes, and open circles represent CD rat hepatocytes. Hepatocytes were incubated with their corresponding rat plasma (a, c) and reversed rat plasma : SD rat hepatocytes were incubated with CD rat plasma and *vice versa* (b). Each point represents the mean and SE (n = 3).

Figure 8. Scatchard plot for the binding of D01-4582 to rat plasma (a) and purified albumin (b) prepared from SD rat (closed circle), CD rat (open circle), EHBR (open diamond), Wistar rat (closed diamond), Lewis rat (open square), and Brown Norway rat (closed square). Each point represents the mean (n = 3)

Figure 9. Unbound plasma concentration profiles of D01-4582 after intravenous administration at a dose of 10 mg/kg. Unbound concentrations were calculated by multiplying the plasma concentrations in Figure 2a by free fraction shown in Table 3. Closed circles represent SD rats, and open circles represent CD rats (n = 3). Each point represents the mean and SD.

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Table 1 Pharmacokinetic parameters of D01-4582 after oral and intravenous administration to SD rats and CD rats

		PO (n=4)	IV (n=3)			
	Dose	AUC	AUC	CL	V_{dss}	BA
	(mg/kg)	(nmol·h/mL)	(nmol·h/mL)	(mL/min/kg)	(L/kg)	(%)
CD	10	21.2 (10.0)	59.1 (1.8)	5.23 (0.17)	0.237 (0.014)	36
SD	10	1.13 (0.43)	10.0 (1.8)	31.5 (5.6)	0.469 (0.028)	11

Figures in parentheses represent the standard deviation

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Table 2 Cumulative biliary excretion ratio of ^{14}C-D01-4582 in 8 h after intravenous administration at a dose of 2 mg/kg in SD rats and CD rats

Rat strain	Unchanged D01-4582	The other metabolites (1)	Total
SD rat (n=3)	60.1 (4.1)	35.4 (5.3)	95.5 (1.8)
CD rat (n=4)	46.1 (1.6)	44.0 (6.1)	90.1 (7.5)

Unit : % of dose

Figures in parentheses represent the standard deviation

(1): Summation of six metabolites detected by thin-layer chromatography analysis

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Table 3 Binding parameters of D01-4582 in plasma from different strains of rats and observed unbound fractions at a concentration of 18.5 μM

	K_d (μM)	nPt (μM)	Unbound fraction (%)
CD	0.077 (0.009)	346 (11)	0.015
EHBR	0.10 (0.012)	354 (12)	0.019
Lewis	0.076 (0.009)	377 (13)	0.015
SD	1.7 (0.3)	538 (41)	0.26
Wistar	1.6 (0.3)	584 (49)	0.25
Brown Norway	1.3 (0.3)	576 (48)	0.20

Figures in parentheses represent the standard error of the estimate

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Table 4 A list of mutation sites found in cDNA of albumin genes from six strains of rats. Sequence results are compared with reference sequence (NM134326). In the table, wt means wild type, where the nucleic acids correspond to those of the reference sequence.

Mutation	Amino Acid Change	CD	Lewis	EHBR	SD	Brown Norway	Wistar
A114→G	(silent)	wt	wt	wt	G/G	wt	wt
T315→C	(silent)	wt	wt	wt	C/C	C/C	C/C
A736→C	(silent)	wt	wt	wt	C/C	C/C	C/C
G784→C	Val238→Leu	wt	wt	wt	C/C	C/C	C/C
T786→C		wt	wt	wt	C/C	C/C	C/C
A846→G	(silent)	wt	wt	wt	G/G	G/G	G/G
C950→T	Thr293→Ile	wt	wt	wt	T/T	T/T	T/T
C1236→A	(silent)	wt	wt	A/A	wt	wt	wt

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(Table 4 continued)

C1257→T	(silent)	wt	wt	wt	T/T	wt	T/T
G1291→A	Val407→Ile	wt(*)	wt	A/A	A/A	A/A	A/A
T1513→C	(silent)	C/C	C/C	C/C	C/C	C/C	C/C

(*): A/G for one of four rats

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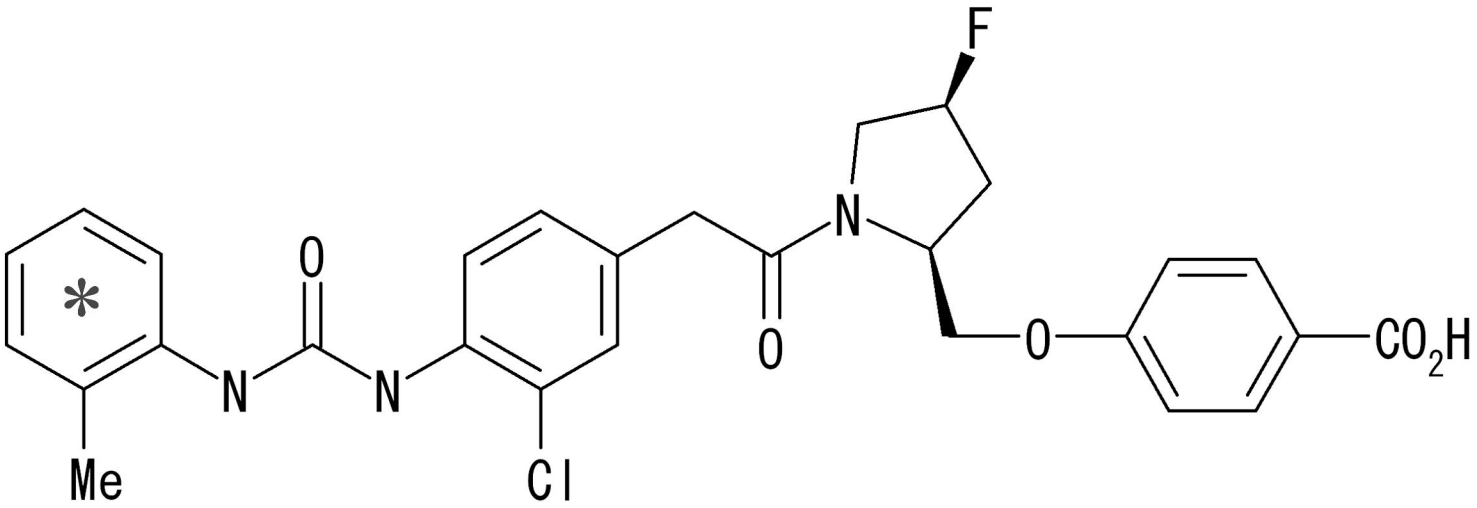
Figure 1

(a)



(b)

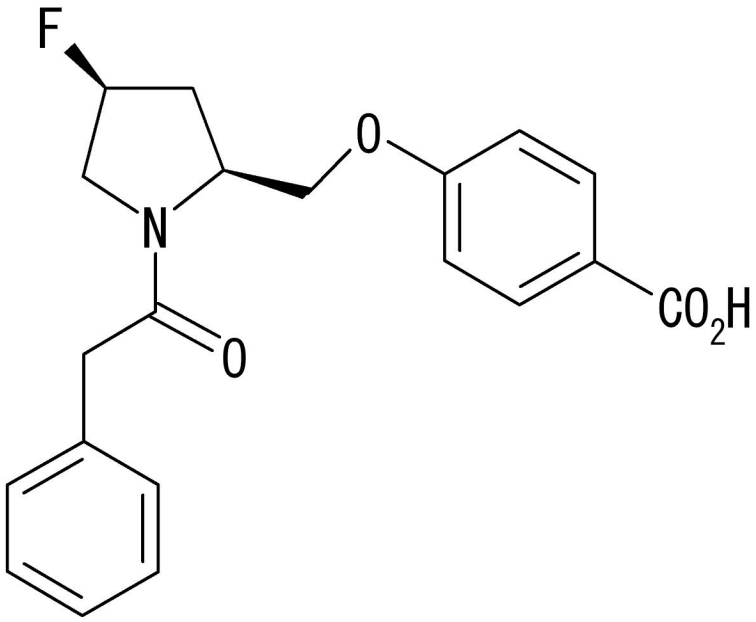


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Figure 2

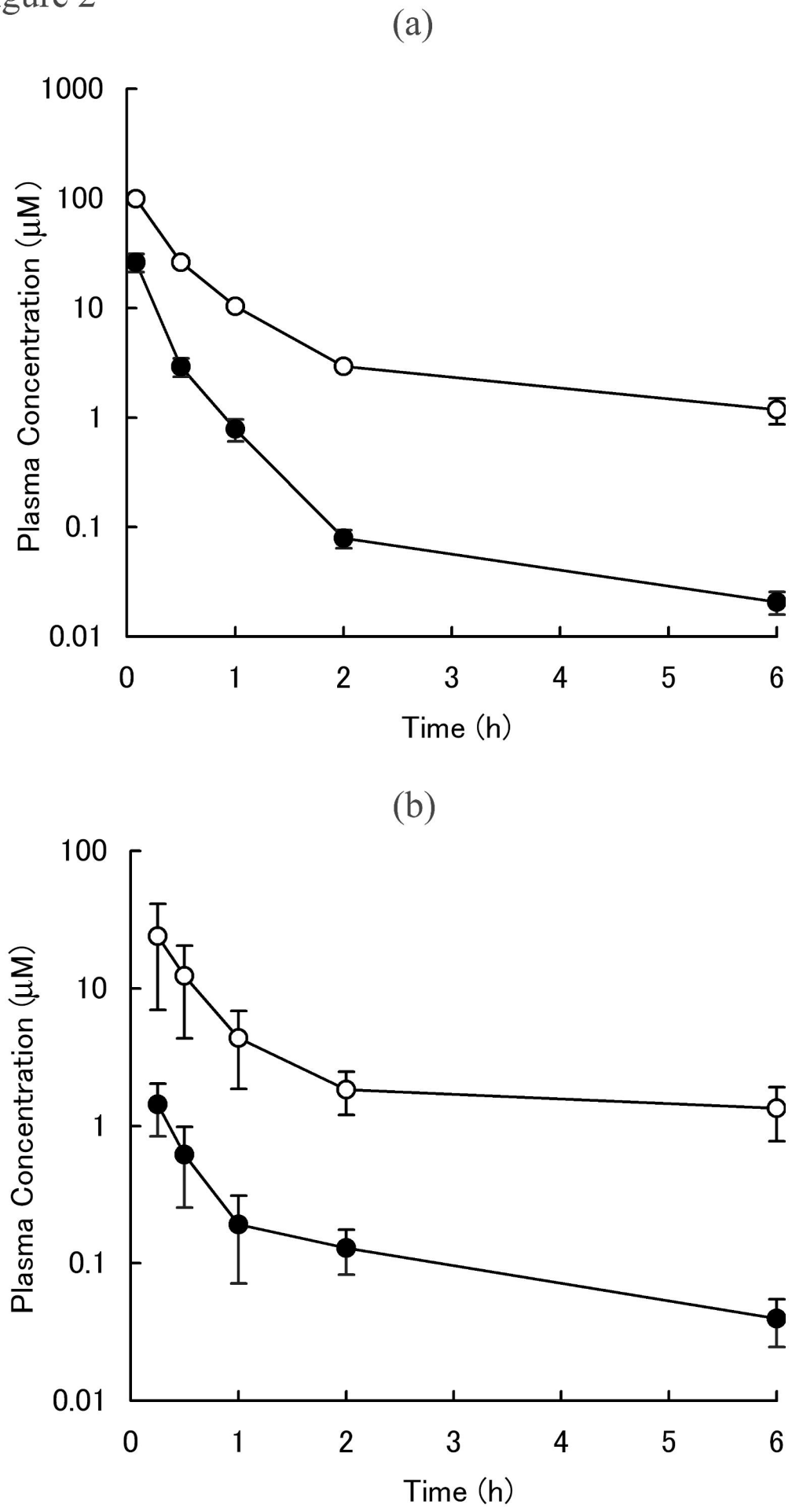

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Figure 3

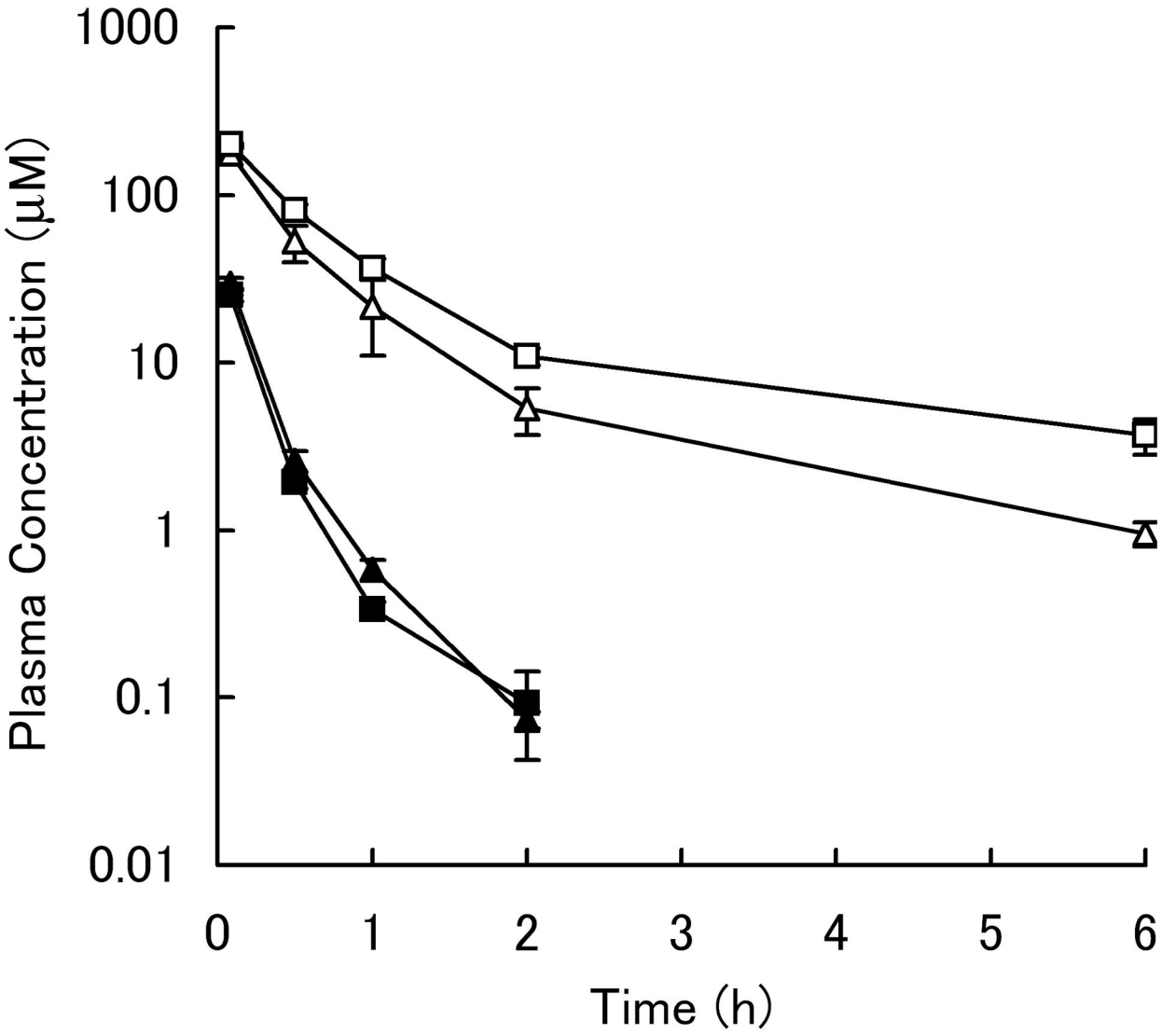

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Figure 4

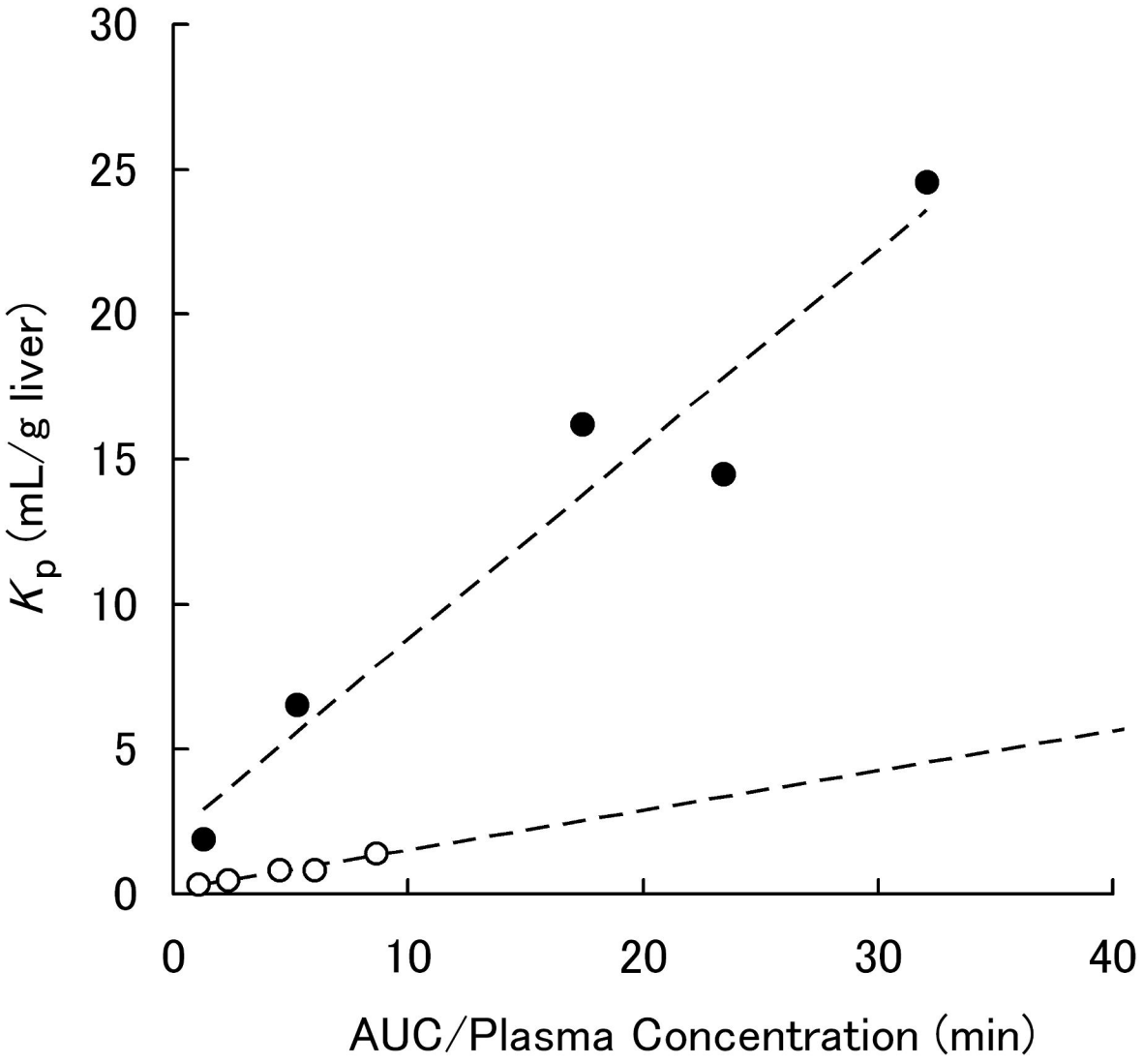

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Figure 5

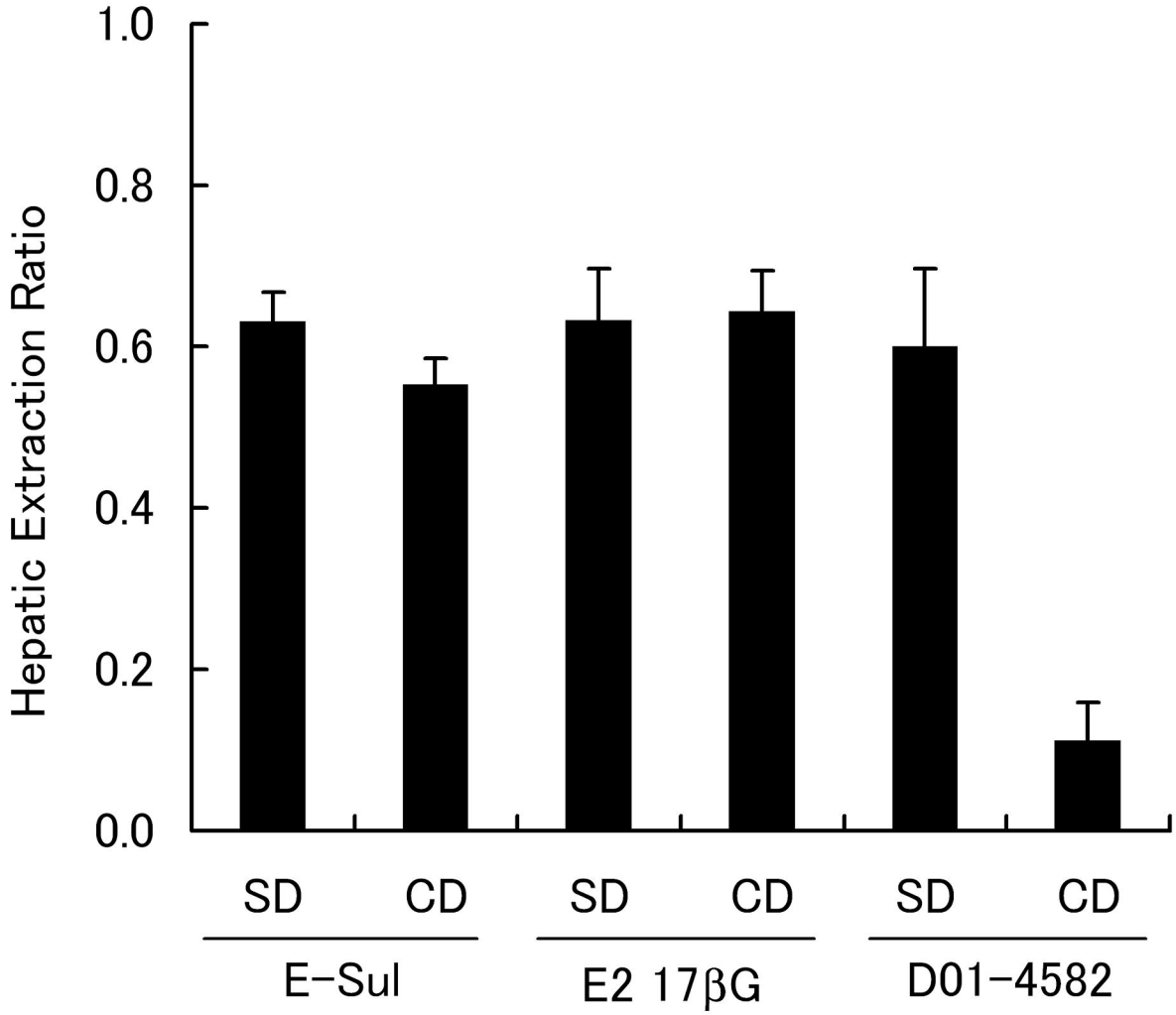

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Figure 6

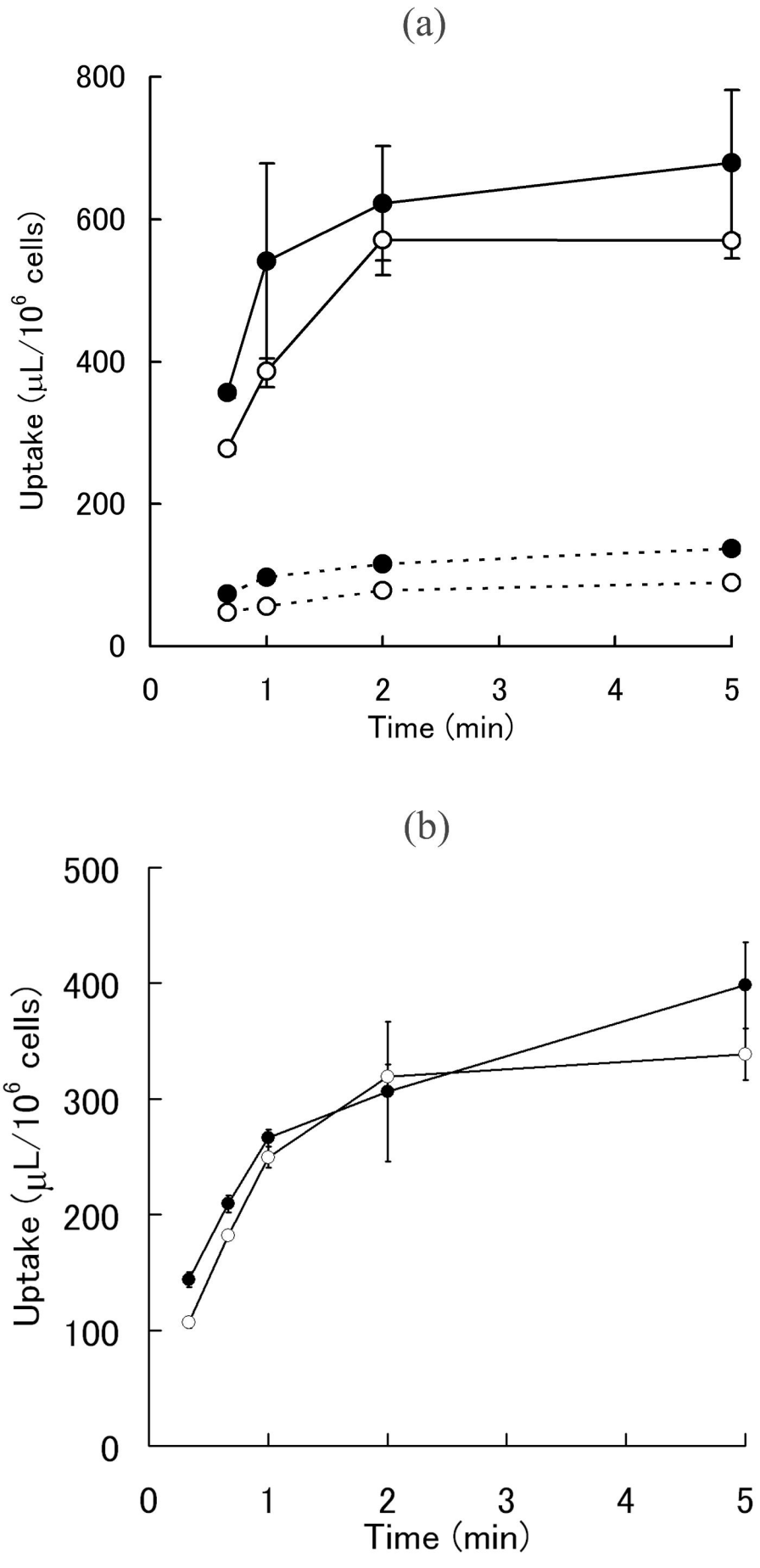

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Figure 7

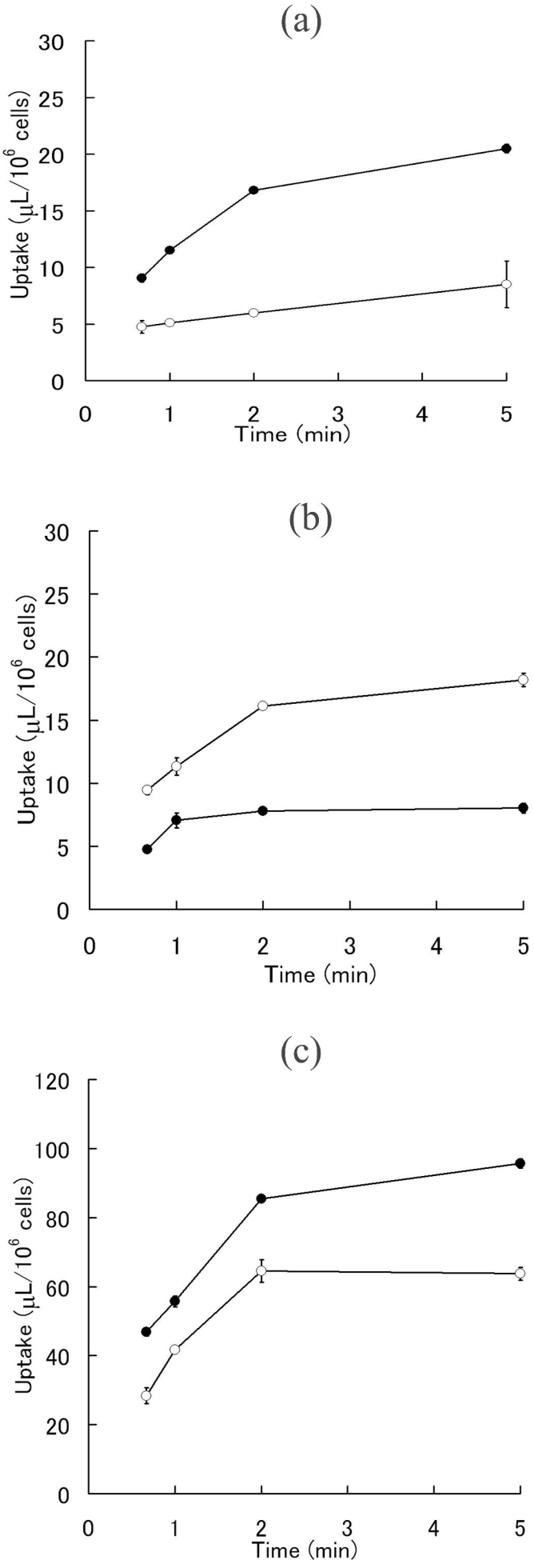

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Figure 8

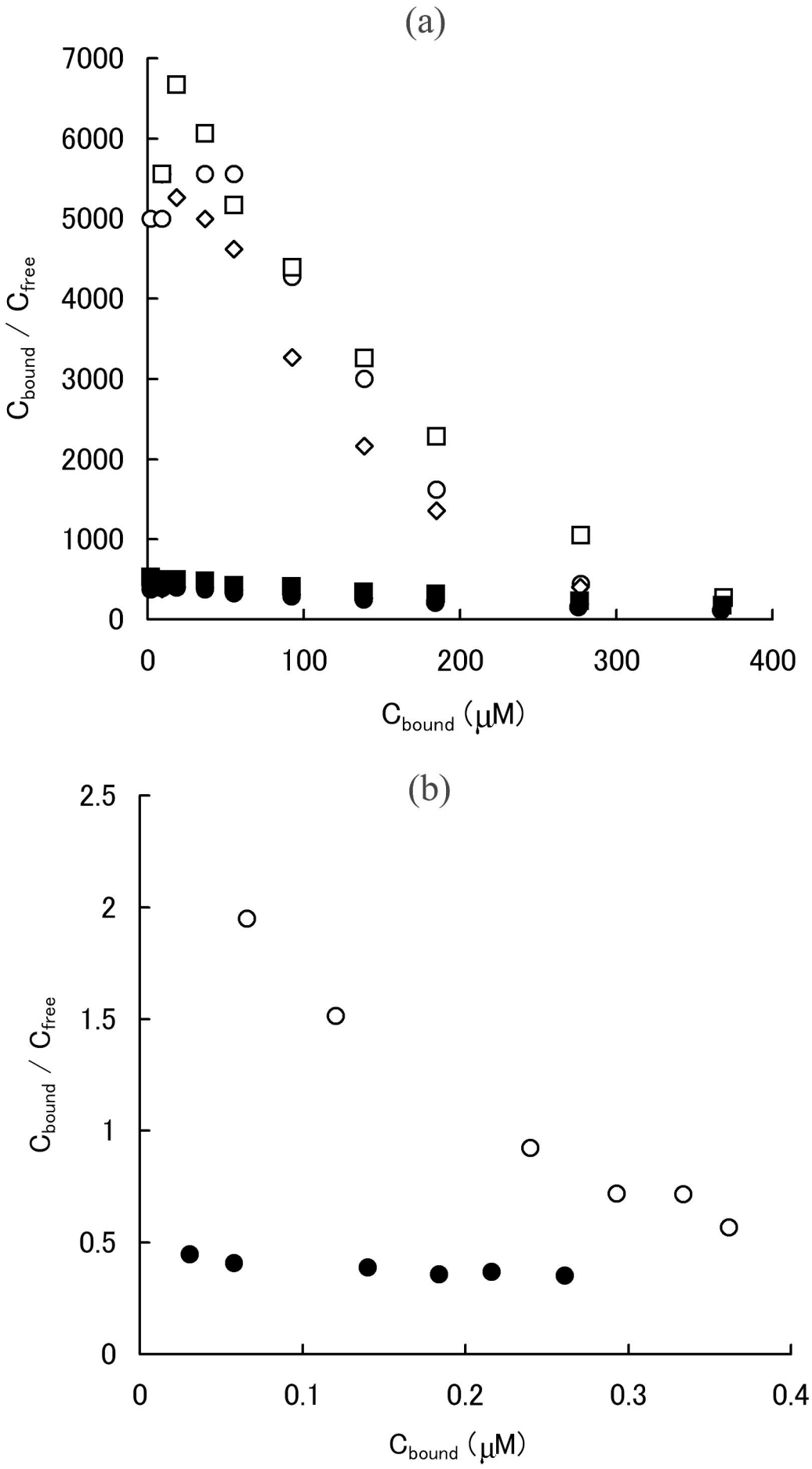

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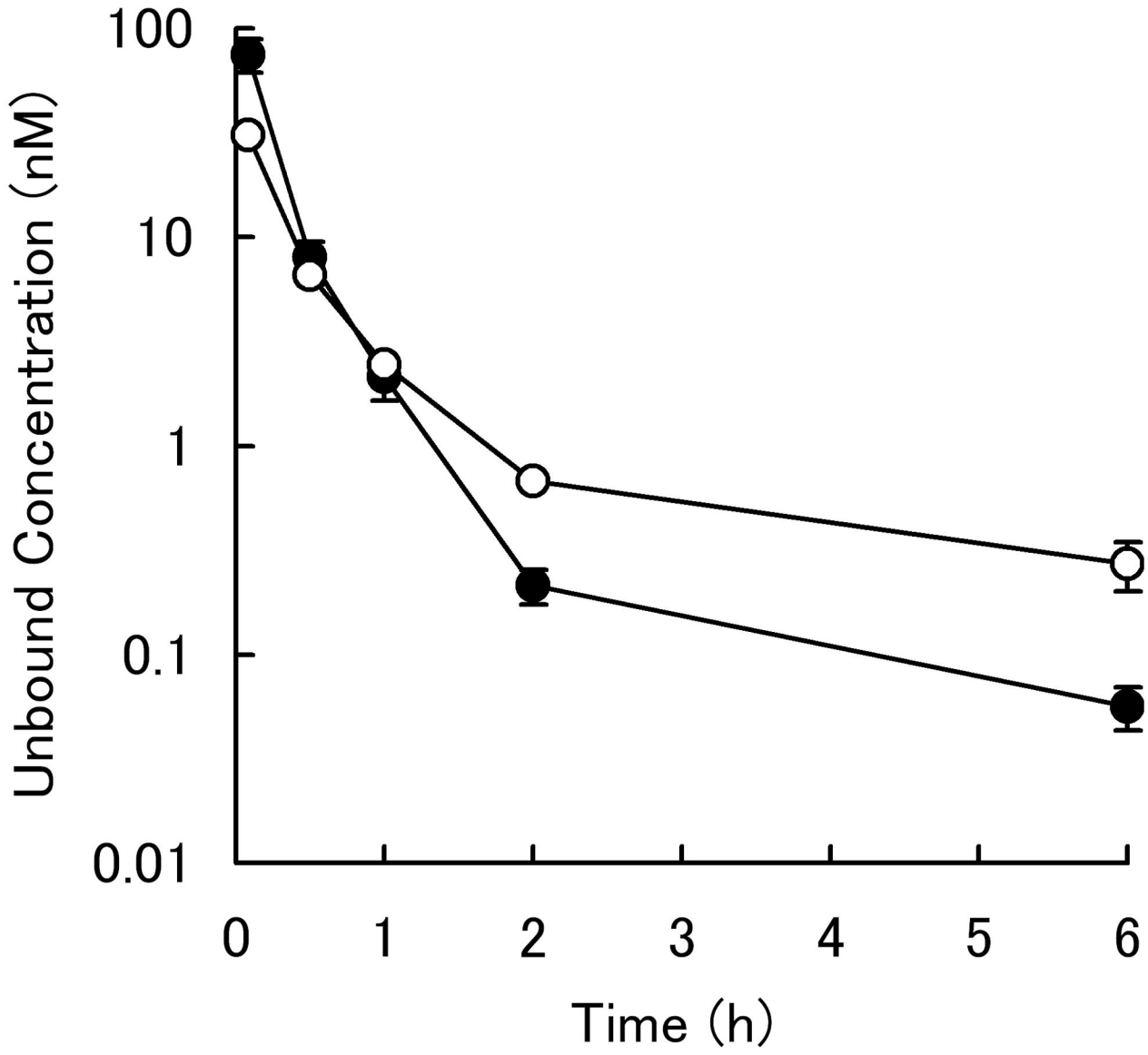
Figure 9



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