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molecular pharmaceutics

Investigation of the Effect of Mutations of Rat Albumin on the Binding Affinity to the $\alpha_4\beta_1$ Integrin Antagonist, 4-[1-[3-Chloro-4-[*N*′-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidine-2-yl]methoxybenzoic Acid (D01-4582), Using Recombinant Rat Albumins

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Received March 11, 2010; Revised Manuscript Received June 7, 2010; Accepted June 15, 2010

Abstract: The authors reported previously rat strain differences in plasma protein binding to $\alpha_4\beta_1$ antagonist D01-4582, resulting in a great strain difference in its pharmacokinetics (19-fold differences in the AUC). The previous study suggested that amino acid changes of V238L and/or T293I in albumin reduced the binding affinity. In order to elucidate the relative significance of these mutations, an expression system was developed to obtain recombinant rat albumins (rRSA) using *Pichia pastoris*, followed by a binding analysis of four rRSAs by the ultracentrifugation method. The equilibrium dissociation constant (K_d) of wild-type rRSA was 210 nM, while K_d of rRSA that carried both V238L and T293I mutations was 974 nM. K_d of artificial rRSA that carried only V238L was 426 nM, and K_d of artificial rRSA that carried only T293I was 191 nM. These results suggested that V238L would be more important in the alteration of K_d. However, since none of the single mutations were sufficient to explain the reduction of affinity, the possibility was also suggested that T293I interacted cooperatively to reduce the binding affinity of rat albumin to D01-4582. Further investigation is required to elucidate the mechanism of the possible cooperative interaction.

Keywords: Albumin; rat; polymorphism; binding affinity

Introduction

It is well recognized that the interindividual variability in the pharmacokinetics of a given drug is caused by different genetic polymorphisms of various proteins. Drug metabolizing enzymes and drug transporters are well-known polymorphic proteins that cause variability, though, causal proteins are not limited to them.[1,2] Therefore, it is worth investigating whether the pharmacokinetics of an investigational drug is affected by genetic polymorphisms of these key proteins during the drug development process to ensure desirable pharmacological efficacy and to avoid undesirable adverse effect as well.

4-[1-[3-Chloro-4-[*N*′-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidine-2-yl]methoxybenzoic acid (D01-4582) is an $\alpha_4\beta_1$ integrin antagonist synthesized in our institute. $\alpha_4\beta_1$ integrin, which is expressed on most leukocytes, plays an important role in the process of the adhesion, migration, and activation of inflammatory leukocytes at sites

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10.1021/mp100061z
Published on Web 06/15/2010

of inflammation by interacting with various adhesion molecules.[3,4] The inhibition of $\alpha_4\beta_1$ integrin is believed to be a promising approach for the treatment of multiple sclerosis, Crohn's disease, and rheumatoid arthritis.[5,6] Since the site of action is in the blood, well-controlled pharmacokinetics is desired to obtain an efficient pharmacological effect. However, marked rat strain differences in the D01-4582 pharmacokinetics were observed in preclinical pharmacokinetic studies where SD rats, Wistar rats, and Brown Norway rats showed relatively lower plasma concentration profiles than CD(IGS) rats (CD rats), Lewis rats, and Eisai hyperbilirubinemic rats.[7] The difference in AUC was approximately 19-fold after an oral administration at the same dose. Since these observations implied interindividual pharmacokinetic variability in humans, the mechanism of the strain differences was investigated in detail. The results suggested that the alteration of the plasma protein binding was associated with the pharmacokinetic strain differences. The three rat strains that exhibited low plasma concentrations showed relatively low plasma protein binding ratios. These alterations in the plasma protein binding were associated with genetic polymorphisms of the rat serum albumin (RSA) gene. Among the three amino acid substitutions found, V238L and T293I were found only from the rat strains that demonstrated low plasma concentrations. Therefore, these two mutations would be one of the causes of strain differences. However, it remains unclear which of the two mutations, or both, caused the strain differences.

Recently, an in vitro expression system that efficiently expresses albumin has been developed using *Pichia pastoris*.[8] This system serves as a standard system for the expression of human serum albumin (HSA) and the subdomain of HSA to investigate drug binding characteristics in detail, and also to investigate the effect of genetic polymorphisms on ligand binding.[9−11] Furthermore, the applicability of this system for the production of HSA has been challenged in regard to transfusion of HSA to patients as a drug.[12] A *Pichia pastoris* expression system has been used for the expression of various proteins, as well as HSA, while rat albumin production has not been reported yet.

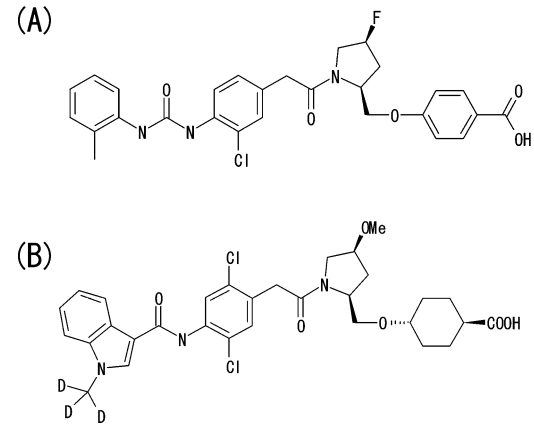

Figure 1. Structure of (a) D01-4582 and (b) the internal standard.

In the present study, an expression system was developed to obtain recombinant rat serum albumin (rRSA) using *Pichia pastoris*. Two types of natural rat albumin and two types of artificial albumin to which a site-directed point mutation was introduced were produced. The significance of the amino acid change was evaluated through a binding study.

Experimental Section

Chemicals. D01-4582 and *trans*-4-[1-[[2,5-dichloro-4-(1-methyl-d_3-3-indolylcarbonylamino)phenyl]acetyl]-(4*S*)-methoxy-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid, an internal standard for the analysis of D01-4582, were synthesized by the Medicinal Chemistry Research Laboratory, Daiichi Sankyo (Tokyo, Japan) (Figure 1). These compounds were obtained through the collaboration with Pharmacopeia (Cranbury, NJ). All other reagents were of analytical grade.

Cloning of the Rat Albumin Gene from SD Rat and CD Rat Liver. Total mRNA was extracted from the liver of each rat strain as described before,[7] and then cDNA

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samples of SD rat albumin and CD rat albumin were obtained by using the following primers: 5′-cgatgaattcatgaagtgggtaacctttctcc-3′ as a forward primer and 5′-tgacgaattcttaggctaaggcttctttgcttc-3′ as a reverse primer. Mature albumin, which does not include a prepro-sequence, was prepared using the following primers carrying an *Xho*I site at the 5′-region and an *Eco*RI site at the 3′-region: 5′-tgcgctcgagaagcacacaagagtgagatcgc-3′ as a forward primer and 5′-agctgaattcggttgtgatgtgtttaggctaaggc-3′ as a reverse primer. The cDNA sequence and amino acid sequence were numbered from the beginning of the mature albumin throughout this study. The two cDNA samples were cloned into pUC19 vector with a leader sequence, L10 (coding for EFMKLLLLLLLLLSSSSSSS). L10 contains an *Eco*RI site at its 5′-region and an *Xho*I site at its 3′-region, and was inserted at the 5′-region to construct L10-CDRSA and L10-SDRSA. Then, each of the inserts was cloned into a *Eco*RI-digested methanol-inducible pHIL-D2 vector (Invitrogen, Carlsbad, CA) to construct expression vectors, pHIL-D2-L10-CDRSA and pHIL-D2-L10-SDRSA. The recombinant albumins that were obtained from these vectors are designated as rCD and rSD, respectively. pHIL-D2-L10-CDRSA and pHIL-D2-L10-SDRSA differ in that the latter carries naturally occurring substitutions of nucleotides that code two amino acid changes, V238L and T293I. All of the cloning procedures were conducted in accordance with the guidelines of the Daiichi Sankyo Recombinant DNA Safety Committee.

Introduction of Mutations into pHIL-D2-L10-CDRSA. The point mutations corresponding to V238L and T293I were introduced separately into pHIL-D2-L10-CDRSA using a Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the detailed protocol provided by the manufacturer. The following primers were used: 5′-caccaaattggcaacagacctctaccaaaatcaacaaggagtgc-3′ for artificial rRSA that carries V238L (VL) and 5′-cccagtgtctcgctgagatagaacatgacaacattcc-3′ for artificial rRSA that carries T293I (TI). As a result, G712C and T714C were introduced for V238L, and C878T was introduced for T293I. The sequences of the products were confirmed by analyzing their sequence with a CEQ8000 (Beckman Coulter, Fullerton, CA) using a CEQ DTCS Quick Start Kit (Beckman Coulter).

Expression and Purification of Recombinant Albumin. Expression vector constructs were introduced into *Pichia pastoris* (strain GS115, Invitrogen) by the electroporation method (BIO-RAD, MicroPulser, Hercules, CA). A yeast clone that contained the expression cassette stably integrated into the chromosomal DNA was isolated. The cells were grown in YPD [1% yeast extract, 2% peptone, 2% dextrose, 0.01% ampicillin] and were then grown to confluence in BMGY [1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, (4×10^{-5})% biotin, 1% glycerol] at 30 °C with shaking at 165 rpm. The volume of BMGY was 20, 12.5, 4, and 4 L for the yeasts that had pHIL-D2-L10-CDRSA, pHIL-D2-L10-SDRSA, V238L mutation, and T293I mutation, respectively. The cells were grown for 12 h or more so that an attenuance of 10-fold diluted yeast suspension became approximately 0.8 at 600 nm. In the induction phase, the growth phase cells were harvested by centrifugation (1500*g*, 5 min, 4 °C), cell pellets were resuspended with BMMY [1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, (4×10^{-5})% biotin, 0.5% methanol], and then the cells were grown for an additional 96 h at 30 °C. The volume of BMMY was 4, 2.5, 0.8, and 0.8 L for the yeasts which had pHIL-D2-L10-CDRSA, pHIL-D2-L10-SDRSA, V238L mutation, and T293I mutation, respectively. Methanol of 1% of the incubation volume was added every 24 h to maintain induction.

Purification of rRSA. The growth medium was separated from the yeasts by centrifugation (10000*g*, 20 min, 4 °C), and the secreted rRSA was isolated from the growth medium as follows. Briefly, ammonium sulfate was added to the medium at room temperature. The temperature was then lowered to 4 °C, and the pH was adjusted to 4.0. The precipitated protein was collected by centrifugation (10000*g*, 60 min, 4 °C) and was resuspended in 250 mL of 200 mM sodium acetate buffer (pH 5.5). Dialysis was performed three times at 4 °C against the sodium acetate buffer. Afterward, the solution was loaded onto a column of Blue Sepharose CL-6B (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The column was washed with a 10-fold bed volume of 200 mM sodium acetate buffer at pH 5.5, and then the rRSA was eluted with 3 M sodium chloride. The eluted rRSA was deionized, freeze-dried, and then stored at −20 °C until use. A small portion of the resulting protein was boiled in 0.025 M Tris buffer, pH 6.8 containing 100 mM dithiothreitol, and then analyzed by sodium polyacrylamid gel electrophoresis (SDS−PAGE). The recombinant albumin was assessed by Western blotting analysis detected with ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, U.K.) using ×5000 diluted sheep anti-rat albumins (Bethyl Laboratories Inc., Montgomery, TX) as the first antibody, and ×2000 diluted peroxidase-rabbit anti-sheep IgG (H+L) (Invitrogen) and ×5000 diluted streptavidin-HRP (Dako, Glostrup, Denmark) as the second antibody. The N-terminal of the amino acid sequence was determined by Edman degradation sequence analysis (PPSQ-33A, Shimadzu, Kyoto, Japan) using 10−20 μg of protein. Recombinant albumins in Sörensen buffer (113 mM Na_2HPO_4, 17 mM KH_2PO_4, pH 7.4) (0.3 μM) were transferred to a 10 mm quartz cell (JASCO; Tokyo, Japan) and subjected to J-720 circular dichroism spectrometer (JASCO) to obtain the spectra between 200 and 250 nm. Each solution was measured 6 times (step resolution 0.2 nm, 1 s each step) at 25 °C, and the mean spectrum was obtained. The spectra were smoothed using a noise reduction feature of the Standard Analysis program (Jasco), which is included in the software package provided with the instrument. For calculation of the mean residue ellipticity,[13] $[\theta]$, the molecular mass of the albumin was assumed to be 65 kDa. Human serum

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albumin (Sigma-Aldrich, St. Louis, MO) was used as a control albumin. The α-helical content of albumins was calculated from the mean residue ellipticity value at 222 nm.[14]

Binding Assay Using Recombinant Albumin. Albumins were solubilized with Sörensen buffer at a concentration of 0.2% w/v. The concentrations of the albumin solutions were quantified with Albumin Test Wako (Wako Pure Chemical Industries, Osaka, Japan) using HSA (Sigma-Aldrich) as the standard protein. After 3 min warming at 37 °C, 5 μL of the D01-4582 standard solution was added to 245 μL of albumin solution and incubated for 15 min at 37 °C. The final concentrations of D01-4582 were 0.5, 1, 2, 5, 10, 20, 30, and 50 μM ($n = 3$). At the end of the incubation period, 210 μL of each sample was centrifuged in a 0.23 PC tube (Hitachi Koki Co., Ltd., Tokyo, Japan) at 200000*g* for 16 h at 4 °C. Then, the supernatant (50 μL) was collected. Samples with high concentrations were diluted with buffer. Methanol (150 μL) containing the internal standard was added to the samples (50 μL), followed by vigorous mixing. Two microliters of the mixture was introduced into the LC−MS/MS to quantify the free concentration of D01-4582. Samples for the calibration curves were freshly prepared by adding several concentrations of the D01-4582 standard solution to the buffer.

LC−MS/MS Analysis. A Waters HPLC system (Aliance 2695, Waters, Milford, MA) coupled to a Quattro Micro (Waters) via an electrospray ionization interface was used for mass analysis and detection. Chromatographic separation was achieved on a Symmetry Shield RP8 column (50 × 2.1 mm i.d.; Waters) 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 sample. The mass spectrometer operated in positive ion mode. The MS−MS transition selected to monitor D01-4582 was from *m*/*z* 540.0 to a product ion at *m*/*z* 402.0. The internal standard was monitored using the transition from *m*/*z* 619.0 to *m*/*z* 493.0. Each transition was alternately monitored at a dwell time of 0.2 s. Calibration curves were obtained with a weighting of $1/x^2$. The coefficient of determination was >0.998. The limit of quantification was 1 nM, and the upper limit of quantification was 3000 nM.

Data Analysis. The binding characteristics were initially examined by a Scatchard plot to determine the applicability of only one principle class of binding site with a nonspecific binding component. The equilibrium dissociation constant (K_d) and the number of binding sites (N) were estimated by fitting the Langmuir binding model to the results as follows.

$$C_b/C_{alb} = NC_u/(K_d + C_u) + \mathrm{NSB} \times C_u \qquad (4)$$

where C_b, C_u, and C_{alb} represent the molar concentrations of bound and unbound D01-4582 and albumin concentration, respectively. N is the number of binding sites per unit of albumin. NSB is a coefficient to account for the nonspecific binding of D01-4582. A nonlinear least-squares program (WinNonlin, Mountain View, CA) was used to fit the model.

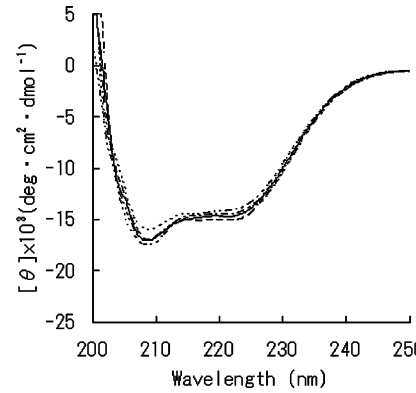

Figure 2. Circular dichroism spectra at 25 °C. The protein concentration was 0.3 μM in Sörensen buffer (pH 7.4). Spectra are shown for rCD (—), rSD (– –), VL (– · –), TI (– · · –), and HSA (···).

Presentation of Binding Site I of HSA. The location of V238 and T293 on rat albumin was speculated using a crystal structure of HSA that was registered in the protein data bank (PDB ID: 1HA2).[15] Viewer Light 5.0 (Accelrys, San Diego, CA) was used to present the structural data.

Results

rRSAs were expressed in the *Pichia pastoris* system. Each recombinant protein was purified and migrated to show the anticipated molecular weight of 65 kDa. The N-terminal amino acid sequence of each protein was EAHKSEIAHR-FKDL, demonstrating that the insert on every expression vector was correctly translated into rRSA with the designed amino acid sequence. The secondary structure of recombinant protein was assessed by measuring the circular dichroism spectra of rRSA (Figure 2). The spectra for rRSA were nearly identical among them, and there was little difference between rRSAs and HSA. α-Helical content for rRSA was in a range of 39% to 41%, and that for HSA was 41%, which was slightly smaller than that described on the product information sheet from the provider (48%).

The binding of rRSA to D01-4582 was examined. Since a nonspecific binding component was observed in samples of high concentration, the results were analyzed by a Langmuir binding model with nonspecific binding (Table 1, Figure 3). The dissociation constant was 4.6-fold higher for rSD than for rCD. The dissociation constant for TI was almost the same as that for rCD. In contrast, the dissociation constant for VL was higher than that for TI and rCD. The

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Table 1. Binding Parameters of Each Recombinant Rat Albumin to D01-4582[a]

specimens	K_d (nM)	*N*	NSB
rSD	974 (17)	0.636 (11)	1.17×10^{-4} (5)
rCD	210 (10)	0.668 (5)	9.50×10^{-5} (4)
VL	426 (17)	1.05 (9)	1.12×10^{-4} (9)
TI	191 (16)	0.920 (7)	1.23×10^{-4} (6)

[a] Figures in parentheses represent the coefficient of variability of the estimate (unit: %).

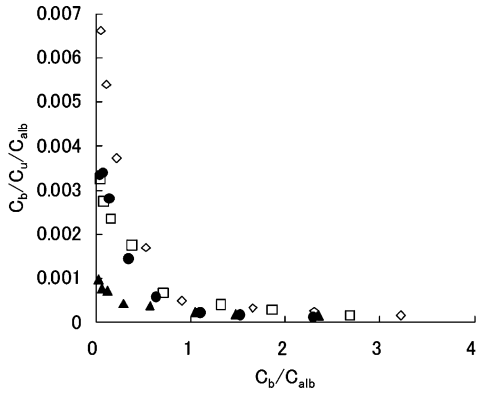

Figure 3. Binding of D01-4582 to the recombinant albumins; rCD (closed circle), rSD (closed triangle), TI (open diamond), and VL (open square). Each plot represents the mean ($n = 3$). C_b, C_u, and C_{alb} represent the molar concentrations of bound and unbound D01-4582 and albumin concentration, respectively.

number of binding sites per unit of albumin (N) for VL and TI was approximately 1.5-fold higher than that of the other albumins.

Disussion

In the present study, rRSAs were produced by an expression system so that the significance of amino acid changes that influence the binding affinity of rat albumin to D01-4582 could be evaluated. The yielded proteins were assessed by SDS−PAGE, Western blotting, and their N-terminal amino acid sequences, demonstrating that the recombinant proteins were in fact the designed proteins. The secondary structure of rRSA was also assessed by measuring the circular dichroism spectra, indicating that the recombinant albumins were folded properly. Accordingly, a *Pichia pastoris* expression system, commonly used to produce HSA and various peptides of the albumin subdomain,[9,10,12,16] was successfully applied to produce rat albumins in the present study to investigate binding characteristics.

The rSD showed lower affinity than rCD, which was in concordance with the finding that the protein binding ratio of D01-4582 in SD rat plasma was lower than that in CD rat plasma. The dissociation constants of SD rat and CD rat plasma were reported to be 1.7 and 0.077 μM, respectively.[7] Although the results of the present study could not reproduce the remarkable difference in K_d observed in SD and CD rat plasma, it was considered that the relative significance of amino acid changes on the alteration of the binding affinity could be evaluated by the recombinant albumins obtained by this system.

The artificial recombinant albumins, VL and TI, were produced by introducing the point mutations, V238L and T293I, into CD rat albumin, respectively. According to the estimated K_d for VL and TI, it was demonstrated that a single mutation of V238L could cause the reduction of the binding affinity of rat albumin to D01-4582, and that T293I had little impact on the affinity. However, none of the single mutations could result in the reduction of the binding affinity as was observed in rSD, suggesting that V238L alone is not sufficient to explain the alteration of K_d. Therefore, it was suggested that a single mutation of V238L would be more important in the alteration of K_d, though, it was possible for T293I to interact with V238L cooperatively to reduce the binding affinity of rat albumin to D01-4582.

The number of binding sites (*N*) for VL and TI was approximately 1.5-fold higher than the other albumins, which might be relevant to the fact that the results showed that nPt for SD rat plasma was higher than CD rat plasma by 1.6-fold.[7] However, since *N* for rSD was not different from *N* for rCD, the effect of the amino acid change on the binding capacity remains unclear.

Although the possibility of cooperative interaction of two mutations was suggested, V238 was considered to play a more important role in the binding of D01-4582 than T293, implying that D01-4582 binds near this amino acid residue. In human serum albumin, the amino acid residue corresponding to V238 of RSA is L238, which is located at binding site I, also known as the warfarin site.[17] Acidic drugs that have a planar group are known to bind to site I. These planar ligands bind snugly between the apolar side chain of L238 and A291.[18] This feature is shown in Figure 4, in which the *S*-(−) enantiomer of warfarin binds to HSA[15] (Figure 4).

Since RSA is homologous to HSA, V238 on RSA, as well as L238 on mutant RSA, they could directly interact with ligands, similar to L238 on the HSA dose. The amino acid change from valine to leucine is just an elongation of the side chain of the amino acid, which appears at first to be insignificant compared with T293I, a change with the loss of the hydroxy moiety in its side chain. However, the amino acid change of V238L alters the binding affinity of ligands, probably because this amino acid determines the space of the binding cavity. Our results imply that leucine might make

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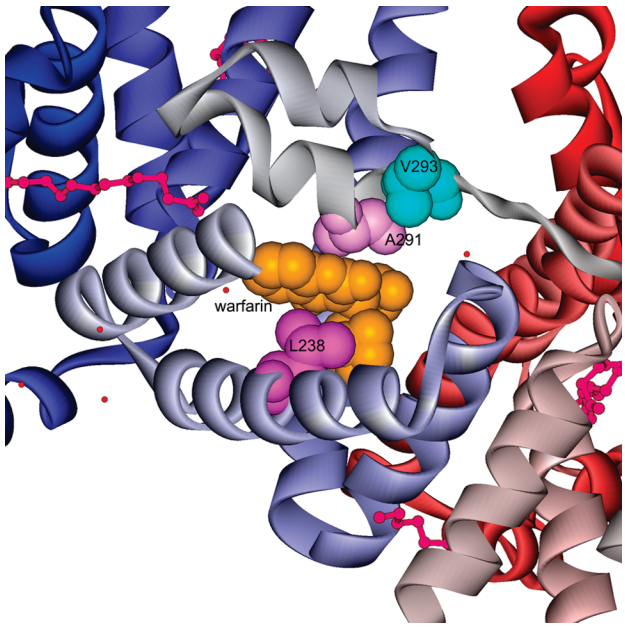

Figure 4. Structure of binding site I in human serum albumin as a typical binding conformation of HSA with a ligand (Protein Data Bank ID: 1HA2). Warfarin and L238, A291 and V293 are shown as a representation with the van der Waals surface. The planar group of warfarin is clipped between L238 and A291, while V293 does not directly interact with warfarin.

the binding cavity narrower, restricting the appropriate positioning of D01-4582. On the other hand, V293 (T293 in RSA) cannot interact with ligands directly because this residue is located at a slight distance from the binding cavity. This could be one of the reasons that TI did not alter the binding affinity. The detail of the mechanism of cooperative interaction between V238L and T293I remains unclear at present, and therefore further study is required. However, the mechanism might be elucidated if mutant albumin is purified from mutant rat plasma and is crystallized to identify the structural conformation.

It is also interesting to consider amino acid changes from the aspect of species differences in the protein binding of various drugs. The binding cavity of site I of HSA is composed of K195, K199, R218, and R222 at the pocket entrance, and Y150, H242, and R257 at the bottom of the pocket. F211, W214, A215, L238, and A291 form the main cavity from the entrance to the bottom.[18] The species differences in the amino acid residues listed above between humans and rats are R195, N242, and V238. V238L might be considered to be a minor substitution, but it resulted in the alteration of the binding affinity. Likewise, the other two sites of species differences of amino acid residues, R195 and N242 that form the binding cavity, might cause alteration of the binding affinity of various drugs. The present study suggests that small changes caused by these substitutions could result in significant changes of the binding affinity, which may lead to overall species differences in the protein binding ratio. However, one should not neglect the possibility that substitution of the amino acid residue, which is located at a slight distance from the binding cavity, could also affect the binding affinity.

The results of the present study demonstrate that albumin genetic polymorphisms can cause interindividual differences in protein binding. Although the binding site of D01-4582 on HSA has not yet been investigated, site I on HSA is a possible binding site since the binding characteristics of site I have been reported to be similar between HSA and RSA.[19] Several polymorphisms that result in amino acid changes have been reported around binding site I of HSA including R218H, K225Q, K240E, Q268R, D269G, and K276N.[20,21] Additionally, the genetic polymorphisms at the sites of both 238 and 293 for HSA have not been reported so far. It is not plausible for interindividual differences to arise in the protein binding, which is caused by the amino acid changes at sites 238 and 293 in humans. However, there is the possibility that other reported polymorphisms could affect the binding affinity of HSA to D01-4582. Site-directed mutagenesis technology coupled with an expression system would be a valuable tool, enabling us to evaluate the risk of interindividual variability in the binding affinity caused by genetic polymorphisms of albumin, by which the interindividual pharmacokinetic variability can be evaluated at an earlier stage of the drug development.

In conclusion, an expression system was developed to produce RSA using *Pichia pastoris*. With this system, the significance of amino acid changes on the alteration of the binding affinity was evaluated. The results suggested that a single mutation of V238L was more important in the alteration of K_d. The possibility was also suggested that T293I interacted with V238L cooperatively to reduce the binding affinity of rat albumin to D01-4582. Further investigation is required to elucidate the possible cooperative interaction of these two mutations in the future.

Abbreviations Used

HSA, human serum albumin; RSA, rat serum albumin; rRSA, recombinant rat serum albumin; K_d, equilibrium dissociation constant; nPt, concentration of the binding site; rSD, recombinant SD rat albumin; rCD, recombinant CD rat albumin; VL, recombinant rat albumin with a site directed mutation of V238L; TI, recombinant rat albumin with a site directed mutation of T293I.

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