Role of Biantennary Glycans and Genetic Variants of Human α_1-Acid Glycoprotein in Enantioselective Binding of Basic Drugs as Studied by High Performance Frontal Analysis/Capillary Electrophoresis

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Purpose. To establish a clear understanding of the role of biantennary branching glycans and genetic variants of α_1-acid glycoprotein (AGP) in enantioselective bindings of basic drug.

Methods. Human native AGP was separated using concanavalin A affinity chromatography into two subfractions, the unretained fraction (UR-AGP, defect of biantennary glycan) and the retained fraction (R-AGP, possessing biantennary glycan(s)). Imminodiacetate–copper (II) affinity chromatography was used to separate human native AGP into A variant and a mixture of F1 and S variants (F1*S variants). The mixed solutions of the (*R*)- or (*S*)-isomer of the model drugs (15 μM disopyramide (DP) or 30 μM verapamil (VER)) and 40 μM of respective AGP species were subjected to high-performance frontal analysis/capillary electrophoresis (HPFA/CE) to determine the unbound drug concentrations.

Results. The unbound concentrations (Cu) of DP in UR-AGP solutions were lower than those in R-AGP solutions, whereas there was no significant difference in the enantiomeric ratios (Cu(*R*)/Cu(*S*)) of DP between UR- and R-AGP solutions. In case of genetic variant, the Cu(*R*)/Cu(*S*) values of DP in F1*S and A solutions were 1.07 and 2.37, respectively. On the other hand, the enantiomeric ratio of VER in F1*S and A variant solutions were 0.900 and 0.871, respectively.

Conclusions. The biantennary glycan structures are related to binding affinity of DP to AGP, but not responsible for the enantioselectivity. Genetic variants give significant effect on the enantioselectivity in DP binding, but not in VER binding.

KEY WORDS: α_1-acid glycoprotein; biantennary branching glycan; genetic variants; capillary electrophoresis; protein binding; enantioselectivity.

INTRODUCTION

Plasma protein binding gives significant effect on the pharmacokinetic and the pharmacodynamic properties because the unbound drug can penetrate the blood vessel wall easily, whereas the bound drug can not (1). In addition, plasma protein binding of a chiral drug often shows enantioselectivity because of the inherent chirality of protein (2). Therefore, the enantioselective plasma protein binding study is indispensable for the rational drug development and for the safe usage of drugs. α_1-acid glycoprotein (AGP) is especially responsible for plasma protein binding of basic drugs (3). AGP, though higher structure is unknown, is highly glycosilated up to about 45% of its molecular weight, and is negatively charged mainly due to the endterminal sialyl residues (4). The microheterogeneity of AGP arises from (i) branching type of glycans (bi-, tri-, and tetra-antenna), (ii) their distribution on the five glycosilation sites, (iii) the fucose content, (iv) extension of branches by a lactosamine and (v) linkage between sialyl and galactose residues (5). It is known that the biantennary glycans are substituted mainly to asparagine 15 and/or asparagine 38 on the polypeptide chain (5). On the other hand, the drug-binding site of AGP is considered to locate between these two glycosilation sites on the peptidic chain (6). Therefore, the biantennary branching glycan structure may contribute to the enantioselective drug binding property. In addition to the microheterogeneity, AGP involves several peptidic variants coded by AGP-A, -B, and -B′ genes locating on three different loci (7). A major variant, A variant, is coded by AGP-B/B′ genes and F1 and S variants are coded by AGP-A gene (8). It is known that 22 amino acids of total 181 residues in A variant differ from those of F1 and S variants, whereas the substitution between F1 and S variants is unknown (7). Among 22 substitutions, only one residue around the drug binding site, namely glutamine 20 of F1*S variants, is replaced with arginine 20 of A variant (7). Such variation shows different drug binding properties as demonstrated by Hervé et al (9,10). Furthermore, the variation in the distribution of glycoforms (4) and genetic variants (11) of AGP as well as in their plasma levels (4,11) is observed in the acute phase response under several pathological conditions, which causes a change in drug–plasma protein binding condition. Therefore, it is important to investigate the difference in the drug binding property among AGP glycoforms and variants.

The conventional methods for binding analyses, such as equilibrium dialysis and ultrafiltration were not suitable for the present binding study, because these methods require relatively large amount of AGPs. In contrast, HPFA/CE requires a very small sample size (<200nL), which is one-thousandth or less of those needed by the conventional methods, and is beneficial to the present binding study. This method was established as a novel analytical method for determination of unbound drug concentration in drug and protein mixed solution (12,13), and has been applied to the binding analyses of AGP (14,15) and plasma lipoproteins (16).

Disopyramide (DP) and verapamil (VER), enantiomeric antiarrhythmic drugs, used as model basic drugs (Fig. 1), are bound mainly to AGP in plasma (17,18), and show enantioselectivity in their pharmacokinetics (19,20). Both are used clinically as racemate, whereas the (*S*)-isomers of respective drugs have much stronger pharmacological activity than the antipodes.

MATERIALS AND METHODS

Materials

Human AGP (purified from cohn fraction VI), DP racemate, (2*S*,3*S*)-(−)- and (2*R*,3*R*)-(+)-tartaric acid were purchased from Sigma (St. Louis, MO). (*R*)- and (*S*)-VER were obtained from Research Biochemicals Int. (Natick, MA). Concanavalin A immobilized on sepharose gel (conA-

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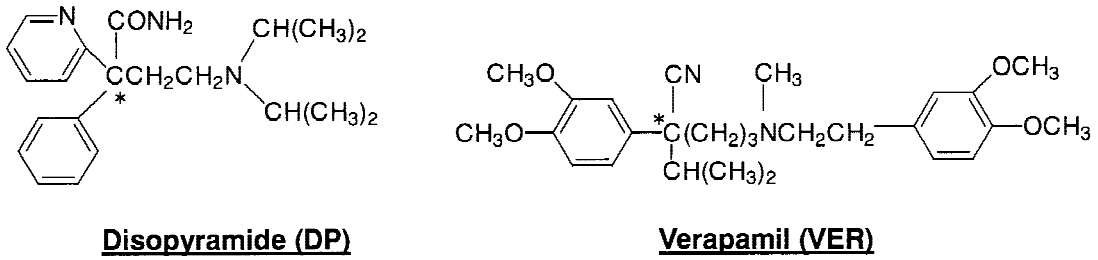

Fig. 1. Structures of disopyramide (DP, pKa 10.2) and verapamil (VER, pKa 8.6).

sepharose) and Chelating Sepharose Fast Flow (inimodiacetate (IDA)-sepharose) were purchased from Pharmacia Biotech (Uppsala, Sweden). Carboxymethyl-β-cyclodextrin (CM-β-CD, <3.5 substitution per ring) was obtained from Cycrolab R&D Laboratory Ltd (Budapest, Hungary). Z-1 methyl (trimethylammoniumpropanesulfonate) was from Millipore (Milford, MA). Spectra/Por 7 dialysis membrane (molecular weight cut off 10,000) was from SPECTRUM (Gardena, CA). Other chemicals of reagent grade were used without further purification.

Preferential Crystallization for Enantioseparation of Disopyramide Racemate

Disopyramide (DP) racemate was enantioseparated using preferential crystallization method reported by Burke *et al.* (21). The optical purity of DP enantiomers was comfirmed by capillary electrophoresis using cyclodextrin derivative as a chiral selector (22). Electrophoretic conditions were as follows, capillary: fused silica capillary (GL Science, Tokyo, Japan) of which inner surface was coated with linear polyacrylamide according to Hjertén (23); total length 82 cm; separation length 70 cm; 75 μm I.D., electrolyte solution: 100 mM pH 4.5 sodium phosphate buffer containing 15 mM CM-β-CD, applied voltage: +15kV, capillary temperature: 25°C, detection: UV absorbance at 205 nm. To decrease noise level of baseline around the migration time of DP enantiomers (55–65 minutes), the electrolyte solution was changed to 100 mM pH4.5 sodium phosphate buffer without CM-β-CD at 40 minutes after start of migration. (*R*)-DP migrated slower than (*S*)-DP. The (*R*)- and (*S*)-DP standards thus prepared showed optical purity of >96% and >98%, respectively.

Fractionation of Native AGP by Concanavalin A (conA) Lectin Affinity Chromatography

Native AGP was fractionated according to Bierhuizen *et al.* using conA-sepharose column chromatography (24). Unretained AGP (UR-AGP) is defect of biantennary branching glycan, whereas retained AGP (R-AGP) possesses one or more biantennary glycan(s). Distribution of UR- and R-AGP is determined from the peak area of the chromatogram. Both fractions thus obtained were dialyzed using Spectra/Por 7 membrane against deionized distilled water at 4°C and lyophilized.

Fractionation of Native AGP by Immobilized Copper (II) Ion Affinity Chromatography

AGP was separated into two fractions by iminodiacetate (IDA)–copper (II) affinity chromatography according to Hervé *et al.* (25,26). One fraction contains both F1 and S variants, and the other contains A variant. Both fractions were dialyzed using Spectra/Por 7 membrane against excess volumes of deionized and distilled water at 4°C, and lyophilized. Because of the background UV absorption of imidazole in the eluent for A variant, the distribution of the AGP variants was not determined from peak area of the preparative chromatography. Therefore, the small size IDA-Cu^{2+} column (13cm × 1.6 cmϕ) was used to determine the distribution of the genetic variants. Five milligrams of UR- or R-AGP was loaded. The amount of F1*S variants was determined from the area of the first peak obtained by using the copper chelated column. In parallel, the amount of total AGP (F1*S variants + A variant) was determined from the peak area obtained from copper free IDA column. The amount of F1*S variants was subtracted from the amount of total AGP to give the amount of A variant.

Determination of Unbound Drug Concentration in AGP Solution

AGP and drug enantiomer were dissolved in sodium phosphate buffer (pH 7.4, ionic strength 0.17) to prepare sample solutions. Two capillary electrophoresis systems were used to determine the unbound drug concentrations in these sample solutions by HPFA/CE (12,13). One system, CAPI-3000 (Otsuka Electronics Co. Ltd., Osaka, Japan) was equipped with a fused silica capillary (total length 42 cm, separation length 30 cm, 75 μm I.D. GL Science Inc., Tokyo, Japan). The other system, ABI 270A (Applied Biosystems, San Jose, CA) was equipped with Z shaped flow cell capillary (total length 43 cm, separation length 22 cm, 75 μm I.D., 3 mm optical path length, LC packings, San Francisco, CA) (27), which enhanced detection sensitivity. Because the unbound concentration of (*S*)-DP in A variant solution was not high enough to be detected using normal optical path length capillary, the latter system was applied to the series of DP–AGP variant mixed solutions. In both systems, capillary temperature was set at 25°C, and detections were achieved by UV absorption intensity at 200 nm. It was necessary to eliminate electroosmosis so as not to introduce AGP into the capillary; otherwise AGP adsorbed on the fused silica surface disturbs binding equilibrium. Therefore, the inner surface of the capillary was coated with linear polyacrylamide according to Hjertén (23). Sodium phosphate buffer (100 mM, pH 4.5) was used as background electrolyte. This buffer was superior, in avoiding the hydrolysis of the coating, to the phosphate buffer of physiological pH, which was used as the solvent in preparing the sample solutions. AGP solutions containing (*R*)- or (*S*)-DP were put in a vial, and were subjected to the electro-

kinetic sample injection (+7kV, 120 s for DP, +12kV, 60 s for VER). Before each sample injection, a very small volume (100 nL *ca.*) of sodium phosphate buffer (pH 7.4) was hydrodynamically introduced into the capillary from the inlet end to avoid direct contact of sample solution with acidic buffer which disturbs drug–protein binding equilibrium state.

Electrophoresis was performed at +7kV for DP and at +10kV for VER. The drug was detected as a zonal peak with a plateau region, and the unbound drug concentration was calculated from the plateau height. A series of the standard DP solutions (0.2 to 100 μM) and standard VER solutions (10 to 100 μM) were used to prepare calibration lines, showing good linearity ($R \geqq 0.999$).

Measurement of Electrophoretic Mobility of AGPs

The electrophoretic mobilities of these AGPs in capillary zone electrophoresis (CZE) mode were measured in duplicate using CAPI-3000 equipped with an uncoated fused silica capillary (total length; 42 cm, effective length; 30 cm, 75 μmID, 375 μm OD, GL Science Inc., Tokyo, Japan). Running buffer was sodium phosphate buffer (pH 7.4, ionic strength 0.17) containing 1.0 M Z-1 methyl which was added in order to prevent undesirable adsorption of AGP onto the inner surface of capillary. Mesityl oxide was used as a marker of electroosmotic flow. The applied voltage was +5 kV, and the temperature of the capillary cartridge was se at 25°C. AGPs were monitored at UV 205 nm.

Statistics

Statistical significance was evaluated using the Student *t* test.

RESULTS AND DISCUSSION

The electrophoretic mobility in the CZE mode was -1.1×10^{-4}cm^2/sV for native AGP, -1.1×10^{-4}cm^2/sV for UR-AGP, -1.1×10^{-4}cm^2/sV for R-AGP, -1.2×10^{-4}cm^2/sV for F1*S variants and -1.2×10^{-4}cm^2/sV for A variant. The mobility of asialo AGP, which was measured for comparison, was -0.502×10^{-4}cm^2/sV, indicating that desialylation did not occur during the fractionation procedures. The circular dichroism spectra and the fluorescence emission spectra (excitation wavelength, 286 nm) showed no significant difference among all AGPs, which meant that these AGPs have similar polypeptide structures. Table I shows the distribution of UR-AGP, R-AGP, F1*S variants, and A variant. The proportion of F1*S and A variants (70:30) were almost the same between UR-AGP and R-AGP, and the proportion of UR-AGP and R-AGP were almost the same (48:52) between F1*S variants and A variant.

Figure 2 shows the typical HPCE/FA profiles of (*R*)-VER and AGP mixed solution. Negatively-charged AGP did not appear in these electropherograms because of suppression of electroosmotic flow. As a result, only the drug with a positive charge was detected as a trapezoidal peak with a plateau region. The plateau height of (*R*)-VER in A variant solution (Fig. 2, right panel) was lower than that in the protein-free solution (Fig. 2, left panel) due to the protein binding. Similar profiles were obtained in all electropherograms of other sample solutions. Judging from the peak area, the amount of drug introduced into the capillary was about 3 pmol, which is smaller by two orders of magnitude than those needed by the conventional methods such as equilibrium dialysis and ultrafiltration. This means that HPFA/CE is beneficial for binding study of scarce protein such as AGP subtypes.

Table I. Distribution of UR-, R-AGP, and AGP Variants

	F1*S variants	A variant	Total
UR-AGP	33.4%	15.0%	48.4%
R-AGP	36.8%	14.8%	51.6%
Total	70.3%	29.7%	100%

Effect of Branching Glycan Structure of AGP on Binding of Drug

Our previous study revealed that the biantennary glycan(s) of AGP did not give any significant effect on enantioselective binding of VER to AGP (15). However, their effect upon DP–AGP binding has been unknown. Table II lists the unbound concentrations of DP enantiomers and their ratios in native AGP solutions, UR-AGP solutions and R-AGP solutions. As shown in Table I, both UR- and R-AGP show similar population of genetic variants (F1*S varians : A variant = 70:30). The unbound DP concentrations in UR-AGP solutions were significantly lower ($P < 0.05$) than those in R-AGP solutions. This suggests that the biantennary branching structure of AGP glycans gives significant effect upon DP–AGP binding. Biantennary branching glycan locates mainly near the drug-binding site of AGP molecule (5,6). Although electrophoretic mobilities of UR- and R-AGP were similar to each other, the number of sialyl residues of a biantennary glycan could be smaller than that of a tri- or tetra-antennary branching glycan. Therefore, it is considered that the local negative charges around the drug-binding site of R-AGP are smaller than those of UR-AGP, and consequently, R-AGP showed weaker binding affinity to the basic drug than UR-AGP.

It is reported that (*S*)-DP is bound more strongly than its antipode to native AGP (20). The unbound concentrations of (*R*)-DP shown in Table II are significantly higher ($P < 0.05$) than that of its antipode in both UR- and R-AGP solutions. The enantiomeric ratios of unbound DP concentrations (Cu(*R*)/Cu(*S*)) in UR-AGP and R-AGP solutions were 1.17 and 1.14, respectively, both of which did not show significant difference to that in native AGP solution (1.16). This result indicates that the biantennary branching structure of AGP glycans is not responsible for the enantioselectivity in DP–AGP binding.

Effect of Genetic Variant of AGP on Enantioselective Drug Binding

Table III lists the unbound concentrations of the model drug enantiomers and their enantiomeric ratios in the AGP variant solutions. The unbound concentrations of DP enantiomers in the F1*S variants solutions were significantly higher ($P < 0.001$) than those in the A variant solutions. These results were in accordance to the previous report by Hervé *et al.* (9,10) that DP is bound selectively to A variant. The

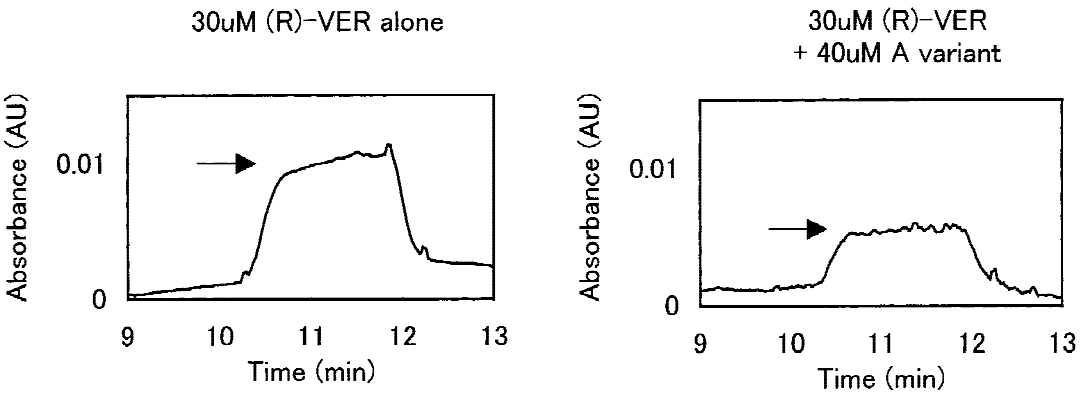

Fig. 2. HPFA/CE profiles of 30 μM (*R*)-VER standard solution (left) and 30 μM (*R*)-VER and 40 μM A variant mixed solution (right). Capillary; 30 + 12 cm, 75 μM ID, electrolyte; sodium phosphate buffer (pH 7.4, ionic strength 0.17), applied voltage; +10 kV, sample injection; +12 kV 60 sec.

enantiomeric ratio of the unbound DP concentrations (Cu(*R*)/Cu(*S*)) in F1*S variants solution was 1.07, indicating significantly less selectivity (P <0.001) than that in native AGP solution (1.16). On the other hand, the enantiomeric ratio in A variant solution was 2.37, which was significantly higher (P <0.001) selectivity than that of native AGP and F1*S variants. The unbound concentrations of VER enantiomers in native AGP solution and those in A variant solution did not show significant difference, whereas that in F1*S variants solution was significantly higher (P <0.01). In addition, the enantiomeric ratio of the unbound VER concentration in each AGP solution did not show significant difference. Because the proportion between UR- and R-AGP is almost the same between F1*S and A variants as shown in Table I, it is considered that the enantioselectivity in DP–AGP binding and in VER–AGP binding arises from the polypeptidic variation. The peptide chain also gives significant effect on VER–AGP binding, whereas it does not play a crucial role in the enantioselectivity of VER–AGP binding.

According to Hervé *et al.* (10), drugs with selective affinity for A variant share structural similarities that most of them contain an amino group linked by a short carbon chain to two aromatic rings that are either bridged to form a tricyclic structure (e.g., imipramine, amitryptyline) or unbridged (e.g., DP, methadone). VER has partial similarity, which includes two aromatic rings linked by a carbon chain containing a basic amino group. The carbon chain of VER between the two aromatic rings, which is longer than that of DP, may be folded to form the structure required by the binding pocket of A variant. However, the selective affinity of VER to A variant was smaller than that of DP, because the folded carbon chain may not be so rigid as that of DP.

In the IDA-Cu^{2+} affinity chromatography, AGP is retained by chelations of copper (II) ions to histidine residues (26). Both F1*S and A variants have three histidine residues. Because AGP variants are divided into two fractions by IDA-Cu^{2+} affinity chromatography, there must be some conformational difference between these variants. However, no significant difference was observed in the fluorescent emission spectra and CD spectra of these variants. Therefore, it is considered that small and local conformational difference around the drug-binding pocket on AGP molecule, which is not detectable by measurements of fluorescence or CD spectrum, causes the selective binding properties. Although Hervé *et al.* (10) demonstrated comparative molecular field analysis (CoMFA) models of drug–AGP variant complex, this approach did not include an enantiomeric factor and was not enough to understand the enantioselective binding aspects. It would be necessary for clear elucidation of the enantioselective drug– AGP binding mechanism that the topology of the protein should be revealed by direct investigation using such as NMR and X-ray crystallography.

Table II. Unbound DP Concentrations in Native, UR-, or R-AGP Solutions[a]

	Cu(R) (μM)	Cu(S) (μM)	Cu(R)/Cu(S)
Native AGP	6.97 ± 0.054	5.87 ± 0.130	1.16 ± 0.024
UR-AGP	6.56 ± 0.163	5.61 ± 0.059	1.17 ± 0.027
R-AGP	7.84 ± 0.099	6.89 ± 0.039	1.14 ± 0.059

[a] Mean ± SD (n = 3). Sample: 15 μM DP enantiomer dissolved in 40 μM native, UR-, or R-AGP solution (pH 7.4, ionic strength 0.17). Cu(R) and Cu(S) represent the unbound concentration of (R)- and (S)-DP, respectively.

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Table III. Unbound Drug Concentrations in AGP Variant Solutions[a]

	Cu(R) (μM)	Cu(S) (μM)	Cu(R)/Cu(S)
Disopyramide			
Native AGP[b]	6.97 ± 0.054	5.87 ± 0.130	1.16 ± 0.024
F1*S variants	10.8 ± 0.031	10.1 ± 0.160	1.07 ± 0.015
A variant	4.16 ± 0.107	1.76 ± 0.032	2.37 ± 0.065
Verapamil			
Native AGP	15.7 ± 1.04	18.6 ± 1.87	0.888 ± 0.0941
F1*S variants	20.1 ± 0.21	22.4 ± 0.92	0.900 ± 0.0330
A variant	14.9 ± 0.70	17.9 ± 0.76	0.871 ± 0.0668

[a] Mean ± SD (n = 3). Sample: 15 μM DP enantiomer or 30 μM VER enantiomer dissolve in 40 μM native AGP or AGP variant solution (pH 7.4, ionic strength 0.17). Cu(R) and Cu(S) represent the unbound concentration of (R)- and (S)-isomer, respectively.

[b] Data also listed in Table II.

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