Enantioselective Binding of Propranolol, Disopyramide, and Verapamil to Human α_1-Acid Glycoprotein

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Received 6 July 1999; revised 6 December 1999; accepted 19 December 1999

ABSTRACT: We investigated the binding of propranolol (PL), disopyramide (DP), and verapamil (VP) enantiomers by human α_1-acid glycoprotein (AGP; also called orosomucoid) and the relationships between the extent of drug binding and lipophilicity, desialylation, and genetic variants of AGP. Desialylation had little effect on the affinity of AGP for the drugs tested. The percentage binding correlated significantly with the partition coefficients for the drugs tested. Each enantiomer was competitively displaced from AGP by another enantiomer of the same drug, suggesting that they bind to the same site. However, the enantiomers bound to AGP with stereospecific affinities; the (–)-isomers of DP and VP had higher Kd values (4.27 and 4.97 μM, respectively) than the (+)-isomers (1.51 and 2.48 μM, respectively). When enantiomers of the different drugs were used in competitive binding experiments, VP binding was only partially inhibited by DP. This result suggested that drug binding is specific to different variants of AGP (A, F1, S). DP was found to specifically bind to variant A, whereas PL and VP bind to both A and F1/S variants. © 2000 Wiley-Liss Inc. and the American Pharmaceutical Association J Pharm Sci 89:751–757, 2000

Keywords: α_1-acid glycoprotein (AGP); stereoselectivity; sialic acid, partition coefficient

INTRODUCTION

The binding of drugs by plasma proteins is an important pharmacokinetic parameter because it influences the size of the free fraction of the drug in plasma. Changes in drug binding by plasma proteins affect the plasma unbound drug concentration and, therefore, influence the pharmacologic effect and/or adverse reaction caused by the drug, particularly for the highly extracted drug.

Many drugs mainly bind to albumin in plasma but α_1-acid glycoprotein (AGP; also called orosomucoid) a plasma transport protein that binds many basic drugs, including β-adrenergic-receptor blockers, antidepressants, and local anesthetics.[1–2] AGP is also well known as an acute-phase reactant protein, and although the physiologic function of AGP is not well understood, some evidence exists that AGP may suppress the immune response.[3] In normal animals AGP is present in the plasma at a relatively low concentration. However, in response to acute inflammation, such as that seen in cases of cancer and trauma, the plasma concentration of AGP increases dramatically as the result of an increase in its specific mRNA.[4] Thus the plasma AGP level is highly variable compared with the albumin level. Therefore, it is important to characterize the mechanism of drug binding to AGP to be able to predict changes in drug disposition and the interactions between drugs.

The molecular interactions involved in AGP-ligand binding are poorly understood. AGP is strongly acidic because it contains sialic acid residues; some laboratories have reported that the

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Journal of Pharmaceutical Sciences, Vol. 89, 751–757 (2000)

sialic acid residues do not affect drug binding,[5] but others have reported slight differences in drug binding between desialylated AGP and native AGP.[6] To analyze the effect of sialic acid residues on the binding of drug enantiomers, we have performed binding studies using desialylated AGP prepared with neuraminidase. Lipophilicity also affects drug binding to plasma proteins. Therefore, we also examined the relationship between lipophilicity and drug binding to human AGP.

Many basic drugs have chiral centers and are available commercially as racemic mixtures. Some drugs show stereoselective pharmacokinetics and pharmacodynamics, including differences in metabolism, tissue distribution, and excretion.[7–9] Many of these stereospecific characteristics may be due to stereoselective protein binding in plasma and tissues; however, few studies have examined the individual enantiomers of drugs. Therefore, quantitative studies of the stereoselective interactions of drugs may help to characterize the preformed ligand-binding sites of serum proteins.

We have studied the relationships between drug binding and lipophilicity, desialylation of AGP, and the genetic variants of AGP, using individual drug enantiomers. Three anti-arrhythmic agents, propranolol (PL), disopyramide (DP), and verapamil (VP), were studied because these drugs are available commercially as racemates and are known to bind with high affinities to human AGP.

EXPERIMENTAL SECTION

Materials

Human AGP, obtained from Sigma (St. Louis, MO; Cohn fraction VI; lots 13H9336 and 125H9329), was a mixture of the three genetic variants of AGP (F1, S, and A). Neuraminidase (type II) and VP hydrochloride were also obtained from Sigma. An immobiline dry plate, low-molecular-mass marker protein kit and chelating Sepharose Fast Flow were obtained from Pharmacia LKB (Uppsala). PL enantiomers were obtained from Aldrich (Milwaukee, WI). Racemic and [^{3}H]-labeled (specific activity, 0.96 TBq/mmol) DP were gifts from Nippon Roussel K. K. (Tokyo). Racemic [*N*-methyl-^{3}H]VP hydrochloride and [4-^{3}H]PL (specific activities, 2.2 and 0.89 TBq/mmol, respectively) were obtained from DuPont-New England Nuclear (Boston, MA). The (+)- and (−)-enantiomers of DP and VP were separated by high-performance liquid chromatography (HPLC),[9] and their stereochemical purities were ascertained by stereospecific HPLC resolution (the stereochemical purities of S(+)-DP, R(−)-DP, S(−)-VP, and R(+)-VP were 98.4, 99.6, 98.6, and 99.3%, respectively). All other reagents used were of analytical grade, unless stated otherwise.

Protein Binding Study

Binding of DP and PL enantiomers to human AGP was studied using an ultrafiltration technique as previously reported.[9] Ten microliters of ^{3}H-labeled drug (80 GBq/mmol for DP, 120 GBq/mmol for PL), 10 μL of nonlabeled drug (final drug concentration: 0.1–100 μM for DP, and PL) and, 10 μL of competitor (final concentration was described in Figure) or Sörensen buffer (0.113 M Na_2HPO_4 and 0.017 M KH_2PO_4, pH 7.4) were added to 0.5 mL of AGP (1 mg/mL in Sörensen buffer), incubated at 37°C for 10 min and ultrafiltered (Ultrafree C3-LGC, Nihon Millipore, Tokyo) at 1000 × *g* for 15 min at 37°C. The radioactivities of both the filtrate and an aliquot of the reaction mixture were measured in a liquid scintillation counter (LSC, LSC-700, Aloka).

Binding of VP to AGP was evaluated by equilibrium dialysis instead of ultrafiltration because of significant adsorption to the filtration device (21–27%). A trace amount (10 μL) of ^{3}H-labeled VP enantiomer (2.5 GBq/mmol), 10 μL of nonlabeled VP enantiomer (final concentration was 0.1–90 μM) and 10 μL of a competitor or Sörensen buffer were added to 0.5 mL of AGP (1 mg/mL in Sörensen buffer) and the solutions were dialyzed (Spectra/Por 2, molecular mass cut-off, 12–14 kDa, Spectrum Inc.) against 3 mL of Sörensen buffer for 6 h at 37°C. After equilibrium had been reached, the radioactivities of the solutions (inside and outside the dialysis tube) were counted in an LSC. Changes of incubation volume during dialysis were corrected according to the changes in the AGP concentration.

Determination of the Buffer-Octanol Partition Coefficient

To explain the relationship between drug binding to AGP and physicochemical properties, the bindings of DP, PL, and VP enantiomers, imipramine, quinidine, atenolol, metoprolol, flecainide, aprindine, pilsicainide, ketoprofen, and tolbutamide to human AGP were determined. The binding as-

sessed by equilibrium dialysis (final drug concentration was 0.1 μM) as described previously and their radioactivities or concentrations were determined using an LSC and by HPLC, respectively.

The partition coefficient of each drug was determined as described previously.[9] Sörensen buffer and n-octanol were used as the aqueous and organic phases, respectively. After equilibration, the radioactivities or concentrations in both the aqueous and organic phases were determined using an LSC and by HPLC, respectively.

Drug Binding to Desialylated Human AGP

AGP (100 mg) was dissolved in 40 mL of 0.05 M acetate buffer (pH 5.5) and added to 10 units of neuraminidase. The mixture was incubated at 37°C for 48 h and then dialyzed (Spectra/Por 2, molecular mass cut-off 12–14 kDa) against deionized water at 4°C. The deionized water was changed three times, and the mixture in the dialysis tube was lyophilized. The purity of the desialylated AGP was determined by SDS-PAGE and isoelectric focusing (IEF).[10] To examine the effect of sialic acid residues on the binding of drugs to AGP, the binding of PL, DP, and VP enantiomers to desialylated AGP was performed as described previously. The nonspecific binding was assumed to be the binding obtained in the presence of excess amounts of nonlabeled drug (100 μM).

Separation of AGP Genetic Variants

The human AGP genetic variants were separated by the method of Herve et al.[11] An IDA Sepharose gel loaded with copper (II) ions and equilibrated in buffer 1 (20 mM sodium phosphate buffer, pH 7.0, containing 0.5 M sodium chloride) was packed into a glass column (2.0 × 30.0 cm L, Pharmacia LKB). A commercial AGP (100 mg was dissolved in 3.0 mL of buffer) was applied to the column at a flow rate of 60.0 mL/h. Fractions (10 mL) were collected and their respective absorbance values were determined spectrophotometrically at 280 nm. As soon as variant A had eluted, elution buffer 2 (buffer 1 containing 20 mM imidazole) was applied to the column to remove both of the bound F1 and S variants (F1/S mixture). The peak fractions of each eluent were collected, concentrated on a YM 10 membrane filter (Amicon, Danvers, MA), dialyzed against deionized water, and lyophilized. The purities of the isolated AGP preparations were checked by IEF followed by incubation at 37°C for 24 h with 1 unit neuraminidase.[11]

Data Analysis

The linear relationships between percentage binding and pKa and partition coefficients were analyzed by use of Pearson's correlation test by computer program SPSS (SPSS Inc., Chicago, IL). The human AGP binding characteristics of each drug enantiomer were calculated using the nonlinear least squares method program NLS.[12] The drug binding characteristics showed one specific (high-affinity) binding site and one nonspecific (low-affinity) binding site, and that the manner of binding can be represented by the equation:

$$Cb = \frac{n \cdot P \cdot Cf}{Kd + Cf} + \alpha \cdot Cf$$

where Cb and Cf are the concentrations of bound and unbound drug, P, n, and Kd are the AGP concentration (the molecular mass was taken as 44 kDa), the number of binding sites, and the dissociation constant, respectively. The calculation was confirmed when the correlation coefficient between observed and predicted values was greater than 0.990. The statistical comparison of enantiomers of the same drug was performed using Student's unpaired t test ($P < 0.05$).

RESULTS

To know the relationship between physicochemical properties and drug binding to AGP, the relationships between drug binding and both drug ionization and lipophilicity were studied. The percentage binding of drugs to AGP did not correlate with the pKa values of the drugs ($r = 0.022$), whereas the correlation coefficient of the fitting to show that a correlation may exist between percentage binding and drug lipophilicity (Fig. 1).

To analyze the effect of sialic acid residues on the binding of drug enantiomers, we studied desialylated AGP that we prepared with neuraminidase (Table 1). All total bindings between native and desialylated AGPs excepted that of R(+)-PL were not statistically different.

The binding of PL, DP, and VP enantiomers to human AGP and interactions between the enantiomers were analyzed with Scatchard plots (Fig. 2). Table 2 summarizes their binding character-

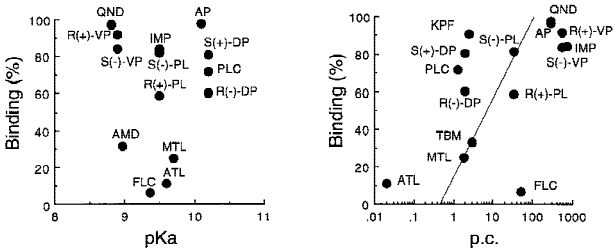

Figure 1. Relationship between percentage binding to human α_1-acid glycoprotein (a mixture of the A, F1, and S variants) and the pKa values and partition coefficients (p.c.) of the drugs. Percentage binding = 40.0 × Log (p.c.) + 15.0 (r = 0.578, $P < 0.05$). AMD = amiodarone; AP = aprindine; ATL = atenolol; DP = disopyramide; FLC = flecainide; IMP = imipramine; KPF = ketoprofen; MTL = metoprolol; QND = quinidine; PL = propranolol; PLC = pilsicainide; TBM = tolbutamide; VP = verapamil.

istics. The dissociation constant (Kd) of S(−)-PL was two times lower than that of R(+)-PL, and the number of binding sites was close to one for each enantiomer; the binding of each enantiomer was completely and competitively inhibited by the other enantiomer. Similarly, the Kd of S(+)-DP was about three times lower than that of R(−)-DP, and the binding of each enantiomer was completely and competitively inhibited by the other enantiomer. The Kd value of S(−)-VP was twice that of R(+)-VP and the number of binding sites close to one for each enantiomer; the binding of each enantiomer was completely and competively inhibited by the other enantiomer. Therefore, each of these drug enantiomer pairs binds to a single site on AGP but with different affinities.

Next we studied the competition between enantiomers of different drugs. Binding of DP enan-

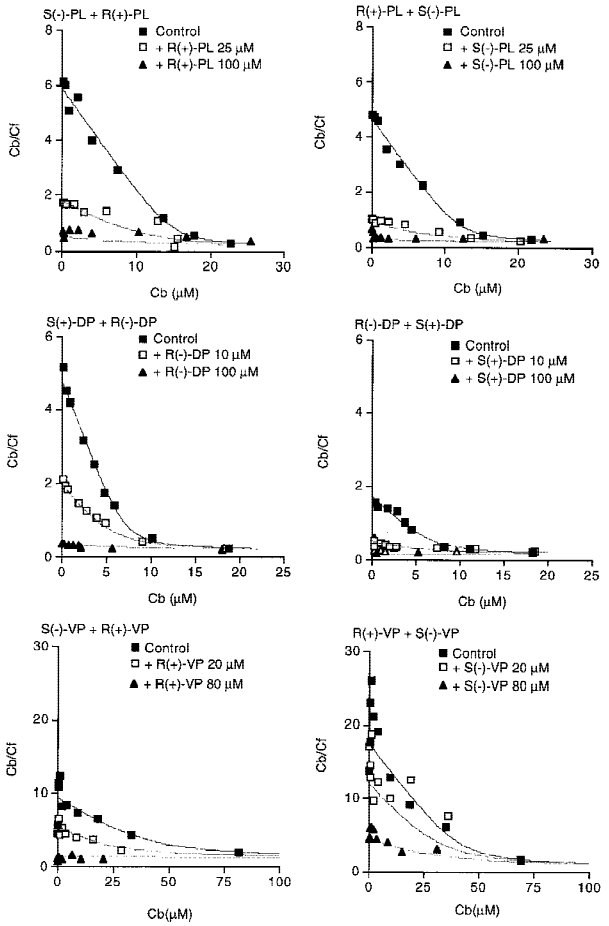

Figure 2. Scatchard plots of the binding to human α_1-acid glycoprotein (a mixture of the A, F1, and S variants) of propranolol (PL), disopyramide (DP), and verapamil (VP) enantiomers in the presence or absence of another enantiomer.

Table 1. Effects of Desialylation on the Binding of Propranolol (PL), Disopyramide (DP), and Verapamil (VP) to Human AGP

	Native AGP		Desialylated AGP	
	Total Binding (%)	Nonspecific Binding (%)	Total Binding (%)	Nonspecific Binding *%)
S(−)-PL	76.9 ± 2.38	34.1 ± 4.36	78.3 ± 0.24	30.6 ± 2.37
R(+)-PL	78.2 ± 5.73	34.9 ± 1.03	65.3 ± 6.32[a]	32.3 ± 2.98
S(+)-DP	73.5 ± 9.07	25.6 ± 1.63	84.6 ± 1.32	31.0 ± 2.33[a]
R(−)-DP	43.3 ± 6.33	22.2 ± 10.0	56.3 ± 4.76	26.7 ± 4.97
S(−)-VP	90.7 ± 2.88	63.3 ± 7.33	93.1 ± 3.56	66.7 ± 4.62
R(+)-VP	95.2 ± 1.67	69.8 ± 8.16	90.2 ± 5.22	70.9 ± 6.19

Each value is the mean ± S.D. (n = 5).
[a] Significantly different from that of native AGP ($p < 0.05$).

Table 2. Binding Characteristics of Propranolol (PL), Disopyramide (DP), and Verapamil (VP) Enantiomers to Human AGP

	S(−)-PL	R(+)-PL	S(+)-DP	R(−)-DP	S(−)-VP	R(+)-VP
n	0.78	0.63	0.37	0.35	1.33	1.41
Kd (μM)	1.58	2.65	1.51	4.27	4.97	2.48
α	0.11	0.13	0.15	0.13	1.10	0.73
r	0.994	0.999	0.999	0.997	0.992	0.999

The AGP concentration, 22.7 μM, was calculated by assuming a molecular mass of 44 kDa. For the VP binding study the AGP concentration was corrected by volume alteration during dialysis.

α, fraction of nonspecific binding; Kd, dissociation constant; *n,* number of binding sites; r, correlation coefficient between observed and predicted values.

tiomers to AGP was competitively inhibited by PL and VP enantiomers (Fig. 3 shows typical data). By contrast, the binding of VP enantiomers was completely inhibited by PL enantiomers but was only partially inhibited by DP enantiomers. S(+)-DP completely inhibited the binding of S(−)-VP to the A variant of AGP but not to the F1/S variant (Fig. 4). These results suggest that VP binds specifically to both the A and F1/S variants, and that DP can displace VP only from the A variant.

DISCUSSION

Drug lipophilicity may influence drug binding to AGP. The percentage binding of drugs used in this study correlated with the partition coefficients of the drugs but not with the pKa. Drug lipophilicity may be one of the determinant factors for the drug binding to human AGP.[13] Thus, binding that is not enantiospecific may partially depend on the lipophilicity of the drug because the partition coefficients of enantiomers are the same.

AGP possesses five *N*-glycan chains with sialic acids in the terminal groups. Desialylation of AGP does not change its affinity for progesterone and dicoumarol[5] but slightly reduces its affinity for PL.[6] These findings and our observation suggest that the contribution of the sialic acid residues of AGP to drug binding is small. Therefore, we studied more closely the enantioselective characteristics of drug binding to human native AGP.

All the drugs we studied had enantiomers that bound to the same site on human AGP. The number of binding sites we detected for the DP enantiomers was low (about 0.4), which conflicted with the number of binding sites. However, Herve et al.[11] recently demonstrated that low number of binding sites for DP is sometimes observed because of the presence of genetic variants of hu-

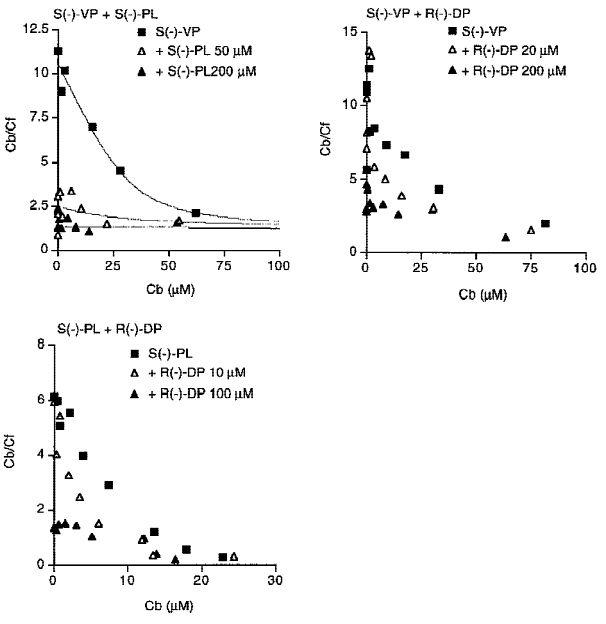

Figure 3. Effects of enantiomers of other drugs on the binding to human α_1-acid glycoprotein (a mixture of the A, F1, and S variants) of propranolol (PL), disopyramide (DP), and verapamil (VP).

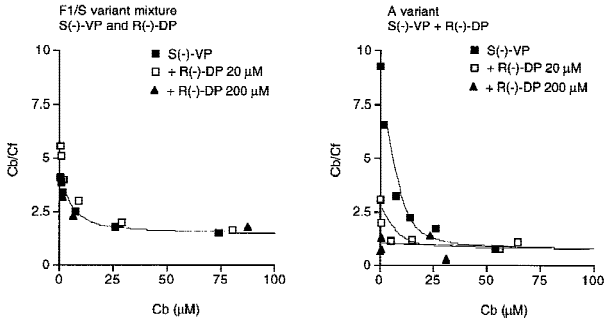

Figure 4. Effects of S(+)-disopyramide on the binding of S(−)-verapamil to human variant A and F1/S mixture of α_1-acid glycoprotein.

man AGP. AGP has three main genetic variants, F1, S, and A, which can be identified by their different isoelectric point values. The F1 and S variants arise from alleles of a single gene, and variant A is encoded by a separate gene.[14] DP binds preferentially and specifically to variant A and nonspecifically to the F1/S mixture. Variant A is thought to account for about 30 to 40% of the total plasma AGP in humans[11,15] (and our unpublished observations), so the concentration of AGP that has a binding site for DP is 30 to 40% of the total AGP concentration. When this factor is taken into account, the number of binding sites on AGP specific for DP is one. On the other hand, PL binds specifically to both variant A and the F1/S mixture with similar binding affinities.[14] In this study, the number of binding sites for PL enantiomers on AGP in the F1/S mixture appeared to be one. We demonstrated that VP also binds to both AGP variants.

AGP possesses five *N*-linked glycan chains, and the carbohydrate content of AGP is about 45%. Recently, several investigators have reported the influence of glycans of AGP glycoforms on drug binding. Kuroda et al.[17] studied the binding of PL and VP enantiomers to AGP separated by concanavalin A lectin affinity chromatography and suggested the branching type of glycan chains of AGP does not play a significant role in the magnitude of the binding of both drugs and enantioselectivity. On the other hand, Kishino et al.[18] studied the influence of glycans of AGP glycoforms, which were separated by hydroxyapatite chromatography, on the binding of DP and showed that the binding decreased with an increase in the extent of branching of the glycan chains. However, the ratio of genetic variants in these AGP glycoforms was not determined. Further study is required to characterize the drug binding site by taking into consideration both genetic variants and glycoforms of AGP.

In summary, enantiomer pairs of drugs bound to human AGP at the same site but with different affinities. DP bound specifically only to one genetic variant of AGP (variant A), as has been reported previously, whereas PL and VP bound specifically to both variant A and to the F1/S mixture.

ACKNOWLEDGMENT

This work was supported in part by a grant from the Japan Research Foundation For Clinical Pharmacology and Therapeutics.

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