Evaluation of Selective Competitive Binding of Basic Drugs to α_1-Acid Glycoprotein Variants

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We examined the binding of various basic drugs to the F_1S and A genetic variants of α_1-acid glycoprotein (AGP), which were isolated from native human commercial AGP (total AGP) by chromatography on an immobilized copper(II) affinity adsorbent. The values of the dissociation constant (K_d) of some basic drugs with the F_1S variant in equilibrium dialysis differed characteristically from those with the A variant. The selective binding to these variants was evaluated by measuring the displacement ratio of dicumarol bound to the F_1S variant or that of acridine orange bound to the A variant, using circular dichroism spectroscopy. There was reasonably good agreement between the K_d values and displacement ratios. There was a characteristic difference between the values of inhibition constant (K_i) of basic drugs towards dipyridamole binding to F_1S and towards disopyramide binding to A in total AGP. We found that the K_i values for dipyridamole binding were well correlated with the K_d values for the F_1S variant, whereas those for disopyramide binding were well correlated with the K_d values for the A variant. In conclusion, the higher the affinity of basic drugs for AGP, the more they inhibit the binding of other basic drugs, and further, the inhibitory potency depends on the selectivity of binding to the AGP variants.

Key words α_1-acid glycoprotein; variant; drug interaction; protein binding; dissociation constant; inhibition constant

The binding of drugs to proteins is an important determinant of their efficacy and/or side-effects in clinical use. Many basic drugs bind mainly to α_1-acid glycoprotein (AGP), an acute phase reactant, in plasma, but the plasma level of AGP varies greatly in various diseases. It is increased by inflammatory reaction, injury, and surgery,[1—3] but decreased by liver cancer, hepatitis and nephritic syndrome.[4—6] We have shown that there is a good correlation between the unbound fraction (f_u) of ropivacaine and AGP concentration after surgery.[3]

Recently, it has been reported that human AGP exists as a heterogeneous population of three genetic variants of ORM1 F_1 and ORM1 S derived from the AGP-A gene and ORM2 A derived from the AGP-B/B′ genes in the plasma of most individuals.[7,8] The variants can be identified by isoelectric focusing assay[9] and separated by chromatography on an immobilized copper(II) affinity adsorbent.[10,11] Fitos *et al.* demonstrated that the two main forms (the F_1S and A variants) have different drug binding properties by means of circular dichroism (CD) spectroscopy using dicumarol and acridine orange as probes.[12] It has been clarified that some drugs bind selectively to different AGP variants: for example, dipyridamole binds selectively to the F_1S variant, and disopyramide to the A variant, whereas lidocaine binds to both the F_1S and A variants.[13—16]

However, there are few reports regarding the effects of drug–drug interaction arising from selective binding to AGP variants on the f_u values of basic drugs. In this study, we evaluated the selective competitive binding of basic drugs to the F_1S and A variants isolated from native human AGP.

MATERIALS AND METHODS

Materials Anapeine injection® (ropivacaine hydrochloride) and Xylocaine injection® (lidocaine hydrochloride) were purchased from AstraZeneca Co., Ltd. (Osaka, Japan). Persantin injection® (dipyridamole) was purchased from Boehringer Ingelheim Co., Ltd. (Tokyo, Japan). Vasolan injection® (verapamil hydrochloride) was purchased from Eisai Co., Ltd. (Tokyo, Japan). Rythmodan P injection® (disopyramide phosphate) was purchased from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Nifejipine and amlodipine besilate were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Herbesser injection® (diltiazem hydrochloride) was purchased from Tanabe Co., Ltd. (Tokyo, Japan). Carvedilol was purchased from LKT Laboratories, Inc. (MN, U.S.A.). Native human α_1-acid glycoprotein (total AGP) was purchased from Sigma-Aldrich Co., Ltd. (MO, U.S.A.). 3,3′-Methylene-bis(4-hydroxycoumarin)(dicumarol), acridine orange hemi(zinc chloride) salt and mepivacaine were purchased from Sigma-Aldrich Co., Ltd. Other chemicals were of reagent grade.

Drug Assays Concentrations of ropivacaine, lidocaine and disopyramide were determined by GC-MS (Model GC-17 system Class 5000, Shimadzu, Kyoto, Japan). The assay for these drugs was carried out by using the procedure described for ropivacaine by Yokogawa *et al.*[3]

Aliquots of 100 μl of sample were mixed with 400 μl of saline, 500 μl of 50 ng/ml mepivacaine in saline as an internal standard, 200 μl of 10% Na_2CO_3 buffer and 4 ml of *n*-heptane : dichloromethane (4 : 1 v/v). The mixture was shaken for 20 min and centrifuged for 5 min at 3000 ***g***. The supernatant organic phase was transferred to another glass tube and preconcentrated under a stream of nitrogen gas at room temperature. Then, 100 μl of *n*-heptane : ethanol (9 : 1 v/v) was added to the residue, and an aliquot (1 μl) of the mixture was injected into the GC-MS system. Analyses were carried out in the selected-ion monitoring mode, with moni-

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toring at *m*/*z* 126, *m*/*z* 86, *m*/*z* 212 and *m*/*z* 98 for ropivacaine, lidocaine, disopyramide and mepivacaine, respectively. Chromatographic separation of lidocaine was achieved with a 5% phenyl-methylpolysiloxane-crosslinked capillary column (DB-5; 30 m×0.315 mm i.d.; J&W Scientific Inc., U.S.A.) in a gas chromatograph equipped with a splitless injector. The oven temperature was set at 60 °C for 1 min and then programmed up to 280 °C at 10 °C/min. The final temperature was maintained for 22 min.

Concentrations of amlodipine, carvedilol, diltiazem, dipyridamole, nifedipine and verapamil were determined with an HPLC system (LC-9A, Shimadzu Co., Ltd., Kyoto, Japan). The assay of these drugs, except for nifedipine, was carried out by modifying the reported procedures for amlodipine.[17] To 100 μl of sample in a glass-stoppered 10 ml centrifuge tube were added 200 μl of methanolic diltiazem solution as an internal standard (1 μg/ml), 100 μl of 1 N NaOH and 2 ml of diethyl ether. After shaking (10 min), the mixture centrifuged for 10 min at 3000 ***g***. The upper layer was separated and dried under nitrogen. The residue was reconstituted with 100 μl of mobile phase and a 50 μl aliquot was injected into the HPLC system.

All samples were analyzed on an HPLC system equipped with a Shim-pack CLC-ODS column (150×6.0 mm i.d., Shimadzu). The absorbance was detected at a wavelength of 230 nm. The mobile phase was a mixture of 0.01 M sodium dihydrogen phosphate buffer and acetonitrile (63 : 37) adjusted to pH 3.5 at flow rate of 1 ml/min. The assay of nifedipine was carried out by modifying the reported procedures.[18]

Circular Dichroism Spectroscopy to Measure Drug Binding Ligand binding to the genetic variants of AGP was evaluated by using a CD spectropolarimeter (J-820, JASCO Co., Ltd., Tokyo, Japan) according to Fitos *et al.*[12] Dicumarol (for the F_1S variant) was dissolved in 1 N NaOH, and diluted with phosphate buffered saline (PBS). Acridine orange (for the A variant) was dissolved in and diluted with PBS. Dicumarol or acridine orange (final concentration 9 μM) was added to AGP solution (final concentration 70 mg/dl) containing a ligand (final concentration 27 μM) and the sample was incubated for 30 min at room temperature. The CD spectrum was measured in the wavelength ranges of 250—370 nm for dicumarol and 400—580 nm for acridine orange.

Isoelectric Focusing of AGP Isoelectric focusing (IEF) assay was carried out according to Eap and Baumann.[9]

a) Sample Preparation: A mixture of 5 μl of plasma and 5 μl of 8 M urea was incubated at 37 °C for 1 h. Then, 1 U of neuraminidase (Wako Pure Chemicals Co., Ltd., Osaka, Japan) in a volume of 20 μl was added, and incubation was continued at 37 °C for 24 h.

b) Gel Preparation: The gel was used Immobiline Dryplate pH 4.5—5.5 (Amersham Pharmacia Biotech Co., Ltd., U.K.). The gel buffer was prepared by mixing 9.6 g of urea, 60 mg of dithiothreitol (DTT, Wako Pure Chemicals Co., Ltd.), 0.5 ml of pharmalyte narrow range 4.5—5.5 (Amersham Pharmacia Biotech Co., Ltd.) and 0.1 ml of Triton X-100 with 12 ml of distilled water. The gel was used after distension with the buffer at room temperature for 18 h. IEF was performed using a NA-1410-R apparatus (Eido Co., Ltd., Tokyo, Japan), with the cooling plate set at 10 °C. A 10 μl aliquot of sample was applied, and run at 500 V for 1 h, followed by 2000 V for 10 h.

c) Immunoblotting: The gel was blotted onto a single sheet of polyvinylidene difluoride (PVDF, Millipore, U.S.A.) in Tris–Glycine buffer (pH 8.3) for 45 min. The sheet was blocked with PBS containing 5% skin milk at 4 °C for 1 h, then incubated overnight at 4 °C with primary antibody, rabbit anti-human orosomucoid (DAKO Co., Ltd., Kyoto, Japan) and for 4 h with secondary antibody, anti-rabbit immunoglobulin G (IgG) (H&L) HRP linked antibody (Cell Signaling Technology, MA, U.S.A.). The sheet was extensively washed with washing buffer [0.1% Tween-20 in Tris buffered saline (TBS)], and immunopositive bands were measured with the ECL plus Western Blotting Detection System (Amersham Pharmacia Biotech). The isoelectric point (p*I*) values of the A, S and F_1 variants of AGP were 5.06, 5.03 and 4.93, respectively.

Fractionation of Genetic Variants of AGP The two main genetic variants (F_1S and A) were isolated from total AGP by chromatography on an immobilized copper(II) affinity adsorbent according to Hervé *et al.*[10,11]

a) Preparation of Sample: Total AGP (final concentration 10 mg/ml) was dissolved in 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl (buffer E).

b) Pre-treatment of Column: To adsorb copper in a small column of iminodiacetate (IDA)-Sepharose gel (1.5 cm i.d., bed volume 10 ml, Amersham Bioscience, Tokyo, Japan), the column was perfused with 0.2 M copper(II) chloride solution. Excess copper was washed out with 0.1 M acetate buffer (pH 3.8) containing 0.5 M NaCl. Then the column was washed and equilibrated with buffer E.

c) Subsequent Treatment: After the fractionation of genetic variants of AGP, copper adsorbed on the column was washed out with 50 mM ethylenediaminetetraacetic acid (EDTA), and the column was washed with distilled water. The flow rate was 10 ml/h.

d) Fractionation of AGP: AGP solution (10 mg/ml) was applied to the copper column and eluted with buffer E. The F_1S variant, which flows through the column, was detected by measuring the absorbance at 280 nm. When the absorbance of the eluate reached approximately zero, the column was eluted with buffer E containing 20 mM imidazole to obtain the A variant. The nature of the AGP variant in the collected fractions was confirmed by IEF measurement. The fractions were concentrated on a Centricom YM-10 (Amicon, CA, U.S.A.) at 4 °C for 30 min, desalted and lyophilized.

Protein Binding Studies The extent of binding of drugs to the AGP variants was measured by the equilibrium dialysis technique. Aliquots of 10 μl of various concentrations of drug were added to 1 ml of PBS containing total AGP, F_1S variant or A variant (final concentration 70 mg/dl). Equilibrium concentrations of drug in the AGP and buffer compartments were reached within 6 h at 37 °C. The f_u was calculated as the ratio of the concentrations of the drug in the buffer and AGP compartments. In this study, volume shifts between the two compartments were negligible. For the inhibition studies, aliquots of 10 μl of various concentrations of various drugs were added to 1 ml of PBS containing total AGP (final concentration 70 mg/dl) and dipyridamole, lidocaine, or disopyramide (final concentration 2.5, 5, 15, or 30 μM).

Data Analysis The dissociation constant (K_d) values of

basic drugs were calculated by means of Scatchard plot analysis. The values of inhibition constant (K_i) for binding of dipyridamole, lidocaine or disopyramide were calculated from the following equation by means of Lineweaver–Burk plot analysis:

$$K_i = K_d \cdot [I]/(K'_d - K_d)$$

where K_d is the dissociation constant in the absence of any other drug, K'_d is the dissociation constant in the presence of another drug, and [I] is the concentration of the other drug.

RESULTS

Fractionation of Genetic Variants of Total AGP The F_1S and A genetic variants of AGP were isolated from total AGP by chromatography on an immobilized copper(II) affinity adsorbent. Figure 1a shows the elution profile. Isoelectric focusing analysis (Fig. 1b) showed that fraction 1 contained 72, 22 and 6% of the F_1, S and A variants, respectively, while fraction 2 contained 4, 9 and 87%, respectively.

Binding of Basic Drugs to the AGP Variants The binding parameters of various basic drugs to total AGP, F_1S variant and A variant (70 mg/dl) were calculated by the Scatchard plot method. Table 1 summarizes the values of the dissociation constants (K_d). For comparison with our data, the literature values of these parameters, where available, are given in Table 1. The K_d values of various basic drugs for total AGP are roughly intermediate between those for the F_1S and A variants. Some drugs had particularly high affinity for one variant, and the ratios of K_d for the F_1S variant to that for the A variant are also shown. Dipyridamole, carvedilol and nifedipine showed ratio values of less than 1, *i.e.*, they were selective for the F_1S variant, with dipyridamole having the lowest value (0.09). The K_d values of carvedilol for the F_1S variant and A variant were 0.29 and 0.79, respectively, so that carvedilol had high affinity for both variants. In contrast, diltiazem, amlodipine and disopyramide showed ratio values higher than 3, *i.e.*, they were selective for the A variant, with disopiramide having the highest value (29). Verapamil, lidocaine and ropivacaine showed ratio values of roughly 1, *i.e.*, they were unselective.

Next, selective binding of basic drugs to the AGP variants was examined with unseparated total AGP by means of CD spectral measurement. Figure 2 shows the induced CD spectra of dicumarol (a) and acridine orange (b) in the presence of various basic drugs with total AGP. The competitive bind-

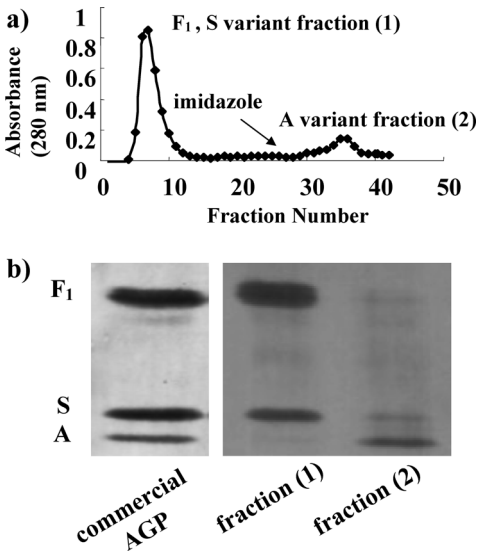

Fig. 1. Elution of Sialylated AGP Variants in Affinity Chromatography of Total AGP on IDA-Cu(II) Gel at pH 7.0

(a) Absorbance of the indicated fraction number. (b) Electrophoretic patterns analyzed by isoelectric focusing after desialylation of AGP variants.

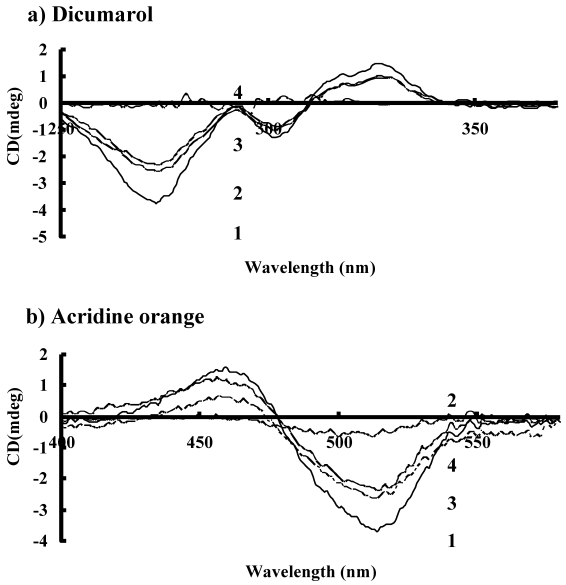

Fig. 2. Induced CD Spectra of Dicumarol (a) and Acridine Orange (b) in the Presence of Various Basic Drugs with Total AGP

1; dicumarol or acridine orange, 2; disopyramide, 3; lidocaine, 4; dipyridamole.

Table 1. Dissociation Constants (μM) of Various Basic Drugs as Determined with Total AGP, F_1S Variant and A Variant

Drug	Total AGP	F_1S variant	A variant	Ratio (F_1S/A)	Reported value[a)]		
					F_1S variant	A variant	Ratio (F_1S/A)
Dipyridamole	0.64	0.44	4.7	0.09	0.38	6.4	0.06
Carvedilol	0.32	0.29	0.79	0.37	N.R.	N.R.	N.R.
Nifedipine	9.8	6.3	16	0.39	N.R.	N.R.	N.R.
Verapamil	1.8	2.5	2.3	1.1	N.R.	N.R.	N.R.
Lidocaine	7.1	9.1	6.9	1.3	37	18	2.1
Ropivacaine	0.76	1.3	0.75	1.7	N.R.	N.R.	N.R.
Diltiazem	9.5	15	4.6	3.3	N.R.	N.R.	N.R.
Amlodipine	5.6	7.2	1.8	4.0	N.R.	N.R.	N.R.
Disopyramide	1.2	6.6	0.23	29	N.D.	0.40	N.D.

N.D., not determined; N.R., not reported. *a*) Value from ref. 15.

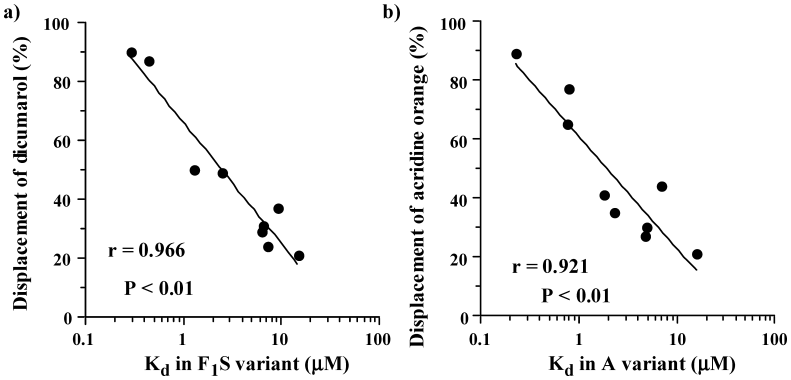

Fig. 3. Correlations between the Displacement Rates of Dicumarol from Total AGP and the K_d Values for the F_1S Variant (a) and between the Displacement Rates of Acridine Orange from Total AGP and the K_d Values for the A Variant (b), for Various Basic Drugs

ing and selectivity were evaluated by measuring the extent of displacement of dicumarol for the F_1S variant and of acridine orange for the A variant. Table 2 shows the percentage displacement of dicumarol or acridine orange by various basic drugs. The order of the extent of displacement of dicumarol and that of acridine orange by the basic drugs were not the same.

Figure 3a shows the correlation between the values of percentage displacement of dicumarol and the K_d values for the F_1S variant, for nine drugs. There was a high correlation, and the regression equation was as follows:

$$y = -41.1 \cdot \log(x) + 65.8 \quad (1)$$

Figure 3b shows the correlation between the values of percentage displacement of acridine orange and the K_d values for the A variant. Again, there was a high correlation, and the following regression equation was obtained:

$$y = -34.8 \cdot \log(x) + 56.5 \quad (2)$$

The two regression equations are quite similar.

Inhibitory Effect of Basic Drugs on Drug Binding to AGP Variants The inhibitory effects of basic drugs on the binding of dipyridamole, lidocaine or disopyramide (10 μM) to total AGP (70 mg/dl) was examined. The values of the inhibition constant (K_i) were calculated from Lineweaver–Burk plots. All of the drugs showed competitive inhibition of binding, and the K_i values towards dipyridamole, lidocaine disopyramide binding are summarized in Table 3. We examined the correlations between the K_i values against dipyridamole, lidocaine or disopyramide binding (Table 3) and the K_d values of the basic drugs for total AGP, F_1S variant or A variant (Table 1). In the case of dipyridamole, the correlation was highest for the F_1S variant ($r=0.959$, $p<0.01$). For disopyramide, the highest correlation was found for the A variant ($r=0.997$, $p<0.01$), while for lidocaine, the highest correlation was found for total AGP ($r=0.904$, $p<0.01$).

Table 2. Displacement of Dicumarol or Acridine Orange Bound to Total AGP by Various Basic Drugs as Determined by CD Spectra Measurement

Drug	Dicumarol (%)	Acridine orange (%)
Carvedilol	90	77
Dipyridamole	87	27
Ropivacaine	50	65
Verapamil	49	35
Lidocaine	37	44
Disopyramide	31	89
Nifedipine	29	21
Amlodipine	24	41
Diltiazem	21	30

Table 3. Inhibition Constants of Various Basic Drugs for Binding of Dipyridamole, Lidocaine or Disopyramide to Total AGP

Drug	K_i (μM)		
	Dipyridamole	Lidocaine	Disopyramide
Carvedilol	1.6	1.5	0.98
Verapamil	4.7	7.8	6.7
Ropivacaine	7.0	4.0	3.1
Nifedipine	8.9	18	39
Amlodipine	9.2	8.4	3.6
Disopyramide	10	8.3	—
Lidocaine	11	—	17
Diltiazem	20	13	11
Dipyridamole	—	4.6	10

DISCUSSION

Our results indicate that drug interaction arising from protein binding to AGP is dependent upon the genetic variant of AGP, at least for some basic drugs.

We separated the genetic variants of AGP from total AGP by using the procedure of Hervé *et al.*[10] The purities of the F_1S and A variant fractions were about 94 and 87%, respectively, as determined by isoelectric focusing analysis. To confirm the absence of mutual contamination, we measured the induced CD spectra of dicumarol and acridine orange with each variant isolated from total AGP. In the case of the A variant, only the characteristic CD spectrum of acridine orange was observed, while with the F_1S variant, only the characteristic CD spectrum of dicumarol was observed (data not shown). These results indicate that contamination of each variant by the other variant was negligible.

We chose nine basic drugs in clinical use to examine drug binding to AGP variants. We used six injections, dipyridamole, disopyramide, lidocaine, diltiazem, ropivacaine, and verapamil. These injections include additives that may affect the protein binding. The K_d values of dipyridamole, disopyramide, and lidocaine were consistent with those of Hervé *et al.* (Table 1),[14] so that the additives of these injections had no effect on the protein binding. Another main additives are

D-mannitol and D-sorbitol. To confirm the effect of these reagents, we measured unbound fraction of dipyridamole with D-sorbitol (300 μg/ml) or D-mannitol (20 μg/ml). Unbound fractions of these values agree with control value (data not shown). These results indicate that the existence of additives in this study was negligible.

We found that the K_d values of the drugs for the F_1S variant differed considerably from those for the A variant, and the values for total AGP were intermediate (Table 1). Selective binding to the F_1S variant is indicated by a low value of the ratio of K_d for the F_1S variant to that for the A variant, while selective binding to the A variant is indicated by a high value. Dipyridamole, carvedilol and nifedipine showed selective affinity for the F_1S variant, but the degree of the selectivity varied, and dipyridamole showed the greatest selectivity. Similarly, diltiazem, amlodipine and disopyramide showed selective affinity for the A variant, but the degree of the selectivity varied, and disopyramide was the most selective. Verapamil, lidocaine and ropivacaine did not exhibit clear selectivity of binding to AGP variants. Our results for dipyridamole and disopyramide are consistent with those of Hervé *et al.*,[14] who suggested that drugs that bind selectively to the A variant contain a tertiary amine group linked by a three- or four-atom chain to aromatic rings, which are bridged to form a tricyclic structure (imipramine) or are unbridged (disopyramide). Disopyramide and diltiazem have these characteristics, but amlodipine contains a primary amine, not a tertiary amine group, linked by four-atom chain to aromatic rings. On the other hand, dipyridamole (F_1S selective) and lidocaine (non-selective) contain tertiary amine, as well as aromatic amines (dipyridamole) or an amido group (lidocaine). Thus, these results suggest that drugs that bind selectively to the A variant require some other structural characteristic, as well as a tertiary amino group.

Moreover, we examined the selectivity of binding to the AGP variants, based on the displacement of dicumarol from the F_1S variant or acridine orange from the A variant, using CD spectroscopy (Fig. 2), and plotted the correlation between the displacement ratio of dicumarol or acridine orange and the K_d values for the F_1S or A variant (Fig. 3). The obtained regression equation for the F_1S variant was quite similar to that for the A variant, suggesting that it is reasonable to compare the results for the two variants.

Next, we examined the inhibitory effects of basic drugs that bind selectively to AGP variants, using dipyridamole and disopyramide as selective binders to the F_1S or A variant, respectively, and the non-selective binder lidocaine for comparison. We confirmed that all of the inhibitory effects are competitive by means of Lineweaver–Burk plots. We found that there are characteristic differences among the K_i values of various basic drugs for dipyridamole, lidocaine or disopyramide binding to total AGP (Table 3). The K_i values towards dipyridamole (F_1S variant) or disopyramide (A variant) were highly correlated with the K_d values for the F_1S variant or A variant, respectively. Also, the K_i values towards lidocaine (non-selective) showed a good correlation with the K_d values for total AGP. These result suggested that the inhibitory effect is strongly related to the selectivity of binding to AGP variants.

Ropivacaine is widely used as an amide-type local anesthetic. We have shown that f_u of ropivacaine gradually decreased as the AGP concentration was increased, but there was large inter-individual variation among the five patients who received epidural infusion of ropivacaine.[3] We then examined the inhibitory effect of dipyridamole, lidocaine or disopyramide on the binding disposition of ropivacaine in blood. Dipyridamole was the most potent inhibitor of ropivacaine binding among three drugs, having the lowest K_i value.[3]

To predict drug interaction arising from binding to AGP and/or variants, it is preferable to obtain the K_i values from examination of the binding disposition of a certain drug in the presence of other drugs, but this is infeasible for all combinations of basic drugs that might be used. We obtained the K_d values for the F_1S and A variants from the changes of the induced CD spectra of dicumarol and acridine orange, respectively. The resulting data on affinity and selectivity for the F_1S and A variants allowed us to estimate the potential for drug–drug interaction. We are planning to use this approach to examine other basic drugs used in combination therapy, such as local anesthetics.

In conclusion, our results indicate that the higher the affinity of basic drugs for AGP, the stronger their inhibitory effect on the binding of other basic drugs to AGP. Further, the inhibitory potency depends upon the selectivity of binding to the AGP variants. Thus, drug–drug interaction may occur *via* selective binding of basic drugs to AGP variants. This suggests that inter-individual differences in AGP genetic variants may influence the efficacy and/or side effects of basic drugs used in combination therapy.

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