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Research Article

Reversal of Signs of Induced Cotton Effects of Dicumarol-α_1-Acid Glycoprotein Systems by Phenothiazine Neuroleptics Through Ternary Complexation

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The interaction of dicumarol and phenothiazine neuroleptics binding to α_1-acid glycoprotein (AGP) was investigated by circular dichroism (CD) and equilibrium dialysis. The induced CD spectra of the dicumarol–AGP complex were affected differently by the different substituents of the phenothiazine molecule. The sign of the induced Cotton effect of dicumarol bound to AGP was reversibly changed with the introduction of the propyldimethylamine substituent at position 10 or chloride group at position 2 of the phenothiazine molecule. Chlorpromazine, which contains both of these substituents reversed the sign of the induced Cotton effect with the highest intensity. The addition of trifluoperazine, fluphenazine, and promethazine containing neither of the two substituents generated a new negative CD band. However, the addition of opromazine, which contains sulfoxide at position 5, decreased the CD intensity of the dicumarol–AGP complex without changing the shape of the CD spectra. Equilibrium dialysis studies revealed that the interaction of dicumarol–AGP with phenothiazine derivatives occurred simultaneously, and the interaction followed a cooperative and anticooperative binding model. Further, among the six phenothiazine derivatives that reversed the signs of the induced Cotton effects of the dicumarol–AGP complex, a linear relationship was observed between coupling constants and the difference in the induced optical ellipticity. The opromazine and dicumarol interaction was competitive for a common binding site on the AGP molecule. Removal of sialic acid did not have any effect on this interaction. These data support the hypothesis that the acidic and the basic drug binding sites overlap each other.

KEY WORDS: α_1-acid glycoprotein; induced circular dichroism; dicumarol; phenothiazine derivatives; ternary complex; binding mode.

INTRODUCTION

α_1-Acid glycoprotein (AGP) functions as a transport protein of basic drugs (1,2). The displacement of the drug bound to AGP by another drug may result in an increase in the free concentration of the drug in serum (3). Circular dichroism (CD) has proven useful for the characterization of drug–AGP complexes (4,5). Recently, we proposed that AGP has a wide and flexible drug binding area which permits two bulky drug molecules such as dicumarol and protriptyline to form a ternary complex with AGP (6). Further, the signs of the induced CD spectra of the dicumarol–AGP complex were reversed by basic tricyclic drugs such as chlorpromazine, which caused the change in the chirality of the dicumarol molecule through the formation of a 1:1:1 ternary complex with dicumarol and AGP (6). This result suggested that the binding sites of dicumarol and the basic tricyclic drug were very close to each other. The present study was intended to characterize further the binding sites on AGP *in vitro*. A series of phenothiazines including chlorpromazine was used to analyze the structure of the ternary complex by measuring the inversion of the CD sign of the dicumarol–AGP complex.

MATERIALS AND METHODS

Materials

Human AGP was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). AGP gave only one band in SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) with an assumed molecular weight of 44,100. Phenothiazine derivatives (Table I) were used as supplied from Yoshitomi Pharmaceutical Co. Ltd (Fukuoka, Japan). Dicumarol was purchased from Tokyo Kasei and was used without further purification. All other materials were of reagent grade, and all solutions were prepared in deionized and distilled water. All protein and drug solutions were prepared in 0.067 *M* phosphate buffer, pH 7.4.

Preparation of AsialoAGP

AGP was enzymatically desialylated as outlined by Pri-

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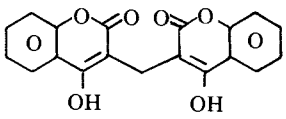
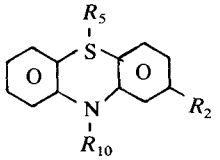
Table I. Chemical Structure of Dicumarol and Phenothiazine Derivatives



Dicumarol



Phenothiazine

Phenothiazine derivative	R_2	R_{10}	R_5
Promazine	$-H$	$-CH_2CH_2CH_2N(CH_3)_2$	—
Chlorpromazine	$-Cl$	$-CH_2CH_2CH_2N(CH_3)_2$	—
Trifluopromazine	$-CF_3$	$-CH_2CH_2CH_2N(CH_3)_2$	—
Methoxypromazine	$-OCH_3$	$-CH_2CH_2CH_2N(CH_3)_2$	—
Prochlorperazine	$-Cl$	$-CH_2CH_2CH_2N$ NCH_3	—
Trifluoperazine	$-CF_3$	$-CH_2CH_2CH_2N$ NCH_3	—
Perphenazine	$-Cl$	$-CH_2CH_2CH_2N$ NCH_2CH_2OH	—
Fluphenazine	$-CF_3$	$-CH_2CH_2CH_2N$ NCH_2CH_2OH	—
Promethazine	$-H$	$-CH_2CH(CH_3)N(CH_3)_2$	—
Opromazine	$-Cl$	$-CH_2CH_2CH_2N(CH_3)_2$	$-O$

mazic and McNamara (7), using an acylnuerammininyl hydrolase enzyme obtained from *Clostridium perfrigens*. AGP in phosphate buffer (1.5 mg/ml) was added to the enzyme (0.357 unit suspended in 5 ml of buffer). This solution was incubated at 37°C in a water bath, while being gently rotated at 30 rpm for 2 hr. The protein was filtered through an 8-μm filter to remove the enzyme. The concentration of sialic acid in the filtrate was determined using the thiobarbituric acid method (8). Approximately 90% of the sialic acid was removed, leaving an average of one sialic acid residue per protein molecule. The molecular weight of asialoAGP was therefore 40,000.

Circular Dichroism (CD)

CD spectra were measured with a Jasco J-600 spectropolarimeter (Tokyo) in a 10-mm cell at 25°C. The induced ellipticities are defined as the ellipticity of the drug–AGP mixture minus the ellipticity of AGP alone and the ellipticity of the dicumarol–AGP–phenothiazine derivatives mixture minus the ellipticity of the AGP–phenothiazine mixture, if any, at the same wavelength and are expressed as degrees.

Equilibrium Dialysis

Equilibrium dialysis experiments were performed using a Sanko plastic dialysis cell (Fukuoka, Japan). The two cell compartments were separated by Visking cellulose membrane. AGP solution (10 μ*M*, 1.5 ml) was poured into one compartment and 1.5 ml of drug solution (0.5–30μ*M*) was poured into the opposite compartment. After 8 hr of dialysis at 25°C, the drug concentrations in each compartment were assayed by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump and Hitachi 655A variable-wavelength UV monitor. Columns of LiChrosorb RP-18 (Cica Merck, Tokyo) for dicumarol derivatives and LiChrosorb CN (E. Merck, Darmstadt, Germany) for phenothiazine derivatives were used as stationary phases. The detection wavelengths were 315 nm for dicumarol and 250 nm for phenothiazine derivatives. The mobile phases consisted of 1.5% acetic acid solution–acetonitrile (6:4, v/v) and 1.5% acetic acid solution–methanol (3:7 v/v) for dicumarol and phenothiazine derivatives, respectively. To determine adsorption on the membrane, concentrations in both compartments were measured. There was no volume shift during equilibrium dialysis. Bound drug concentrations and bound fractions were calculated as follows:

bound concentration (D_b) = drug concentration in protein compartment (D_{b+f}) − drug concentration in buffer compartment (D_f)

bound fraction (%) = D_b/D_{b+f}

Data Analysis

The binding of two ligands to protein could not be explained by a simple competition interaction alone. For example, dicumarol and protriptyline each increased the binding of the other drug. In the present study we treated the data according to the cooperative and anticooperative interaction as described by Kragh-Hansen (9).

Competitive binding was analyzed with the following equations:

$$\frac{[PA]}{[P_t]} = \frac{K_A[A_f]}{1 + K_A[A_f] + K_B[B_f]} \quad (1)$$

$$\frac{[PB]}{[P_t]} = \frac{K_B[B_f]}{1 + K_A[A_f] + K_B[B_f]} \quad (2)$$

where $[P_t]$ is the total AGP concentration, $[A_f]$ and $[B_f]$ are the concentrations of the free ligands, $[PA]$ and $[PB]$ are the

concentrations of the bound ligands. K_A and K_B are binding constants for ligand A and ligand B, respectively.

In case of two ligands binding to AGP simultaneously, we used cooperative and anticooperative binding models as illustrated in Scheme I, where P represents AGP possessing one binding site for ligand A and one binding site for ligand B with the indicated binding constants. In this case $K_{BA} = x \cdot K_A$ and $K_{AB} = x \cdot K_B$, where factor x defines a coupling constant. If A and B bind independently to AGP, then $x = 1$. Cooperative binding and anticooperative binding are characterized by $x > 1$ and $x < 1$, respectively. Competitive binding is represented by $x = 0$. The value of x was calculated as follows (9):

$$[P_t] = [P] + [PA] + [PB] \quad (3)$$

where $[P_t]$ is the total concentration of AGP and $[P]$ is the free concentration of AGP.

This relationship is readily transformed to

$$[P_t] = [P] + K_A \cdot [P][A_f] + K_B \cdot [P][B_f] + x \cdot K_A \cdot K_B \cdot [A_f][B_f][P] \quad (4)$$

where $[A_f]$ and $[B_f]$ are the free concentrations of A and B, respectively.

The concentration of bound $A(A_b)$ is given by

$$[A_b] = [A_t] - [A_f] = K_A \cdot [P][A_f] + x \cdot K_A \cdot K_B \cdot [A_f][B_f][P] \quad (5)$$

where $[A_t]$ represents the concentration of total A. Subtracting Eq. (5) from Eq. (4) gives

$$[P_t] - [A_b] = [P] + K_B \cdot [B_f][P] \quad (6)$$

Since $[P_t]$, $[A_f]$, $[A_t]$, K_B, and $[B_f]$ are known, it is possible to calculate $[P]$, and the known values for K_A and $[A_f]$ in Eq. (4) give x.

RESULTS

Effects of Phenothiazine Neuroleptics on the CD Spectra of Dicumarol Bound to AGP

The binding of dicumarol to AGP generated polyphasic extrinsic Cotton effects (Fig. 1). The extrinsic CD spectra of the dicumarol–AGP complex were affected differentially by the phenothiazine neuroleptics with different substituents. The detail of the phenothiazine effect on the CD spectra of the dicumarol–AGP complex are given in Table II. The signs of the CD spectra of dicumarol bound to AGP were reversed by the addition of the phenothiazines, except for trifluperazine, fluphenazine, promethazine, and opromazine. Spectra obtained with asialoAGP complexes were similar to those of AGP. Trifluoperazine, fluphenazine, and promethazine generated new negative CD bands near 300 nm, but opromazine only decreased the intensity of the Cotton effects of dicumarol bound to AGP and asialoAGP. Methoxypromazine and promethazine were used at higher concentrations than the other drugs, as their effects on the induced Cotton effects of dicumarol–AGP and –asialoAGP systems were small. The binding of all the phenothiazines to AGP or asialoAGP did not produce any significant Cotton effects at wavelengths longer than 260 nm under the experimental conditions.

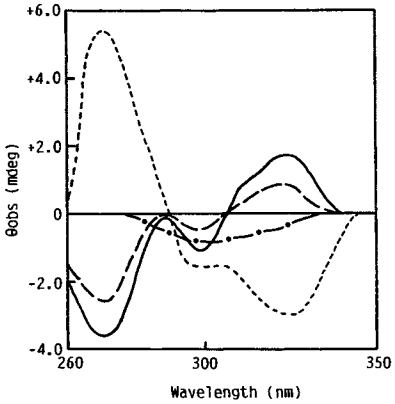


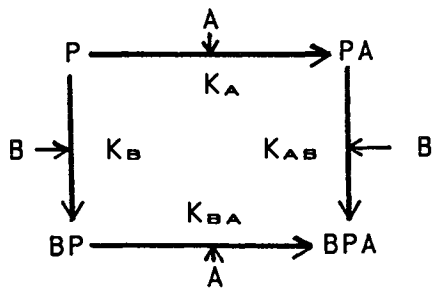
Fig. 1. Effects of chlorpromazine, trifluperazine, and opromazine on the induced CD spectra of dicumarol bound to AGP at pH 7.4 and 25°C. (——) Without drug: (-----) with chlorpromazine; (— · —) with trifluoperazine; (— —) with opromazine. The following concentrations were used: AGP, 10 μM; dicumarol, 10 μM; phenothiazine derivatives, 20 μM.

Effects of Phenothiazine Neuroleptics on the Bound Fraction of Dicumarol

Figure 2 shows the changes of binding fraction of a constant concentration of dicumarol to AGP in the presence of a constant concentration of phenothiazine derivatives. The filled bars represent the observed data, and the open bars the theoretical values calculated under the assumption of competition between two drugs for a common binding site on AGP. With the exception of opromazine, phenothiazines supported dicumarol binding less than would have been expected from direct competition (Fig. 2). Opromazine decreased the bound fraction of dicumarol to the theoretical competitive level. The asialoAGP complex gave the same behavior as did the AGP system (not shown).

These binding interactions were studied in detail with the use of different models (as described under Materials and Methods). The selected drugs have different substituents at position 10 (chlorpromazine and perphenazine) or 5 (chlorpromazine and opromazine) of the phenothiazine molecule. Figure 3A shows the bound fraction of a constant concentration of dicumarol in the presence of various concentrations of chlorpromazine. The bound fraction of dicumarol was lowly changed with rising chlorpromazine concentrations (Fig. 3A), and vice versa (Fig. 3B). These data were treated according to the cooperative and anticooperative binding models. The coupling constant x was calculated to be 1.0 ± 0.18.

Figure 4 shows the bound fraction of dicumarol in the presence of perphenazine, and vice versa. The bound fraction of dicumarol was decreased by the addition of perphena-



Scheme I

Table II. CD Characteristics of Dicumarol Bound to AGP and AsialoAGP in Presence of Phenothiazine Derivatives

Phenothiazine derivatives	AGP λ (nm)	AGP θ obs	AsialoAGP λ (nm)	AsialoAGP θ obs
None	325	+1.8	325	+1.4
	300	−1.0	300	−0.8
	270	−3.7	270	−3.0
Promazine	325	−1.6	325	−1.3
	295	−1.0[a]	295	−0.6[a]
	270	+2.7	270	+2.5
Chlorpromazine	325	−3.0	325	−3.1
	295	−1.6[a]	295	−1.6[a]
	270	+5.5	270	+5.8
Trifluopromazine	325	−1.3	325	−1.4
	295	−1.0[a]	295	−1.0[a]
	270	+3.2	265	+2.0
Methoxypromazine[b]	325	−2.9	320	−1.0
	295	−2.0[a]	300	−0.8[a]
	270	+5.0	270	+1.3
Prochlorperazine	320	−0.7	310	−0.7
	295	−0.6[a]	295	−0.8[a]
	270	+0.8	270	+1.0
Trifluoperazine	320	−0.7	320	−0.6
	300	−0.8	300	−0.8
Perphenazine	320	−1.0	320	−0.9
	295	−0.8[a]	295	−0.8[a]
	270	+2.3	270	+2.0
Fluphenazine	315	−0.8	315	−0.7
	265	+0.7	265	+0.6
Promethazine[b]	320	−1.4	320	−0.6
	300	−2.2	300	−0.6
Opromazine	325	+0.9	325	+0.8
	300	−0.5	300	−0.3
	270	−2.6	270	−2.3

[a] Shoulder; the following concentrations were used: AGP, asialoAGP, 10 μM; phenothiazine derivatives, 20 μM (AGP system), 10 μM (asialoAGP system); dicumarol, 10 μM.

[b] AGP, asialoAGP, 50 μM; methoxypromazine, promethazine, 100 μM; dicumarol, 50 μM.

zine (Fig. 4A). However, the observed curve did not fit the theoretical curve assuming competitive binding model (Fig. 4A), and vice versa (Fig. 4B). These data, when analyzed according to the cooperative and anticooperative binding modes, gave a coupling constant x of 0.4 ± 0.24.

Figure 5A represents the bound fraction of dicumarol in the presence of opromazine, and vice versa (Fig. 5B). These observed data did fit well the theoretical curve assuming competitive binding as predicted from the results in Table II and Fig. 2. Similar results were obtained with asialoAGP (not shown), and the coupling constants, x, between dicumarol and chlorpromazine or perphenazine were 1.0 ± 0.20 and 0.7 ± 0.05, respectively.

DISCUSSION

We suggested previously [6] that the signs of the induced CD spectra of the dicumarol–AGP complex were reversed by the addition of basic tricyclic drugs such as protriptyline and chlorpromazine, because of the formation of a 1:1:1 ternary complex of dicumarol, basic tricyclic drug, and AGP. In this paper, the interaction of dicumarol, AGP, and

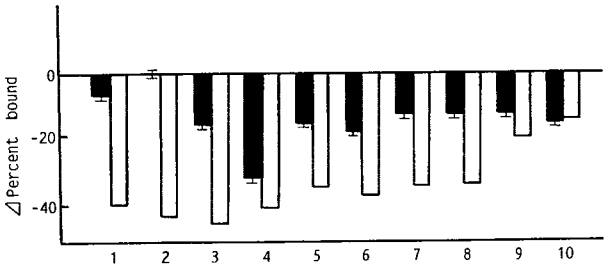


Fig. 2. Percentage dicumarol bound to AGP after the addition of phenothiazine derivatives at pH 7.4 and 25°C. (■) Observed value; (□) calculated value assuming competition. 1, Promazine; 2, chlorpromazine; 3, triflupromazine; 4, methoxypromazine; 5, prochlorperazine; 6, trifluoperazine; 7, perphenazine; 8, fluphenazine; 9, promethazine; 10, opromazine. The following concentrations were used: AGP, 10 μ*M*; dicumarol, 10 μ*M*; phenothiazine derivatives, 20 μ*M*.

various phenothiazine neuroleptics was examined by CD spectroscopy and equilibrium dialysis to elucidate the cause and nature of the interaction of dicumarol with phenothiazine derivatives with respect to the binding to AGP.

Table II shows that the signs of the induced Cotton effects of dicumarol bound to AGP and asialoAGP were reversed by the addition of all phenothiazine derivatives except for trifluoperazine, fluphenazine, promethazine, and opromazine. Trifluperazine, fluphenazine, and promethazine generated new negative CD bands. The sign and magnitude of an induced Cotton effect depend on the spatial relationship between the asymmetric center and the perturbed chromophore (10). Therefore, it was considered that the chirality of the dicumarol molecule was changed by the conformational change in the AGP molecule induced by all phenothiazine derivatives except for opromazine. The different effects on the induced CD spectra of the dicumarol–AGP complex might depend on the different substituents of the phenothiazine molecule. The propyldimethylamine substituent at position 10 and the chloride group at position 2 of the phenothiazine molecule, as in chlorpromazine, completely reversed the signs of the Cotton effects. The presence of only one of these substituents also reversed the sign, but the observed CD intensity was small. Trifluperazine, fluphenazine, and promethazine, containing neither of the above two substituents, did not reverse the signs of the CD spectra of the dicumarol–AGP complex, suggesting that the symmetri-

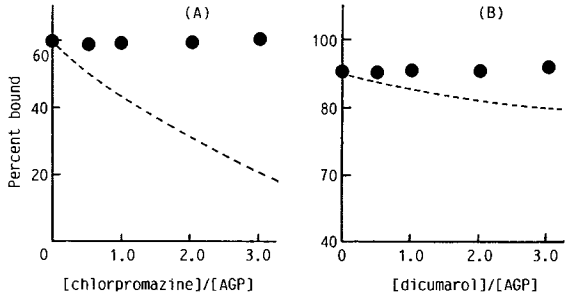


Fig. 3. Percentage dicumarol bound in the presence of chlorpromazine (A) and percentage chlorpromazine bound in the presence of dicumarol (B) at pH 7.4 and 25°C. (●) Observed value; (----) theoretical curve assuming competition. The following concentrations were used: AGP, 10 μ*M*; dicumarol (A), 10 μ*M*; chlorpromazine (B), 10 μ*M*.

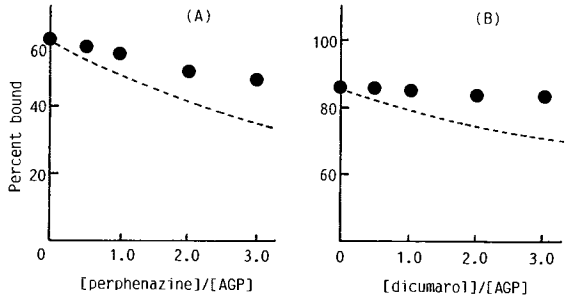


Fig. 4. Percentage dicumarol bound in the presence of perphenazine (A) and percentage perphenazine bound in the presence of dicumarol (B) at pH 7.4 and 25°C. (●) Observed value; (----) theoretical curve assuming competition. The following concentrations were used: AGP, 10 μM; dicumarol, 10 μM (A); perphenazine, 10 μM (B).

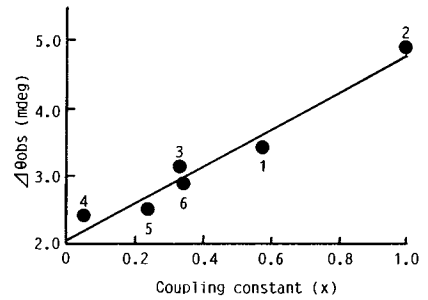


Fig. 6. Relationships between the coupling constant and the difference of $\theta_{obs,325nm}$. 1, Promazine; 2, chlorpromazine; 3, triflupromazine; 4, methoxypromazine; 5, prochlorperazine; 6, perphenazine. In the case of methoxypromazine the θ_{obs} value used in the above curve was obtained by dividing the original θ_{obs} value by 5, as the concentration of methoxypromazine was 5 times higher than that used in the case of other phenothiazine compounds.

cal chromophore of the dicumarol molecule perturbed by the asymmetrical center of AGP near 300 nm was affected more easily than near 270 nm. This change subsequently induced the binding of basic tricyclic drugs to AGP. Opromazine, having a substituent at the 5 position, only decreased the CD intensity and did not affect the shape of the CD spectra of the dicumarol–AGP complex.

From equilibrium dialysis studies, it was revealed that, except for opromazine, all phenothiazine derivatives and dicumarol bound to AGP simultaneously following a cooperative and anticooperative binding modes (9). Opromazine and dicumarol competed with each other for the common binding site on the AGP molecule. In the observed reversal of the signs of the Cotton effects in the dicumarol–AGP system by six of the phenothiazines, a linear relationship was observed between the difference in the induced optical ellipticity ($\Delta\theta_{obs} = \theta_{obs,without\ basic\ drug} - \theta_{obs,with\ basic\ drug}$) and the coupling constant (x) (Fig. 6). The fitted lines gave

$$\Delta\theta_{obs,325nm} = 2.63x + 2.05 \quad (n = 6, r = 0.98) \qquad (7)$$

This result suggested that the intensity of the difference of θ_{obs} did not depend on the distance between the asymmetric center and the purturbed chromophore; rather, it depended on the amount of ternary complex. Therefore, the reversal of the signs of induced CD spectra of dicumarol bound to AGP by addition of these six phenothiazine derivatives seemed to be occurring by the same mechanism. In the asialoAGP system behavior similar to that of the AGP system ($\Delta\theta_{obs,325nm} = 3.67x + 0.21$ ($n = 6$, $r = 0.92$)) was observed.

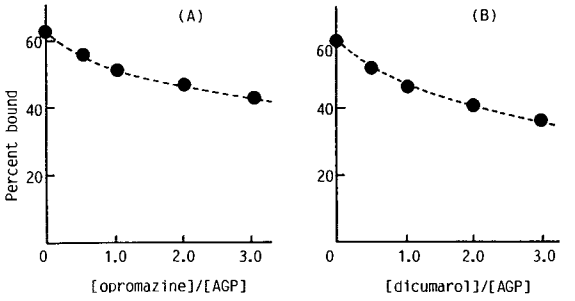


Fig. 5. Percentage dicumarol bound in the presence of opromazine (A) and percentage opromazine bound in the presence of dicumarol (B) at pH 7.4 and 25°C. (●) Observed value; (----) theoretical curve assuming competition. The following concentrations were used: AGP, 10 μM; dicumarol, 10 μM (A); opromazine, 10 μM (B).

In conclusion, the substituents at positions 2, 5, and 10 of the phenothiazine molecule were significant for the reversal of the sign of the CD spectra of the dicumarol–AGP complex. The phenothiazine derivatives, except for opromazine, and dicumarol bound to AGP simultaneously according to the cooperative and anticooperative binding modes, whereas opromazine and dicumarol competed with each other for common binding site. These data suggested that the AGP has a wide and flexible ligand binding area, as the binding property was affected by changing the substituent of ligands.

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