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EFFECTS OF TRICYCLIC DRUG ON INDUCED CIRCULAR DICHROISM SPECTRA OF DICUMAROL BOUND TO α_1-ACID GLYCOPROTEIN

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Abstract—Effects of both tricyclic and non-tricyclic drugs on the extrinsic Cotton effects of dicumarol bound to human α_1-acid glycoprotein (AGP) have been investigated. Basic tricyclic drugs caused the reversal of the signs of the induced Cotton effects of the circular dichroism (CD) spectra of the dicumarol–AGP system while the basic drugs not possessing tricyclic rings and acidic drugs decreased the observed ellipticities without changing the signs of its CD spectra. There was no reversal of the CD signs of the drugs not containing two hydroxycoumarin rings bound to AGP by basic tricyclic drugs. Raising of pH and temperature, and the addition of guanidine hydrochloride decreased the observed ellipticities of the CD spectra of the dicumarol–AGP system without showing any change in the signs of the Cotton effects. The mutual displacement data showed that protriptyline increased its own binding and that of dicumarol with AGP. The results of CD titration and equilibrium dialysis experiments suggest that dicumarol–AGP and dicumarol–AGP–protriptyline form a 1:1 binary complex and a 1:1:1 ternary complex, respectively.

Recent studies have shown that anionic drugs as well as basic drugs are strongly bound to human α_1-acid glycoprotein (AGP) [1–4]. The drugs seem to bind with AGP at a single high affinity site with high association constants [5–7]. Dicumarol, bishydroxycoumarin, is known to give strong polyphasic extrinsic Cotton effects when bound to AGP [8], and the drug is found to be displaced by most of the basic drugs from its binding site on AGP [9]. Preliminary experiments revealed that the basic tricyclic drugs, protriptyline and chlorpromazine, caused the reversal of the signs of the induced Cotton effects of the dicumarol–AGP system. Acidic tricyclic drugs or other basic and acidic drugs did not invert the signs of the Cotton effects produced by the dicumarol–AGP system. Interestingly, only basic tricyclic drugs were found to undergo cooperative binding with AGP in the presence of dicumarol by forming a 1:1:1 ternary complex. The present work was undertaken to study the origin of the Cotton effects generated by the dicumarol–AGP–basic tricyclic drug system. Attempts have been made to relate the changing of signs of the induced Cotton effects with the ternary complex formation.

MATERIALS AND METHODS

Materials. Human AGP was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). AGP (M_r 44,100) gave only one band in SDS–PAGE. Potassium warfarin (Eisai Co., Tokyo, Japan), protriptyline, chlorpromazine, propranolol (Yoshitomi Pharmaceutical Co., Fukuoka, Japan), prothizinic acid (Mochida Pharmaceutical Co., Tokyo, Japan), and carprofen (Nippon Roche K.K., Tokyo, Japan) were used as supplied. Acenocoumarin, coumetarol and phenprocoumon were gifts from Prof. L. H. M. Janssen of Utrecht University. Dicumarol was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All other materials were of reagent grade and all solutions were prepared in deionized and distilled water.

Methods. All solutions were prepared in 0.067 M phosphate buffers at pH 7.4 at 25 ± 1°. CD measurements were made on a Jasco model J-50A spectropolarimeter (Tokyo, Japan), using 5 and 10 mm cells. AGP solutions of 0.5–2.0 μM were used in CD measurements. The induced ellipticity is defined as the ellipticity of the drug–AGP mixture minus the ellipticity of AGP alone at the same wavelength and is expressed in degrees. Equilibrium dialysis experiments were performed in Sanko plastic dialysis cells (Fukuka, Japan). An AGP solution of 10 or 20 μM (2 mL) was poured into one compartment and 2 mL of drug solution with or without displacer into the other.

Absorption of any drug onto membrane was negligible. After 8 hr of dialysis at 25°, the concentration of free drug was assayed by high performance liquid chromatography (HPLC). The HPLC system consisted of a Hitachi 655A-11 pump and Hitachi 655A variable wavelength UV monitor. A column of LiChrosorb RP-18 (7 mμ) (Cica Merk, Tokyo, Japan) was used as the stationary phase. The detector was set at 315 nm with the sensitivity of 0.005 A.U.F.s for dicumarol and 290 nm with the same sensitivity of 0.005 A.U.F.s for protriptyline and propranolol. The mobile phases consisted of 1.5% acetic acid aqueous solution (A) and acetonitrile (B) (A:B, 6:4 for dicumarol; 3:7 for protriptyline and propranolol). The sample was injected onto the column using a 20 μL loop.

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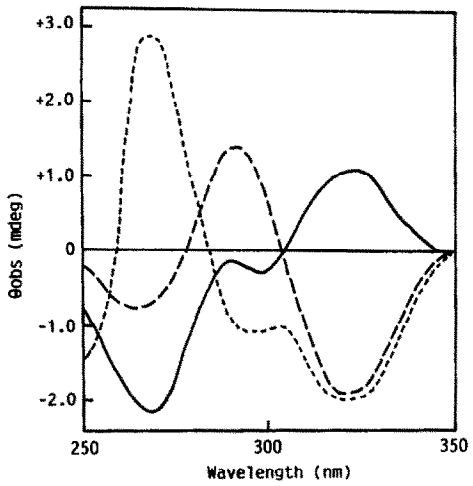

Fig. 1. Effects of chlorpromazine and protriptyline on the induced CD spectra of the dicumarol–AGP system at pH 7.4 (1/15 M phosphate buffer) and 25°. (——) Without basic drug; (— —) with protriptyline; (– – – –) with chlorpromazine. The following concentrations were used: AGP, 10 μM; dicumarol, 10 μM; basic drug, 10 μM. Measurements were made in 10 mm cells.

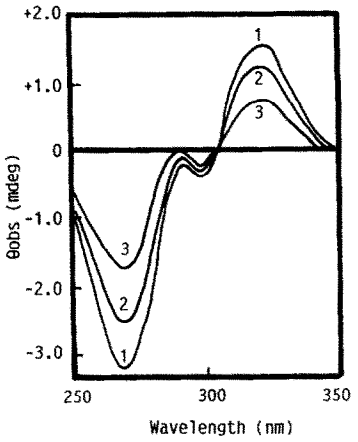

Fig. 2. Temperature dependence of induced CD spectra of the dicumarol–AGP system at pH 7.4. Curve 1, 10°; curve 2, 25°; curve 3, 40°. Measurements were made in 10 mm cells with an AGP concentration of 10 μM and dicumarol concentration of 10 μM.

Treatment of data. A simple competition between two drugs A and B for identical protein binding sites was analysed by the following equations [10]:

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

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

$$[A_t] = [PA] + 2[A_f]$$
$$[B_t] = [PB] + 2[B_f] \quad (3)$$

where:

K_a = association constant for drug A;
K_b = association constant for drug B;
$[A_f]$ = concentration of free drug A;
$[B_f]$ = concentration of free drug B;
$[PA]$ = concentration of bound drug A;
$[PB]$ = concentration of bound drug B;
$[A_t]$ = total concentration of drug A;
$[B_t]$ = total concentration of drug B;
$[P_t]$ = total concentration of protein.

Because $[P_t]$, K_a and K_b are known, it is possible to calculate theoretically $[A_f]$, $[PA]$, $[B_f]$ and $[PB]$ from Eqns (1)–(4). Thus, the theoretical value of free fraction (F) can be calculated as follows.

$$F_A(\%) = \frac{[A_f]}{[PA] + [A_f]} \times 100 \quad (5)$$

$$F_B(\%) = \frac{[B_f]}{[BA] + [B_f]} \times 100. \quad (6)$$

RESULTS

The binding of dicumarol to AGP generated polyphasic extrinsic Cotton effects by exhibiting two negative maxima at 270 and 300 nm, with a large positive maximum at 325 nm (Fig. 1). It can readily be seen (Fig. 1) that the signs of the induced CD spectrum of the dicumarol–AGP system were changed by the basic tricyclic drugs, chlorpromazine and protriptyline. Upon the addition of chlorpromazine, a positive band at 325 nm and a negative band at 270 nm were reversed with some changes in the ellipticities. Protriptyline, another basic drug also reversed the signs of the Cotton effects at 325 and 300 nm, but the addition of protriptyline decreased the band intensity at 270 nm without changing the sign of the Cotton effect under the experimental conditions. Binding of either chlorpromazine or protriptyline to AGP did not generate any measurable extrinsic Cotton effects at wavelengths longer than 260 nm under the given experimental conditions.

In order to elucidate the mechanism of the sign changes of the extrinsic Cotton effects of the dicumarol–AGP system, various effects on the induced Cotton effects of the dicumarol–AGP system were examined. Figure 2 shows the dependence of the induced ellipticities of the dicumarol–AGP system on temperature. With the rise of temperature, the observed ellipticities were decreased without changing the signs of the Cotton effects. Guanidine hydrochloride, a protein denaturant, and pH also caused similar CD spectral changes of the dicumarol–AGP system without showing any change of the signs of the Cotton effects (figure not shown). Changes in temperature and pH affected the binding process of dicumarol to AGP to a greater extent than did the addition of guanidine hydrochloride, as shown in Table 1. The free concentration (D_f) of dicumarol was increased with the increase of pH, temperature and guanidine hydrochloride concentration (Table 1). Mutual displacement experiments between dicumarol and different tricyclic and non-tricyclic drugs bound to AGP were carried out using the equilibrium dialysis technique to further confirm the interaction between dicumarol and basic tricyclic drugs. Figure 3 shows the free

Table 1. Effects of pH, temperature and guanidine hydrochloride on dicumarol free fraction of dicumarol–AGP system

		Free fraction (%)
pH	6.0	28.0 ± 0.5
(25°)	7.4	39.5 ± 0.5
	9.0	76.3 ± 2.5
Temperature (°C)	10	33.2 ± 0.4
(pH 7.4)	25	39.5 ± 0.5
	40	68.7 ± 2.1
Guanidine hydrochloride(M)	0.1	41.5 ± 1.5
(pH 7.4, 25°)	0.2	46.5 ± 1.0
	0.5	47.5 ± 0.5

[AGP] = [dicumarol] = 10 μM.

fraction of a constant concentration of dicumarol in the presence of various concentrations of a non-tricyclic drug, propranolol (curve A), and an acidic tricyclic drug, prothizinic acid (curve B). Both propranolol and prothizinic acid increased the unbound fractions of dicumarol. The observed data fitted the theoretical curves well, as shown in Fig. 3. In contrast, the basic tricyclic drug protriptyline decreased the unbound fraction of dicumarol, causing marked deviations from the theoretical curve as shown in Fig. 4. In data, not reported here, chlorpromazine, another basic tricyclic drug, gave similar results as did protriptyline. Intersections of the plateaus and tangents of the two curves for the dicumarol–protriptyline–AGP system obtained by equilibrium dialysis were found at a drug to AGP ratio of 1.0 (Fig. 4). In the case of the CD titration of either dicumarol–AGP with protriptyline or protriptyline–AGP with dicumarol, the intersections of the plateaus and tangents to the two curves were also obtained at a drug to AGP ratio of 1.0 (Fig. 5). The basic tricyclic drugs chlorpromazine and protriptyline produced similar CD spectral changes in the coumetarol–AGP system to those observed with the dicumarol–AGP system (Fig. 6). Upon the addition of chlorpromazine, the observed positive ellipticity band at 325 nm and the negative ellipticity band at 275 nm were changed to corresponding negative and positive bands (Fig. 6).

Protriptyline also caused the reversal of the signs of the Cotton effects observed at 325 and 300 nm. In contrast, the basic tricyclic drugs did not change the signs of the Cotton effects of the induced CD spectra of warfarin, phenprocoumon or acenocoumarin bound to AGP. Moreover, the acidic tricyclic drugs such as prothizinic acid and carprofen decreased the ellipticity of the CD spectrum of the dicumarol–AGP system without changing the signs of the Cotton effects (Fig. 7).

DISCUSSION

When dicumarol was added to a solution of AGP, polyphasic Cotton effects appeared at 270, 300 and 325 nm (Fig. 1). Since dicumarol is not optically active and AGP does not produce any Cotton effects at these wavelengths, there is no doubt that the observed Cotton effects are extrinsic in origin [9]. Dicumarol produced polyphasic extrinsic Cotton effects with AGP in sharp contrast to the monophasic extrinsic Cotton effect it produced following interaction with human serum albumin [11]. The basic tricyclic drugs, chlorpromazine and protriptyline reversed the signs of the Cotton effects of the CD spectrum of the dicumarol–AGP system (Fig. 1). Since the binding of chlorpromazine and protriptyline to AGP did not produce any extrinsic Cotton effects at wavelengths longer than 260 nm under the experimental conditions, these CD spectral changes observed above 260 nm can be explained either by allosteric modification of the dicumarol binding site on AGP by the basic tricyclic drugs, or by the formation of ternary complexes between dicumarol, AGP and basic tricyclic drugs. Chlorpromazine inverted the signs of the Cotton effects of the CD spectrum of the dicumarol–AGP system (Fig. 1). Protriptyline also did the same with the exception of the band at 270 nm (Fig. 1). The Cotton effect at 270 nm was decreased upon the addition of protriptyline without changing the sign of the Cotton effect under the current experimental conditions, this probably indicates a difference in the binding

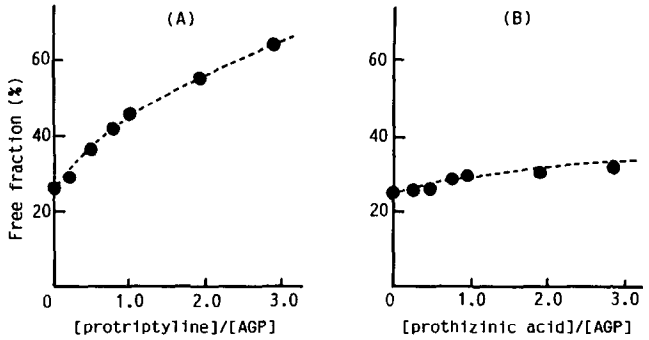

Fig. 3. Dicumarol free fraction with propranolol (A) and prothizinic acid (B) at pH 7.4 and 25°. (– – – –) Theoretical curve calculated assuming competition. The following concentrations were used: AGP, 20 μM; dicumarol, 20 μM.

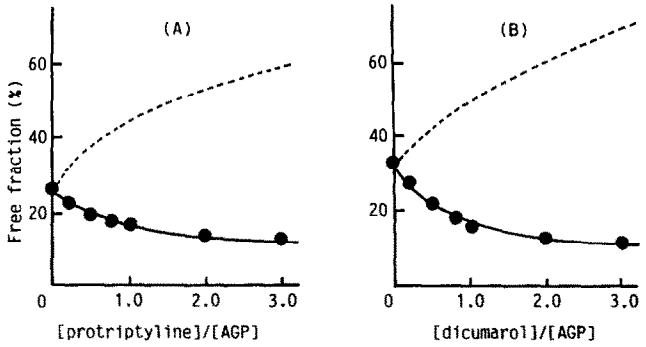

Fig. 4. Dicumarol free fraction with protriptyline (A) and protriptyline free fraction with dicumarol (B) at pH 7.4 and 25°. (- - - -) Theoretical curve calculated assuming competition. The following concentrations were used: AGP, 20 μM (A, B); dicumarol, 20 μM (A); protriptyline, 20 μM (B).

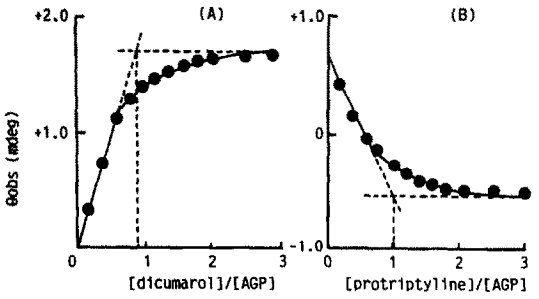

Fig. 5. Plots of induced ellipticities vs drug to AGP ratios for the dicumarol–AGP system (A) and the dicumarol–AGP–protriptyline system (B) at 325 nm, pH 7.4 and 25°. AGP concentration (A, B) of 10 μM and dicumarol concentration (B) of 10 μM were used throughout.

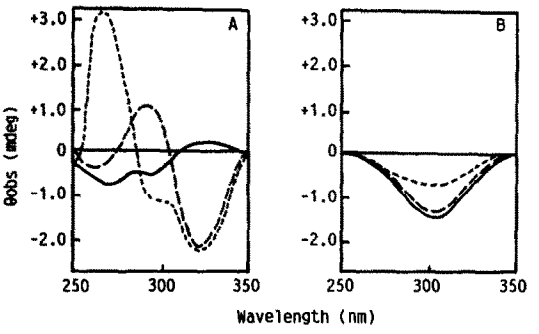

Fig. 6. Effects of basic tricyclic drugs on induced CD spectra of coumarin anticoagulants (A, coumetarol; B, warfarin) bound to AGP at pH 7.4 and 25°. (——) Without basic tricyclic drugs; (- - - -) with chlorpromazine; (— —) with protriptyline. The following concentrations were used: AGP, 10 μM; coumarin anticoagulant, 10 μM; basic tricyclic drugs, 10 μM.

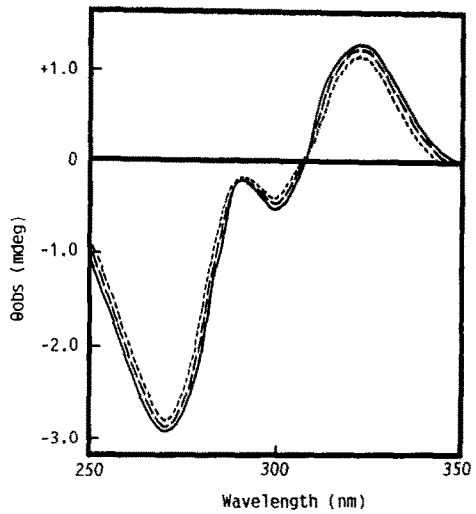

Fig. 7. Effects of tricyclic acidic drugs on induced CD spectra of the dicumarol–AGP system at pH 7.4 and 25°. (——) Without acidic tricyclic drug; (- - - -) with carprofen; (— —) with prothizinic acid. The following concentrations were used: AGP, 10 μM; dicumarol, 10 μM; acidic tricyclic drugs, 10 μM.

mode of protriptyline and chlorpromazine with AGP, even though they belong to the same class of drug.

The involvement of the secondary binding site to the observed ellipticities was not considered since AGP has only one binding site capable of inducing optical activity in the dicumarol molecule under the given experimental conditions [8]. In order to elucidate the mechanism of the sign changes of the extrinsic Cotton effects of the dicumarol–AGP system by basic tricyclic drugs, various effects on the induced ellipticities were examined. Temperature decreased the observed ellipticities without changing the signs of the Cotton effects (Fig. 2). Guanidine hydrochloride and pH were also found to impart similar effects on the CD spectrum of the dicimarol–AGP system as did temperature (not shown).

Temperature and pH had the more profound effects in decreasing the intensity of the CD spectrum of the dicumarol–AGP system than guanidine hydrochloride. Since the free concentration (D_f) of dicumarol was increased with the increase of pH, temperature and guanidine hydrochloride concentration (Table 1), there is no doubt that the reduced intensity of the CD spectrum was due to a lowering of binding affinity rather than to a change in the geometry of the binding site as a result of conformational changes locally produced in AGP. As these studies did not show changes in the signs of the Cotton effects, the reversal of the signs of the Cotton effects of the CD spectrum of the dicumarol–AGP system can be explained by other factors such as ternary complexation. To confirm the interaction between dicumarol and basic tricyclic drugs when binding with AGP, mutual displacement experiments using equilibrium dialysis were carried out. The basic non-tricyclic drug, propranolol, and the acidic tricyclic drug prothizinic acid increased the unbound fractions of dicumarol (Fig. 3). When two drugs like dicumarol and propranolol or dicumarol and prothizinic acid compete for a common high affinity binding site on AGP, the observed data should fit a theoretical curve. It can readily be seen (Fig. 3) that the experimental curves follow the theoretical curves, suggesting that dicumarol and propranolol or dicumarol and prothizinic acid do compete for the same binding site on AGP. In contrast, the basic tricyclic drug, protriptyline, increased the fraction of its own binding and that of dicumarol to AGP (Fig. 4). So the experimental curves were markedly deviated from the theoretical curves (Fig. 4), assuming competition between drugs for a common high affinity binding site. Chlorpromazine also showed similar behavior. The above results indicate clearly the mutual increase in binding of dicumarol and protriptyline or dicumarol and chlorpromazine to AGP when they are used in combination. Thus, dicumarol and protriptyline or dicumarol and chlorpromazine are most likely to be bound cooperatively to AGP. These results are in close accord with the previous findings that AGP may have a wide and flexible drug binding area [12]. Intersections of the plateaus and the tangents to the two curves for the dicumarol–protriptyline–AGP systems were obtained at a drug to AGP ratio of 1.0 (Fig. 4). These inflection points indicate the formation of a 1:1:1 ternary complex and the characteristics of the two curves can be explained by a ternary complex equilibrium. Thus, the free fractions of dicumarol or protriptyline were decreased with the increased concentration of the ternary complex, and the changes in free fractions reached a plateau with the saturation of the ternary complexation. The concept of a ternary complex was also supported by the CD titration of AGP with the same drugs, the intersections of the plateaus and the tangents to the two curves were obtained at a drug to AGP ratio of 1.0 (Fig. 5), indicating the formation of 1 : 1 binary and 1 : 1 : 1 ternary complexes by the dicumarol–AGP and the dicumarol–AGP–protriptyline systems. Chlorpromazine and protriptyline showed similar behavior. To substantiate the validity of this finding, the effects of some tricyclic drugs on the binding of some other coumarin compounds to AGP were studied using the CD and the equilibrium dialysis techniques. Basic tricyclic drugs showed similar effects on the induced CD spectra of the coumetarol–AGP system as did with the dicumarol–AGP system (Fig. 6). Basic tricyclic drugs did not change the signs of the Cotton effects of warfarin, phenprocoumon or acenocoumarin, but did decrease their observed induced ellipticities. Although it has been assumed that all coumarin compounds bind to the same area on AGP, the nature of the change of the extrinsic Cotton effects caused by basic tricyclic drugs clearly reveals that there are fundamental differences even among compounds having similar structures. The above results indicate that basic tricyclic drugs are structurally important for the inversion of the signs of the Cotton effects of only those coumarin compounds containing two hydroxycoumarin rings.

In conclusion, AGP seems to have a wide and flexible drug binding area, it appears from all the above results that the change of the signs of the induced Cotton effects of the dicumarol–AGP system by basic tricyclic drugs is due to the results of a change in the dicumarol chirality by the formation of a 1:1:1 ternary complex as the basic tricyclic drug enhances its own binding and that of dicumarol when bound to AGP.

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