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BINDING OF VINCA ALKALOID ANALOGUES TO HUMAN SERUM ALBUMIN AND TO α_1-ACID GLYCOPROTEIN

ILONA FITOS, JULIA VISY and MIKLÓS SIMONYI*
Central Research Institute for Chemistry of the Hungarian Academy of Sciences, Budapest, P.O. Box 17, H-1525, Hungary

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Abstract—The binding of a series of vinca alkaloid analogues having eburnane or indolo[2,3-*a*]quinolizidine skeletons was studied with human serum albumin (HSA) by affinity chromatography and with α_1-acid glycoprotein by means of competition experiments. On HSA the binding occurs at the benzodiazepine-indole binding site via hydrophobic interaction and shows slight stereoselectivity preferring the *trans* isomers. The binding to α_1-AGP proved to be highly stereoselective in favour of the *trans* isomers having 3(*S*),16(*R*)eburnane or 1(*R*),12b(*S*)indolo[2,3-*a*]quinolizidine absolute configurations.

Some of the natural vinca alkaloids, e.g. (+)-vincamin and (+)-eburnamonine have important pharmacological effects. The synthetic derivative (+)-apovincaminic acid ethyl ester is a cerebral vasodilator [1] registered under the trade name Cavinton®.

In this work the serum protein binding of compounds possessing pentacyclic eburnane or tetracyclic indolo[2,3-*a*]-quinolizidine skeletons was studied with human serum albumin (HSA) and α_1-acid glycoprotein (α_1-AGP). In a series of (+)-*cis*-apovincaminic acid esters we studied the effect of substitution. Since these molecules have two or three chiral centres, the binding of different stereoisomers has also been compared.

MATERIALS AND METHODS

Compounds **1–24** synthesized according to Refs 2–6, diazepam as well as [^{3}H]**3** (752 mCi/mmol) and [^{3}H]**24b** (3.78 Ci/mmol) were obtained from Chemical Works of Gedeon Richter Ltd (Budapest). (*R*)- and (*S*)-Oxazepam acetate were obtained as described previously [7]. *rac*-Warfarin was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and was resolved according to Ref. 8. [^{14}C]*rac*-Warfarin (55 mCi/mmol) and [^{3}H]diazepam (86 Ci/mmol) and *rac*-[^{3}H]propranolol.HCl (20 Ci/mmol) were purchased from Amersham International plc (Amersham, U.K.). Na-salicylate was obtained from Reanal (Budapest). HSA (fatty acid free) and α_1-AGP were purchased from Miles Labs (Elkhart, IN, U.S.A.) and Sigma, respectively. Binding experiments were made in Ringer buffer, pH 7.4, containing 0.01% sodium azide, at room temperature.

Binding to HSA was studied by affinity chromatography following the method of Lagercrantz *et al.* [9]. HSA in about 1% concentration was immobilized on CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The gel was filled into a glass column (diameter 12 mm, length about 3 cm). Elution was made by buffer, the flow rate was about 1 mL/min. Samples of 2–5 μg were applied in 10–20 μL ethanol solution and elution volumes (V_e) were measured by UV detection at 263 nm. The small negative peak caused by the solvent (ethanol) indicated V_o. $V_e - V_o$ values are directly proportional to binding affinities. Reference compounds with known binding constants were measured in order to evaluate the binding constants of the investigated compounds.

Binding to α_1-AGP was studied by means of competitive displacement of bound *rac*-[^{14}C]warfarin measured by ultrafiltration in Amicon MPS-1 system by centrifugation using YM-10 membranes. We chose this marker ligand because it was not adsorbed by the membranes. In our experimental circumstances its binding was not found [10] to be stereoselective, with $K = 3–5 \times 10^4\ M^{-1}$ association constant. We performed a few displacement experiments also with *rac*-[^{3}H]propranolol.HCl marker ligand. Although propranolol was partially adsorbed to the membrane, the compounds investigated showed displacing abilities similar to those found with warfarin. From the 1 mg/mL ethanolic stock solutions of the compounds to be investigated 20 μL was added to 1 mL of standard solution containing *rac*-[^{14}C]warfarin and α_1-AGP. The reference contained 20 μL ethanol. The measurements were repeated twice using fresh solutions in the same concentration.

Calculations for binding to α_1-AGP were performed according to the following scheme describing the competition of two ligands (L_1 and L_2) for a common binding site of the protein (P):

$$L_1 + P \overset{K_1}{\rightleftharpoons} L_1P, \quad (1)$$

$$L_2 + P \overset{K_2}{\rightleftharpoons} L_2P, \quad (2)$$

* To whom correspondence should be addressed.

$$K_1 = \frac{1-\alpha_1}{\alpha_1[c_p-(1-\alpha_1)c_1]} = \frac{1-\alpha_1'}{\alpha_1'[c_p-(1-\alpha_1')c_1-(1-\alpha_2')c_2]}, \quad (3)$$

$$K_2 = \frac{1-\alpha_2'}{\alpha_2'[c_p-(1-\alpha_1')c_1-(1-\alpha_2')c_2]}, \quad (4)$$

where c_1, c_2, c_p denote the total concentrations of L_1, L_2 and P; α_1' and α_2' are the free fractions of L_1 and L_2 in the presence of the second ligand, respectively. If the binding of the first ligand can be detected, (i.e. α_1 and α_1' are measured), K_2 can be calculated according to Eqn 5:

$$K_2 = \frac{B}{A(c_2-B)}, \quad (5)$$

where

$$A = \frac{1-\alpha_1'}{K_1\alpha_1'} \; (= \text{free protein concentration})$$

and

$$B = c_p - (1-\alpha_1')c_1 - A^{-1}$$

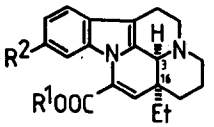
(= protein concentration occupied by L_2).

RESULTS

Substituent effect in the binding of (+)-cis-apovincaminic acid esters to HSA

In Table 1 the elution volumes obtained on a HSA-Sepharose column (V_o = 3.5 mL) for apovincaminic acid (**1**) and for a series of different esters are collected. It can be seen that V_e values vary in a broad range (7–130 mL). The binding constants can be estimated considering the V_e values obtained for some reference compounds on the same column. Because of the wide variation in binding affinities ($K = 10^4$–10^6 M^{-1}) the larger V_e values were approximated. Hence, instead of comparing uncertain K values we think that the elution volumes themselves are informative for the nature of substituent effects. The following tendencies can be observed: (a) increasing hydrophobicity of R^1 markedly enhances the binding affinity (cf. nos. **1**–**8**); (b) in position R^2 acylamino groups decrease (cf. nos. **11**, **12**, **16** vs **2**, **3**, **4**), while NO_2 and Br substituents significantly enhance (nos. **9** vs **3**, and **10** vs **2**) the binding. Table 2 shows the results of some binding interaction studies in order to get information for the binding site of the investigated compounds. It can be seen that Cavinton could be displaced by diazepam and Na-salicylate but its binding was

Table 1. Binding affinities of (+)-*cis*-apovincaminic acid esters to HSA characterized by elution volumes (V_e) measured on HSA-Sepharose column (V_o = 3.5 mL) with buffer eluent



Compounds	R^1	R^2	V_e (mL)
1	H	H	7
2	CH_3	H	28
3	C_2H_5	H	55
4	CH_2—CH=CH_2	H	60
5	$(CH_2)_2$—OH	H	8
6	$(CH_2)_3$—OH	H	7
7	$(CH_2)_2$—Cl	H	55
8	CH_2—CF_3	H	70
9	C_2H_5	NO_2	≧120
10	CH_3	Br	≧130
11	CH_3	$NHCOCF_3$	11
12	C_2H_5	$NHCOCF_3$	18
13	*n*-C_3H_7	$NHCOCF_3$	25
14	*n*-C_4H_9	$NHCOCF_3$	60
15	*n*-C_5H_{11}	$NHCOCF_3$	≧100
16	CH_2—CH=CH_2	$NHCOCF_3$	21
17	CH_2-Ph	$NHCOCF_3$	90
18	C_2H_5	NHCOPh	22
19	CH_3	NHCOPh	15
20	CH_3	NHCO—*o*-F-Ph	14
21	CH_3	NHCO—*p*-NO_2-Ph	26

Evaluation of binding constants (K)

Reference compounds	V_e (mL)	$K(M^{-1})$	Ref.
(*R*)-Oxazepam acetate	7	1.1×10^4	[7]
(*S*)-Oxazepam acetate	25	5.5×10^4	[7]
Diazepam	45	1.8×10^5	[11]

Table 2. Binding interaction studies on HSA-Sepharose column (V_0 = 3.5 mL)

Sample	Eluent	V_e (mL)
[^{3}H]Cavinton (**3**)	Buffer	50
[^{3}H]Cavinton (**3**)	10^{-4} M Diazepam	20
[^{3}H]Cavinton (**3**)	10^{-4} M (R)-Warfarin	55
[^{3}H]Cavinton (**3**)	10^{-4} M (*S*)-Warfarin	50
[^{3}H]Cavinton (**3**)	10^{-4} M Na-salicylate	30
[^{3}H]Diazepam	Buffer	60
[^{3}H]Diazepam	10^{-4} M (**8**)	20

unchanged in the presence of warfarin enantiomers. It suggests that Cavinton and its analogues bind to the benzodiazepine-indol binding site [12] on HSA.

Substituent effect in the binding of (+)-cis-*apovincaminic acid esters to* α_1-*AGP*

Table 3 shows the binding affinities of some apovincaminic acid esters to α_1-AGP characterized by the K_2 inhibition constants according to Eqn 5. Similarly to the binding to HSA (cf. Table 1), the affinities vary in broad range and the weak binding of apovincaminic acid (**1**) is in accordance with the acidic nature of this glycoprotein. The effect of substitution can be characterized by the following observations: (a) increasing hydrophobicity of R^1 enhances the binding (cf. nos. **1–7**); (b) in position R^2 the presence of Br strongly enhances the binding (**10** vs **2**), the NO_2 group does not change (**9** vs **3**) and the acylamino substituent has no unequivocal influence.

Stereoselectivity in the binding to HSA and to α_1-*AGP*

In Table 4 the binding of the stereoisomers can be compared in case of six different molecules (**3**, **22–26**). The binding studies were performed as described for the apovincaminic acid esters to both proteins. In case of the compounds having pentacyclic skeleton the steric positions of 3(H) and 16(Et) groups (**3**, **22**, **23**) were varied, while in the molecule **24** there was a third chiral center in position 14. In case of the tetracyclic compounds **25** and **26** the *cis* and *trans* orientations of the corresponding 1 and 12b positions were varied.

According to the binding data obtained with HSA the following observations can be made: in cases of the strongly bound **3** and the weakly bound **25** no significant differences were found between the binding of the *cis* and *trans* stereoisomers. In the cases of **22**, **23** and **26** the *trans* isomers, regardless of the configurations of the two centers, have two to four times higher affinity than the corresponding *cis* isomers. The binding of **24** is weak due to the hydrophilic substitution, the binding of **24b**, however, is significantly higher than found for the other stereoisomers. It looks as if structures having (*R*)-configuration in position 14 and *trans* geometry of 3(H) and 16(Et) groups were better bound by HSA.

The binding to α_1-AGP showed much more pronounced stereoselectivity. Compounds **3b**, **23b**, **25b** and **26b** have 20–35 times higher affinity than their (*S*,*S*) *cis* isomers. The above *trans* isomers have 3(*S*),16(*R*)eburnane (**3b**, **23b**), as well as 1(*R*),12b(*S*) indolo[2,3-*a*]quinolizidine (**25b**, **26b**) absolute configuration. Compounds **22b** and **26c**, which are *trans* isomers with opposite chirality, have however weaker binding than their 3(*S*),16(*S*) and 1(*S*),12b(*S*) *cis* isomers, respectively. The binding of **24a–f** supports the above conclusion: **24a** and **24b**, both having 3(*S*),16(*R*) configurations, exhibited about 100 times higher affinity for α_1-AGP than the enantiomeric *trans*, or both (*S*,*S*)- and (*R*,*R*)-*cis* isomers. The configuration of the third chiral centre in **24** does not seem to make much difference in the binding.

Table 3. Binding affinities of (+)-*cis*-apovincaminic esters (cf. Table 1) to α_1-AGP measured by competitive displacement of bound *rac*-[^{14}C]warfarin measured by ultrafiltration (cf. Eqn 5)

Compounds	$\frac{\alpha_1'}{\alpha_1}100$ (%)	K_2 (M^{-1})
1	107	2.7×10^3
2	152	7.8×10^4
3	172	2.7×10^5
4	164	1.8×10^5
5	127	2.6×10^4
7	166	4.1×10^5
8	130	3.6×10^4
9	161	3.5×10^5
10	189	$\geqq 6.0 \times 10^6$
11	123	2.1×10^4
12	140	7.4×10^4
13	160	4.5×10^5
14	150	1.6×10^5
16	165	6.4×10^5
17	140	1.0×10^5

$c_w = 3.2 \times 10^{-5}$ M, $c_{AGP} = 6 \times 10^{-5}$ M.
α_1 (free fraction of warfarin) = 0.44 ± 0.03.
α_1': free fraction of warfarin in the presence of additives ($3.5–7 \times 10^{-5}$ M).

DISCUSSION

Competition studies indicated (Table 2) that apovincaminic acid esters bind to the "indol-benzodiazepine" binding site on HSA. This binding site was found [13] to be a hydrophobic pocket with a cationic centre near the surface of the protein. Results in Table 1 and 4 support the important role of the hydrophobic interaction, while the weak binding of apovincaminic acid (**1**) suggests that the carboxyl group in this molecule cannot reach the cationic centre on the protein. The very strong binding of compounds **9** and **10** having NO_2 and Br substituents in position R^2 is unexpected since these substituents in similar position on 1,4-benzodiazepines exerted opposite effects [14]. The stereoselectivity of this binding site has been seen for tryptophan as well as for benzodiazepines [15]. In the present study the slight preference found for the binding of the *trans* isomers independently of the absolute configuration of the chiral centres (Table 1) suggests that the protein prefers the flat molecular geometry (cf. Fig. 1).

The binding of vinca alkaloid analogues to α_1-AGP showed very considerable dependence on their structures. According to the accumulated binding

Table 4. Stereoselectivity in binding to HSA and to α_1-AGP

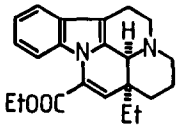
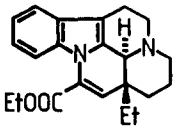
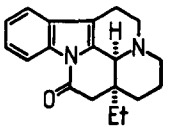
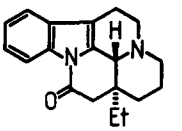
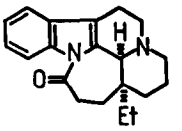
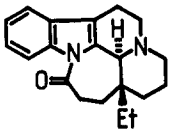
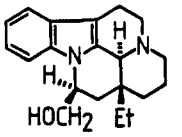
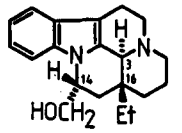
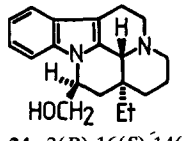
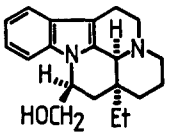
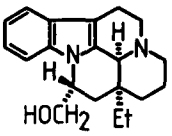
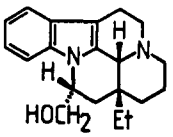
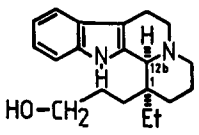
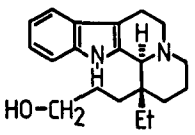
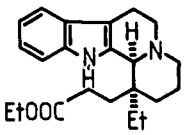
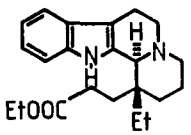
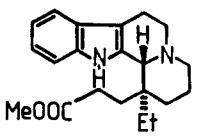
Compounds	HSA			α_1-AGP		
	V_e (mL)	K^*_{HSA} (M^{-1})	$\frac{K_{trans}}{K_{cis}}$	$\frac{\alpha}{\alpha_0}$ 100 (%)	K_{AGP}† (M^{-1})	$\frac{K_{trans}}{K_{cis}}$
 **3a** (+)-*cis*-Cavinton	55	$3 (\pm 1) \times 10^5$	1 (±0.2)	182	2.6×10^5	30 (±10)
 **3b** (+)-*trans*-Cavinton	60	$4 (\pm 1) \times 10^5$		228	7.8×10^6	
 **22a** (−)-*cis*-Eburnamonine	11	$2 (\pm 0.5) \times 10^4$	2 (±0.5)	140	3.9×10^4	0.3 (±0.1)
 **22b** (−)-*trans*-Eburnamonine	19	$4 (\pm 1) \times 10^4$		121	1.2×10^4	
 **23a** (+)-*cis*-lactam	20	$4 (\pm 1) \times 10^4$	4 (±2)	153	5.1×10^4	20 (±10)
 **23b** (−)-*trans*-lactam	44	$1.6 (\pm 0.2) \times 10^5$		218	1.04×10^6	
 **24a** 3(*S*),16(*R*),14(*S*)-E‡	4	$2 (\pm 1) \times 10^3$		214	2.9×10^6	
 **24b** 3(*S*),16(*R*),14(*R*)-E	7.5	$1 (\pm 0.2) \times 10^4$ (8×10^3)§	5‖	210	1.8×10^6 (1.4×10^6)§	100‖
 **24c** 3(*R*),16(*S*),14(*S*)-E	4	$2 (\pm 1) \times 10^3$		125	1.5×10^4	

Table 4. Continued

Compounds	HSA			α_1-AGP		
	V_e (mL)	K^*_{HSA} (M^{-1})	$\frac{K_{trans}}{K_{cis}}$	$\frac{\alpha}{\alpha_0}$ 100 (%)	K_{AGP}† (M^{-1})	$\frac{K_{trans}}{K_{cis}}$
 **24d** 3(*S*),16(*S*),14(*S*)-E	4	2 (±1) × 10^3		146	4.6 × 10^4	
 **24e** 3(*S*),16(*S*),14(*R*)-E	5	4 (±1) × 10^3		127	1.7 × 10^4	
 **24f** 3(*R*),16(*R*),14(*R*)-E	5	4 (±1) × 10^3		146	4.6 × 10^4	
 **25a** (−)-*cis*-alcohol	4	2 (±1) × 10^3	1 (±0.2)	150	4.5 × 10^4	35 (±10)
 **25b** (−)-*trans*-alcohol	4	2 (±1) × 10^3		225	1.6 × 10^6	
 **26a** (−)-*cis*-Et-ester	4.5	3 (±1) × 10^3	3 (±1)	175	1.9 × 10^5	29 (±10)
 **26b** (−)-*trans*-Et-ester	6	8 (±2) × 10^3		223	5.5 × 10^6	
 **26c** (+)-*trans*-Me-ester	6	8 (±2) × 10^3	3¶	158	8.5 × 10^4	0.5¶

* Estimated from V_e values, cf. Table 1.
† Calculated assuming competitive displacement, cf. Table 3.
‡ E: 14,15-dihydro-14-hydroxymethyl-eburnamonine.
§ Obtained from ultrafiltration measurements using radioactive **24a** ($c = 2 \times 10^{-6}$ M, $c_{AGP} = 2.5 \times 10^{-5}$ M, $\alpha = 0.03$).
‖ These values refer to enantioselectivity.
¶ Using **26a** as *cis* analogue.

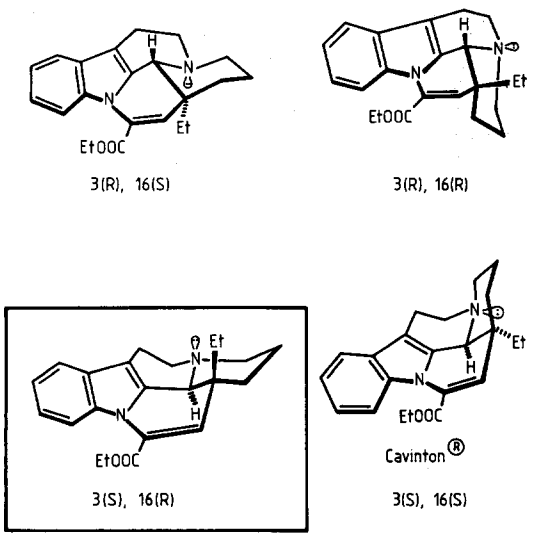


Fig. 1. Stereoisomers of ethyl-apovincaminate. The 3(*S*),16(*R*) stereoisomer (in the frame) is highly favoured by α_1-AGP.

data [16] this protein prefers basic drugs, but neutral and acidic drugs are also bound here via hydrophobic interactions. The conclusions concerning the number of binding sites are also variable. In our experimental approach we supposed competition for one specific binding site, as suggested previously by Müller *et al.* [17]. We applied an acidic ligand, warfarin as a marker, but as mentioned in the experimental part, similar results could be obtained with propranolol. We think that the computed K_2 binding constants are reliable enough to reveal the important structural characteristics of the molecules, which influence the binding strength. Results in Tables 3 and 4 suggest two factors provoking very strong binding: (a) the Br substituent in position R^2 of apovincaminic acid esters; (b) 3(*S*),16(*R*) eburnane or 1(*R*),12b(*S*) indolo[2,3-*a*]-quinolizidine absolute configurations. Up to now the binding to α_1-AGP was found to display only slight stereoselectivities [18], the highest reported value is about 4 [10]. Thus, the K_{trans}/K_{cis} = 20–35 factors in Table 4, as well as the enantioselectivity of about 100 from K_{24b}/K_{24c} seem to indicate a unique interaction of α_1-AGP with these type of molecules. The high stereoselectivity is connected with the overall shape of the molecules, as can be seen in Fig. 1 for the four stereoisomers of ethyl-apovincaminate. While HSA somewhat prefers the flat *trans*isomers independently from the configurations of the chiral centres, α_1-AGP selects the one, having 3(*S*),16(*R*) configurations. It looks as though the benzene ring as well as both nitrogens took part in the binding process, leading to chiral discrimination, and the unfavourable steric position of the 16-ethyl group explains the weak binding of the other *trans*isomer.

The presented binding study of vincaalkaloid analogues indicated marked structure-dependency both with HSA and α_1-AGP.

While in the strong plasma protein binding of Cavinton [19] both proteins participate, there are related compounds like **24b** (RGH-0537) the strong plasma binding of which occurs almost exclusively on the acute phase protein, α_1-AGP.

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