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In vitro binding studies of the peroxisomicine A1–BSA and –HSA interactions

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Abstract

Peroxisomicine A1 (PA1) is a dimeric hydroxyanthracenone isolated from fruits of plants belonging to the genus *Karwinskia*. Showing selective toxicity between malignant and benign cell lines, it is currently under screening as an antineoplastic agent. Very little is known about its mechanism of action. In the present work the extent of binding of this substance with Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA) at pH 7.2 and 7.4 has been evaluated using the spectrophotometric method. Absorbance of PA1 was altered by the presence of albumin and this property was used to generate binding isotherms. The investigation was carried out at four different temperatures. The data were analyzed by assuming two types of binding sites. Results indicated that PA1 binds to both albumins at physiological pH, which is reflected by the affinity constants of the order of 10^5. There are two types of binding sites in the albumin for PA1; with the electrostatic forces being discarded, the hydrophobic and hydrogen bond are more probable. Binding with HSA is stronger than with BSA.

Keywords: Binding; Peroxisomicine; Hydroxyanthracenones; *Karwinskia*; Albumin; UV–Vis spectroscopy; Interactions

1. Introduction

Peroxisomicine A1 (PA1, previously known as T514, Fig. 1) is a dimeric hydroxyanthracenone isolated from plants of the genus *Karwinskia* (Dreyer et al., 1975). It has been demonstrated that it causes severe damage to lung, kidney and liver (Bermúdez et al., 1986). This toxicity has been correlated with findings in liver and skin cell cultures, where a greater sensitivity of hepatocytes was obtained (Garza-Ocañas et al., 1992). Selective toxicity of PA1 was found in studies performed with several human tumor cell lines (Piñeyro et al., 1994), suggesting a possible use as an antineoplastic agent; the substance was assayed with other known anticancer drugs in normal and malignant cell lines; normal cell lines were several times less sensitive than cancer cells. These results were recently corroborated by the National Cancer Institute (USA). Very little knowledge on its mechanism of action is available; however, it is known that it produces, at nonlethal doses, irreversible and selective damage of yeast peroxisomes in vivo (Sepúlveda et al.,

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1992). Hence, it was renamed as peroxisomicine; the letter A comes from its Cotton effect in CD curves and was adopted from the nomenclature proposed by Steglich for similar compounds (Gill and Steglich, 1987; Waksman and Ramírez, 1992). We reported the inhibitory effect in vitro of PA1 and structurally related hydroxyanthracenones on liver catalase activity, using the purified enzyme (Moreno et al., 1995).

It is well known that the extent by which a drug binds to plasma proteins can be an important determining factor for its overall pharmacology and pharmacokinetics. For electrically neutral and anionic drugs, the most important protein in this respect is serum albumin (Kalant and Roschlau, 1989).

The purpose of the present work was to investigate for the first time, the interaction of PA1 with Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA) in order to establish the presence or absence of binding and to characterize it if binding has occurred. Thermodynamic properties were also determined to gain an understanding on the mechanism of binding.

Several methods for the determination of drug protein binding have been described. Most involve equilibration of the drug with a solution containing the protein and then separation of the free drug by dialysis, ultrafiltration or ultracentrifugation. Several chromatographic approaches have also been reported. On the other hand, spectrophotometric procedures do not need a previous separation of the protein–drug complex, but require that either the spectral characteristic of the ligand or the macromolecule change to an appreciable degree when ligand binding occurs (Harris and Bashford, 1988). In order to select the best method, we took into account the fact that PA1 is an unstable substance, and so it was proposed to undertake measurements both rapidly and in situ under experimental conditions reasonable for the protein involved.

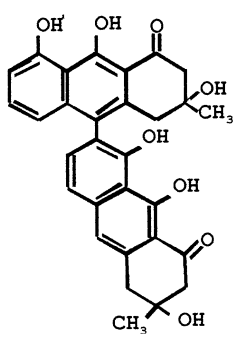

Fig. 1. Peroxisomicine A1 (T514).

2. Material and methods

2.1. Materials

Crystalline BSA was obtained from Sigma Chemical Co. and was used without further purification. HSA was purchased as a 25% solution from Fustery (lot M65205) free from preservatives. This solution was dialyzed with double distilled deionized water and then lyophilized. The crystals were maintained in a dessicator for 48 h to eliminate any remaining water.

Double distilled deionized water was used to prepare all the buffer solutions. BSA and HSA were dissolved in 0.018 M phosphate buffer. Ionic strength of the buffer was maintained at 0.054 M by the addition of KCl. The concentration of the BSA solution was determined spectrophotometrically by means of a Beckman DU-7500 assuming $\varepsilon^{1\%}$ 6.67 at 280 nm and a MW of 66 000 (Halfman and Nishida, 1972); for HSA an $\varepsilon^{1\%}$ of 5.8 was considered and a MW of 66 500 (Lentner, 1984).

PA1 was obtained from the production laboratory of the pharmacology department. Its purity was checked by its melting point and chromatography (TLC and HPLC) (Salazar et al., 1996). Since PA1 has extremely poor solubility in aqueous solutions, a stock solution in spectroscopical grade ethanol was prepared. The final concentration of ethanol in the measured sample solutions was, however, less than 1.9%.

2.2. Equipment

All pH measurements were made using a Beckman digital pH meter model 3500 and a Ag/AgCl combination glass electrode. The electrode was calibrated at the temperature of the sample using

standard buffers of known pH at the same temperature. The pH of the solutions was checked randomly before and after the addition of PA1 and albumin to the buffer. Absorbance measurements were recorded with a diode array Beckman DU 7500 equipped with a Peltier temperature controller. Stock solutions were maintained at the desired temperature using a model HP 1000 Craft thermostated water bath.

2.3. Methods

The binding of peroxisomicine to albumin was characterized by the change in absorbance that accompanied the formation of the complex. Spectrophotometric titrations were conducted as follows: 3.0 ml of PA1 solution of the appropriate concentration was placed in a 1 cm quartz cell. Absorbance was read at 422 nm (A_0); 100 μl of 0.31 mM albumin solution was added. The contents were mixed by inversion; 50 s after the initial reading (A_0), a second reading of absorbance was obtained (A_{50}). The final concentrations of albumin and PA1 were 10 μM and between 17.5 and 46 μM, respectively. Fresh aliquots of the protein and drug solutions were used for each point of the titration curve. The ΔAbs versus [PA1] was plotted. Titrations were performed three times.

In order to obtain ΔAbs max the following procedure was performed: 3 ml of a 21.04 μM PA1 solution (final concentration, 20 μM) was placed in a 1 cm quartz cuvette and absorbance was recorded at 422 nm; an aliquot of a 1.55 mM albumin solution was added (concentration between 20 and 60 μM); the content was mixed by inversion and 50 s after the first reading, a second absorbance was recorded. 1/ΔAbs versus 1/[albumin] was plotted, which approaches linearity at a high albumin concentration and crosses the ordinate at 1/ΔAbs max. With this value we calculated the ΔAbs produced by adding 1 μM of PA1 (α), where $\alpha = \Delta\text{Abs max}/[\text{PA1}]$ total. With this value, the amount of PA1 bound was calculated as $\Delta\text{Abs}/\alpha$ at each point of the titration curve. In all cases, spectral blanks were obtained with the buffer used. The same procedure was performed at two different pH values of 7.2 and 7.4 for both albumins tested; experiments were carried out at four different temperatures for BSA—15, 20, 25, and 30 °C, and three temperatures for HSA—20, 25 and 30 °C.

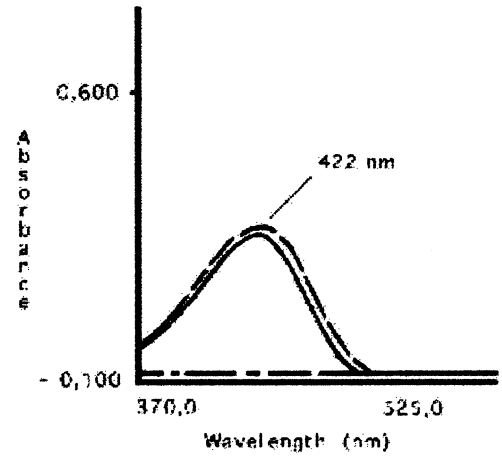

Fig. 2. UV–Vis spectra obtained from PA1 before (– – –) and after (—) addition of albumin.

Regression lines were obtained by means of the statistical package MYSTAT for Macintosh. Significant differences were checked by means of student's t test.

3. Results and discussion

In order to know if the interaction of BSA (HSA) and PA1 displayed spectroscopic differences, we compared the spectra of albumin, PA1 and PA1–albumin. PA1 absorbs in the visible region, and the presence of the albumin produced a hypochromic effect (Fig. 2). At 422 nm the ΔAbs, although small, was reproducible (VC: 4.56% after 50 s delay) and this λ was selected for the titration curves. In order to verify that neither PA1 nor albumin changed the pH of the buffer, the pH of the solutions were checked randomly before and after the addition of PA1 and/or albumin; the changes of pH measurements were not significant, being less than 1% in all the cases. The equilibration period of 50 s was chosen after an initial experiment (run in triplicate) in which the change in absorbance was measured for every 5 s during the 1 min period.

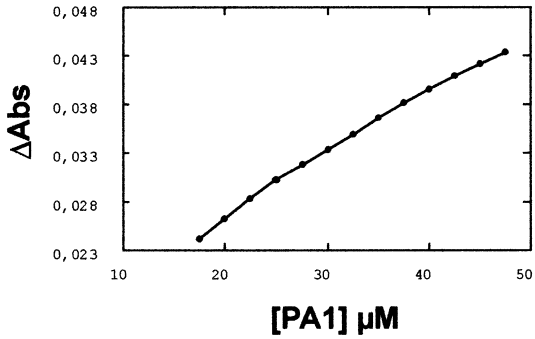

Fig. 3. Titulation of BSA (10 μM) with PA1: pH 7.2 and 15 °C.

Binding studies in which a variety of ligand concentrations are studied at a fixed acceptor concentration have been argued to be more acceptable that those in which the acceptor (albumin) concentration is varied and that of ligand fixed (Nickol et al., 1967).

The calibration plots of ΔAbs versus PA1 were linear, with good correlation coefficient, suggesting that no side reactions took place. A typical titration curve for the drug–albumin interaction is seen in Fig. 3. Since a fresh aliquot of drug and albumin solution was used for each point of the titration curve, photo and thermal decomposition were minimized. In order to proceed to the subsequent calculations, it was necessary to know the ΔAbs max, which is the change in absorbance at saturation (Harris and Bashford, 1988). This is proportional to the concentration of total PA1 binding sites. With this value, α for all the systems examined was calculated (Table 1).

With the values of α bound and free PA1 in each point of the titration curve was calculated; by using the Scatchard equation, $r/[PA1]_{free}$ versus

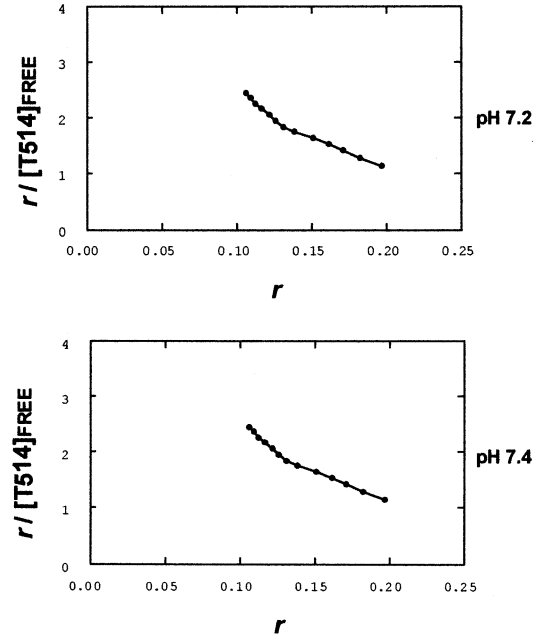

Fig. 4. Scatchard plots obtained for the PA1–BSA binding at 15 °C.

r was plotted; these plots resulted in curves like those shown in Fig. 4. In all cases, two lineal tendencies could be observed, which were treated independently. From each one, a line was obtained with correlation coefficients ranging from 0.982 to 0.998. K_a was obtained from the slope, and n from the intercept. Values obtained are shown in Table 2, these being the means of three replicate analyses. From these values, it can be determined that both BSA and HSA have two types of binding sites: n_1 and n_2. The first one has higher affinity, but less capacity, while the other has a moderate affinity, but more capacity. In all cases, PA1 has a strong interaction reflected by K_a of the order of 10^5.

Table 1
Absorbance produced/mole of PA1 bond (α)

Temperature (°C)	PA1–BSA pH 7.2 α (a.u.)	PA1–HSA pH 7.2 α (a.u.)	PA1–BSA pH 7.4 α (a.u.)	PA1–HSA pH 7.4 α (a.u.)
15	1.852	–	1.020	–
20	2.000	1.613	1.220	1.163
25	2.174	1.852	1.282	1.282
30	2.273	2.000	1.389	1.389

Table 2
Binding parameters of PA1 with BSA and HSA

	BSA				HSA			
	K_{a_1} ($\times 10^6$ l mol^{-1})	n_1	K_{a_2} ($\times 10^6$ l mol^{-1})	n_2	K_{a_1} ($\times 10^6$ l mol^{-1})	n_1	K_{a_2} ($\times 10^6$ l mol^{-1})	n_2
pH 7.2 (15 °C)	0.094 ± 0.005	3.24	0.039 ± 0.002	5.05	–			
pH 7.4 (15 °C)	0.580 ± 0.011	3.05	0.211 ± 0.008	5.01	–			
pH 7.2 (20 °C)	0.079 ± 0.003	2.97	0.032 ± 0.003	5.00	0.295 ± 0.001	3.15	0.112 ± 0.003	4.60
pH 7.4 (20 °C)	0.543 ± 0.004	3.29	0.187 ± 0.006	4.78	1.179 ± 0.016	2.80	0.211 ± 0.007	4.80
pH 7.2 (25 °C)	0.068 ± 0.006	2.88	0.024 ± 0.002	5.37	0.246 ± 0.002	3.14	0.087 ± 0.001	4.60
pH 7.4 (25 °C)	0.497 ± 0.010	3.24	0.157 ± 0.007	5.24	0.731 ± 0.010	3.26	0.161 ± 0.008	5.14
pH 7.2 (30 °C)	0.045 ± 0.001	3.04	0.020 ± 0.001	5.05	0.176 ± 0.005	2.90	0.055 ± 0.003	4.94
pH 7.4 (30 °C)	0.468 ± 0.010	3.09	0.119 ± 0.006	5.26	0.561 ± 0.011	3.03	0.137 ± 0.006	4.64

It should be noted that n values are not integers. This might be either due to the experimental errors or that the albumin viewed is not a homogeneous molecule with respect to its binding properties, as pointed out earlier.

In order to have some idea about the nature of the binding forces between PA1 and albumin that allow the calculation of the thermodynamic functions, the titrations were conducted at four different temperatures. Thermodynamic parameters were calculated from the K_a utilizing usual equations. The ΔH was obtained from a van't Hoff plot (Fig. 5) of $\ln K_a$ versus $1/T$. The linearity obtained indicated that ΔH was essentially independent of the temperature over the range investigated.

The calculated thermodynamic parameters at 25 °C are shown in Table 3. The decrease in the values of binding constants with temperature results from an exothermic process. PA1 is an acidic compound with $pK_a = 7.34$. At the pH used, the molecule is partially ionized (50%); therefore, an electrostatic mechanism of binding could be assumed. However, an electrostatic mechanism would imply that the principal source of ΔG arises from ΔS (O'Reilly and Kowitz, 1967) and, in the present case the principal contribution to ΔG comes from ΔH (between 40 and 90%). Moreover, an increase in the interaction between 83 and 90% with BSA and between 50 and 75% with HSA is observed, when the pH is increased from 7.2 to 7.4. The difference is statistically significant ($\alpha = 0.05$). These experimental results, discarding the electrostatic mechanism if any, where there is an increase in negative charges of the protein should lead to a decrease in the ability of the proteins to attract anions (ionized PA1) (O'Reilly, 1969). These results suggest that a significant portion of the binding energy is derived from nonionic sources. The large positive ΔS associated with many interactions involving proteins is usually attributed to unfolding of the protein molecule. This does not offer a satisfactory explanation in the present work, because the process of unfolding should result in an endothermic reaction of appreciable magnitude. A positive value of ΔS probably resulted in this case from hydrophobic interactions, which has been demonstrated to favor hydrogen bonding (O'Reilly, 1973). In all cases, the magnitude of ΔH is suggestive of hydrogen bonding.

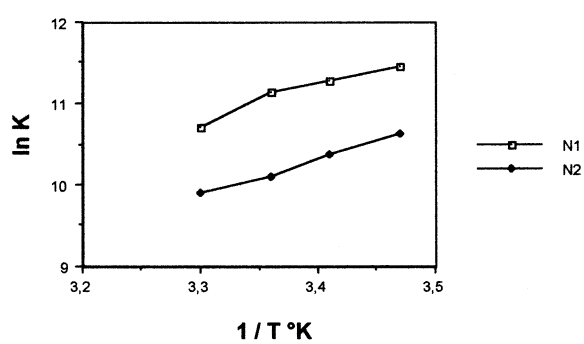

Fig. 5. Van't Hoff plot for the PA1–BSA binding, pH 7.2.

Table 3
Thermodynamic parameters for the bonding of PA1 to BSA and HSA at 25 °C

Albumin	pH	Site	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (ue)
BSA	7.2	n_1	-6.585 ± 0.049	-8.149 ± 0.451	-5.246 ± 1.416
BSA	7.2	n_2	-5.980 ± 0.053	-7.896 ± 0.466	-6.429 ± 1.479
BSA	7.4	n_1	-7.766 ± 0.012	-2.551 ± 0.194	$+17.503 \pm 0.683$
BSA	7.4	n_2	-7.084 ± 0.027	-6.635 ± 0.214	$+1.522 \pm 0.619$
HSA	7.2	n_1	-7.348 ± 0.006	-4.631 ± 0.188	$+9.116 \pm 0.651$
HSA	7.2	n_2	-6.734 ± 0.009	-6.443 ± 0.400	$+0.977 \pm 1.347$
HSA	7.4	n_1	-7.995 ± 0.008	-13.040 ± 0.178	-16.931 ± 0.627
HSA	7.4	n_2	-7.097 ± 0.030	-7.625 ± 0.270	-1.772 ± 1.002

4. Conclusions

PA1 has a strong interaction both with HSA as well as with BSA, which is reflected by the affinity constants of the order of 10^5. There are two types of binding sites in the albumin for PA1. While binding is spontaneous, discarding electrostatic forces, it is more probable that it occurs through hydrophobic interactions and hydrogen bonding. Binding with HSA is stronger than with BSA.

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