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Binding of ring-substituted indole-3-acetic acids to human serum albumin

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Abstract—The plant hormone, indole-3-acetic acid (IAA), and its ring-substituted derivatives have recently attracted attention as promising pro-drugs in cancer therapy. Here we present relative binding constants to human serum albumin for IAA and 34 of its derivatives, as obtained using the immobilized protein bound to a support suitable for high-performance liquid chromatography. We also report their octanol-water partition coefficients ($\log K_{ow}$) computed from retention data on a C_{18} coated silica gel column. A four-parameter QSPR (quantitative structure–property relationships) model, based on physico-chemical properties, is put forward, which accounts for more than 96% of the variations in the binding affinities of these compounds. The model confirms the importance of lipophilicity as a global parameter governing interaction with serum albumin, but also assigns significant roles to parameters specifically related to the molecular topology of ring-substituted IAAs. Bulky substituents at ring-position 6 increase affinity, those at position 2 obstruct binding, while no steric effects were noted at other ring-positions. Electron-withdrawing substituents at position 5 enhance binding, but have no obvious effect at other ring positions.

1. Introduction

Human serum albumin[1] (HSA), the most abundant protein in blood plasma, reversibly binds many endogenous and exogenous compounds, including a large number of drugs. The protein is composed of three homologous domains (I–III) each of which is divided into two subdomains (A and B).[2,3] Two important drug-binding sites, also known as the warfarin (Sudlow's site I) and the indole-benzodiazepine binding sites (Sudlow's site II), are located in subdomains IIA and IIIA, respectively.[4,5] These binding sites are elongated hydrophobic pockets with cationic amino acid residues near their entrances. Dicarboxylic acids and bulky heterocyclic compounds with a negative charge bind mostly to Sudlow's site I, while small aromatic carboxylic acids, such as indole-3-acetic acid (IAA), bind preferably to Sudlow's site II.

The binding affinities of drugs to HSA often strongly affect their absorption, distribution, metabolism, and excretion, and thus their therapeutic effect. Therefore, testing the binding affinities of new drug candidates to serum albumin has become an important early screening step in the drug discovery process.[6]

Indole-3-acetic acid is mostly known as one of the 'auxins' which, in cooperation with other plant hormones, coordinate plant growth and development.[7] Recently, IAA and its ring-substituted derivatives[8] also attracted attention as experimental prodrugs for use in cancer therapy as it was observed that oxidants, such as horseradish peroxidase, convert these compounds to products toxic to human tumor cells.[9,10]

In spite of long-term efforts, the mechanism of auxin action is still not fully understood. A number of plant proteins are able to bind auxins,[11] but research interest has so far been focused on the 'auxin-binding protein 1' (ABP1)[11] and the 'transport inhibitor response 1' protein (TIR1),[12,13] which have been proposed to function as auxin receptors.[14] Exploring the molecular interaction of indole-3-acetic acids with human serum albumin is thus of interest not only for better understanding of auxin pharmacodynamics in humans, but also because it provides preliminary insight into the general structural features of auxin-binding sites in plant proteins, which

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have so far been difficult to access. It is worth noting, in the present context, that bovine serum albumin shares a sequence of 18 amino acids with an auxin-binding protein from rice seedlings (regulator of plasma membrane ATPase).[15]

Here we report the relative binding constants to serum albumin and the octanol-water partition coefficients for 35 indole-3-acetic acids, as obtained by high-performance liquid chromatography. We also put forward a QSPR (quantitative structure–property relationship) model, which provides insight into the physico-chemical factors affecting the affinity of these compounds to the protein.

2. Results and discussion

2.1. Determination of $\log K_{ow}$ values by reversed phase high-performance liquid chromatography

Lipophilicity plays an important role in ligand–receptor interactions and is usually expressed using the logarithm of the partition coefficient of the respective solute in the octanol-water system ($\log K_{ow}$). The determination of $\log K_{ow}$ by the standard shake-flask method is time consuming and technically demanding. An alternative, which we used in the present work, is estimation of the partition coefficient by reversed phase high-performance liquid chromatography (RP-HPLC).[16] This technique is simpler and more rapid than the shake-flask method, but is applicable primarily to congeneric compounds with similar hydrogen-bonding properties. Recently, Yamagami et al.[17] reported that an eluent containing approximately 50% methanol minimizes the differences in the hydrogen-bonding capabilities of weak hydrogen acceptors and non-hydrogen bonders, thus permitting simultaneous chromatographic determination of their $\log K_{ow}$ values. As most of the indole-3-acetic acids (Fig. 1) listed in Table 1 contain substituents which are either non-hydrogen-bonders (i.e., alkyls, halogens) or weak hydrogen acceptors (i.e., the methoxy group), we adopted 50% methanol as the eluent, adding 0.1 mol/L formic acid to keep the solutes in their undissociated forms. A set of seven indolic compounds, for which the $\log K_{ow}$ values are known, were used to construct a calibration curve: the published[28] $\log K_{ow}$ values of these reference compounds were regressed against the logarithms of their isocratic retention factors, $\log k_{C18}$. Then the retention factors of the tested indole-3-acetic acids were determined and the corresponding $\log K_{ow}$ values were obtained from the calibration curve.

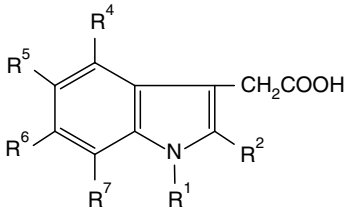

Figure 1. General structure of IAA derivatives.

Because of different hydrogen-bonding properties the $\log K_{ow}$ values obtained for 7-aza-IAA, IAA ethyl ester, 5-OH-IAA, and *N*-methyl-IAA are not expected to be comparable with those obtained by the shake-flask method. Therefore, in the subsequent structure–property analysis we used their calculated $\log K_{ow}$ values (see Table 1), instead of the experimental data.

To check the quality of the obtained $\log K_{ow}$ values we compared them with those for nine mono- and dichloro derivatives of IAA, previously determined by the shake-flask method.[18] Although the published values were determined at pH 7.4, at which indole-3-acetic acids are mostly in their ionized form, they should be correlated with our results, (determined at an estimated pH of 2.4), because all compounds involved had similar dissociation constants. [There appear to be no published data for pK_a values of ring substituted indole-3-acetic acids, but, as their carboxyl group is separated from the indole ring by a methylene group, it is reasonable to assume that they do not differ considerably from the pK_a of unsubstituted IAA ($pK_a = 4.54$)[19]]. Indeed, high correlation was found between our results and the $\log K_{ow}$ values obtained[18] by the shake-flask method ($r^2 = 0.97$).

2.2. Quantitative structure–property relationships

The relative binding constants ($\log k_{HSA}$) for IAA and its 34 derivatives to human serum albumin, determined by HPLC using a column of the immobilized protein, are listed in Table 1, together with the physico-chemical parameters which appear in the 'best' regression model (Eq. 3). A total of 14 descriptor variables were screened while developing the model: $\log K_{ow}$ values, Hammett's electronic sigma constants (σ_m and σ_p) for substituent positions 4–7, and Taft's steric parameters (E_s) for substituent positions 2–7. Compounds **1**–**30** in Table 1 were used in developing the model and compounds **31**–**35** were saved for its external validation. Regression analysis revealed that the HSA-binding affinities of indole-3-acetic acids are influenced by their lipophilicities ($\log K_{ow}$):

$$\log k_{HSA} = -0.558\ (\pm 0.284) + 0.472\ (\pm 0.117) \times \log K_{ow}$$
$$n = 30, \quad r^2 = 0.709, \quad s = 0.221,$$
$$F^{1,28} = 68.2, \quad q^2 = 0.670, \quad \text{PRESS} = 1.543. \qquad (1)$$

The proposed mathematical model is in line with the results of a recently reported crystallographic study[3] on ligand binding to human serum albumin, which shows that the indole ring of indoxyl sulfate is placed in the center of the largely hydrophobic cavity of Sudlow's site II. It should be pointed out that $\log K_{ow}$ also appears as a key parameter in some previously published QSPR models dealing with ligand-serum albumin binding interactions.[20–24] In general, more lipophilic indole-3-acetic acids bind more strongly to human serum albumin. However, analysis of the residuals showed that the binding strength for 2-alkyl-indole-3-acetic and 6-substituted indole-3-acetic acids is

Table 1. Partition coefficients, Taft's steric parameter, and Hammett's electronic constants plus observed and calculated relative binding constants for IAA and 34 of its ring-substituted derivatives on a column of immobilized human serum albumin

No.	Compound	$\log K_{ow}$	E_s (R^2)	E_s (R^6)	σ_m (R^5)	$\log k_{HSA}$		
						Exp.	Eq. 2	Eq. 3
1	IAA	1.42	0.00	0.00	0.00	0.102	0.132	0.102
2	2-Me	1.72	−1.24	0.00	0.00	−0.040	−0.082	−0.081
3	2-Et	2.09	−1.31	0.00	0.00	0.031	0.051	0.050
4	2-Pr	2.56	−1.60	0.00	0.00	0.150	0.166	0.166
5	4-Me	1.84	0.00	0.00	0.00	0.155	0.304	0.269
6	4-Et	2.33	0.00	0.00	0.00	0.393	0.506	0.464
7	4-F	1.66	0.00	0.00	0.00	0.305	0.230	0.198
8	4-Cl	2.13	0.00	0.00	0.00	0.458	0.423	0.385
9	5-Me	1.99	0.00	0.00	−0.07	0.217	0.366	0.298
10	5-Et	2.54	0.00	0.00	−0.07	0.490	0.592	0.517
11	5-Pr	3.21	0.00	0.00	−0.06	0.809	0.867	0.788
12	5-Bu	3.89	0.00	0.00	−0.08	1.050	1.147	1.050
13	5-F	1.72	0.00	0.00	0.34	0.261	0.255	0.374
14	5-Cl	2.38	0.00	0.00	0.37	0.646	0.526	0.650
15	5-Br	2.57	0.00	0.00	0.39	0.808	0.604	0.735
16	5-OMe	1.23	0.00	0.00	0.12	0.185	0.053	0.080
17	5-OH	0.74[a]	0.00	0.00	0.12	−0.125	−0.148	−0.115
18	6-Me	1.96	0.00	−1.24	0.00	0.689	0.748	0.738
19	6-Et	2.56	0.00	−1.31	0.00	1.044	1.017	1.000
20	6-F	1.74	0.00	−0.46	0.00	0.308	0.409	0.386
21	6-Cl	2.41	0.00	−0.97	0.00	0.863	0.847	0.825
22	7-Me	1.95	0.00	0.00	0.00	0.288	0.349	0.313
23	7-F	1.81	0.00	0.00	0.00	0.247	0.292	0.257
24	7-Cl	2.32	0.00	0.00	0.00	0.623	0.501	0.461
25	4,5-Cl_2	2.92	0.00	0.00	0.37	0.799	0.748	0.865
26	4,6-Cl_2	3.29	0.00	−0.97	0.00	1.082	1.209	1.176
27	4,7-Cl_2	3.02	0.00	0.00	0.00	0.840	0.789	0.739
28	5,6-Cl_2	3.20	0.00	−0.97	0.37	1.436	1.172	1.305
29	5,7-Cl_2	3.42	0.00	0.00	0.37	0.983	0.954	1.064
30	6,7-Cl_2	3.16	0.00	−0.97	0.00	1.090	1.155	1.124
31	5-OBz	3.20	0.00	0.00	n.r.[b]	1.186	0.863	—
32	2-Me, 5-OMe	2.14	−1.24	0.00	0.12	0.228	0.091	0.140
33	Et ester	2.34[a]	0.00	0.00	0.00	0.292	0.510	0.468
34	1-Me	1.87[a]	0.00	0.00	0.00	0.243	0.316	0.281
35	7-aza	0.45[a]	0.00	0.00	0.00	−0.314	−0.267	−0.284

[a] In the QSPR calculations, for reasons explained in the text, the $\log K_{ow}$ values experimentally determined for these compounds [i.e., 0.00 (**17**), 2.74 (**33**), 2.19 (**34**), and −0.84 (**35**)] were replaced by values calculated using a web utility.[32]
[b] Not reported.

additionally influenced by other factors: the predicted $\log k_{HSA}$ values for 2-alkylindole-3-acetic acids were significantly larger and those for 6-substituted indole-3-acetic acids were markedly smaller than those found by experiment. Regression analysis (Eq. 2) suggests that the reasons for these discrepancies are steric in nature:

$$\begin{aligned}\log k_{HSA} = &-0.452\ (\pm 0.140)\\ &+ 0.411(\pm 0.059)\ \log K_{ow}\\ &+ 0.271(\pm 0.097) E_s\ (R^2)\\ &- 0.318\ (\pm 0.096)\ E_s\ (R^6) \qquad (2)\end{aligned}$$

$$n = 30, \quad r^2 = 0.937, \quad s = 0.107, \quad F^{3,26} = 128.6,$$

$$q^2 = 0.920, \quad \text{PRESS} = 0.373.$$

It appears that an alkyl group at ring-position 2 interferes with the accessibility of the carboxyl group and thus obstructs the proper orientation of the respective indole-3-acetic acid within the binding cavity. Interestingly, the plant-growth-promoting activities of 2-alkylindole-3-acetic acids are also reduced,[25] as compared to unsubstituted IAA, possibly for similar reasons. In contrast to the 2-alkyl derivatives, their 6-substituted analogs (with the exception of the fluoro derivative) are more strongly bound to the protein than the indole-3-acetic acids with the same substituents in positions 4, 5 or 7. This positive steric effect may be a consequence of bulky 6-substituents accessing a particular narrow cleft within the lipophilic cavity.

The statistical parameters of Eq. 2 show that the model not only well fits the relative binding constants of the compounds in the training set (only the binding constants for compound **28** are predicted beyond two standard deviations) but its predictive ability (internal validation) is also good. Further improvement in accuracy and predictive ability of the model was obtained by introduction of the electronic parameter σ_m for substituents in position 5. Although the three-parameter model accounts for more than 93% of the variations in the relative binding constants, considerable reduction of the standard deviation (about 25%) and PRESS

(approximately 40%) in Eq. 3 justifies this increase of model complexity.

$$\begin{aligned} log k_{HSA} = &-0.463\ (\pm 0.106) + 0.398\ (\pm 0.045) \log K_{ow} \\ &+ 0.244\ (\pm 0.074) E_s\ (R^2) \\ &- 0.339\ (\pm 0.073)\ E_s\ (R^6) \\ &+ 0.448\ (\pm 0.202)\ \sigma_m\ (R^5) \end{aligned} \quad (3)$$

$$n = 30, \quad r^2 = 0.966, \quad s = 0.080, \quad F^{4,25} = 175.6,$$

$$q^2 = 0.953, \quad \text{PRESS} = 0.222.$$

The positive coefficient of σ_m indicates that electron-withdrawing substituents enhance the binding affinity of indole-3-acetic acids, likely by reducing the electron density in the indole ring, making possible its interaction with electron-rich amino acid residues. The reason for not observing this effect at positions 4, 6, and 7 is most likely the low substituent variability at these positions.

A plot of the observed $\log k_{HSA}$ versus the $\log k_{HSA}$ calculated by Eq. 3 is given in Figure 2. The correlation matrix (Table 2) shows that the colinearity between predictor variables in Eq. 3 is negligible.

We also tested the probability of chance correlation for the derived model. According to Topliss et al.,[26] for 30 observations some 22 variables can be screened in developing a model (with $r^2 > 0.9$), keeping the risk of chance correlation below 1%. Taking into account that some of the examined variables are highly intercorrelated (the value of the correlation coefficient (r) between Hammett's constants, σ_m and σ_p, for substituents at positions 4, 6 or 7 exceeds 0.9) the probability of chance correlation for the derived model is well below 1%.

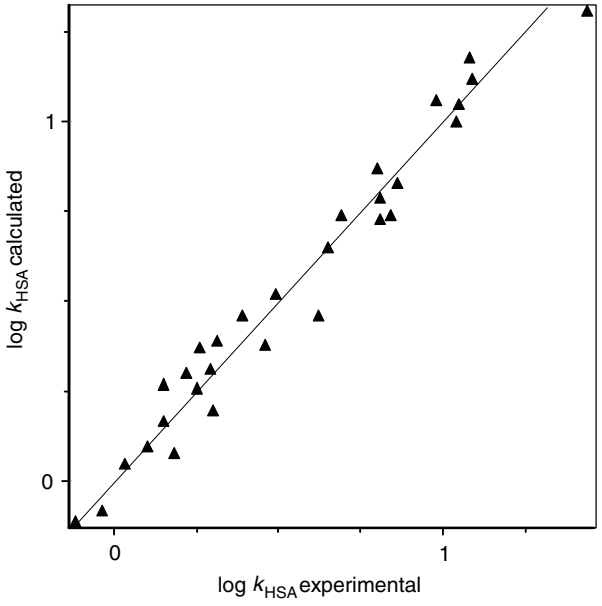

Figure 2. The observed versus calculated $\log k_{HSA}$ values for the studied IAA derivatives using Eq. 3.

Table 2. Correlation matrix (r) between the predictor variables in Eq. 3

	$\log K_{ow}$	E_s (R^2)	σ_m (R^5)	E_s (R^6)
$\log K_{ow}$	1.00			
E_s (R^2)	0.08	1.00		
σ_m (R^5)	0.11	0.16	1.00	
E_s (R^6)	0.25	0.18	0.07	1.00

The quality of the model was further checked by external validation using the validation set of five compounds (**31**–**35**). The high value of q^2 (0.917) obtained in this test confirms the robustness of the model. The relative binding constants of compounds **33** and **34** indicate that neither a free carboxyl group nor a free NH group is necessary for binding, since both the ethyl ester and the *N*-methyl derivative are bound to HSA even somewhat more strongly than the parent IAA. In the case of the ethyl ester, this could mean that the carbonyl oxygen can engage in hydrogen bonding[27] with surrounding amino acid residues. A methyl group attached to the indole ring nitrogen appears to enhance the affinity through interaction with the lipophilic environment of the binding site, in a similar fashion as a methyl group at another ring position.

In conclusion, the association constant for the interaction of human serum albumin with the compounds studied was estimated (data not shown) from the published[34] value for IAA ($K = 2.2 \times 10^5\ M^{-1}$) and the relative affinities (k) listed in Table 1, assuming proportionality of K and k (an assumption corroborated by a large number of published examples), and found to cover the same range (approximately 5×10^4–$5 \times 10^6\ M^{-1}$) as common drugs[34] known to bind to Sudlow's site II, such as diazepam ($3.8 \times 10^5\ M^{-1}$) and diclofenac ($3.3 \times 10^6\ M^{-1}$). From this point of view, the compounds studied here would thus appear to deserve consideration for drug use in humans, and more detailed pharmacological and pharmacokinetic studies could be promising.

3. Conclusion

The relative binding constants to human serum albumin and the octanol-water partition coefficients for 35 indole-3-acetic acids were determined by high-performance liquid chromatography. QSPR analysis confirmed that the interactions of indole-3-acetic acids with the hydrophobic amino acid residues, lining the indole-benzodiazepine binding site (Sudlow's site II), contribute significantly to their binding affinities. Substituents of increasing size lowered affinity when attached to C-2, but enhanced it when bound to C-6. In contrast, no steric effect was observed at C-5, even though the most bulky substituents tested were attached to that site. Electron-withdrawing groups at the 5-position did, however, enhance the affinities of the respective compounds to human serum albumin. These data indicate that the compounds examined assume a defined orientation in the indole-binding site and provide first leads for targeted structural modifications aimed at improving pharmacological properties.

4. Experimental

4.1. Chemicals

All chemicals were of analytical reagent grade or better. Redistilled organic solvents and deionized water were used in preparing the mobile phases. Because of their low water-solubility, the indolic compounds were first dissolved in a small volume of 2-propanol and then diluted by the eluent, to an approximate concentration of 0.1 mg/mL. Before use, all eluents were filtered through a 45 μm polyamide membrane filter (Schleicher & Schüll, D-37582 Dassel, Germany).

4.2. HPLC instrument

High-performance liquid chromatography was performed using a system (Knauer, Berlin, Germany) composed of two WellChrom K-501 pumps, a WellChrom K-5004 solvent degasser, a K-6 Electric Valve Drive injection port, a Jet-Stream Plus Peltier column thermostat, and a UV–VIS WellChrom K-2800 diode array detector. The system was controlled by ChromGate software (version 2.8 Build 861).

4.3. Determination of $\log K_{ow}$ by RP-HPLC

The following reference compounds were used in the determinations of $\log K_{ow}$ values: indole (2.14), 5-methylindole (2.68), 5-methoxyindole (2.06), 5-chloroindole (3.25), IAA (1.41), indole-3-propionic acid (1.75), and indole-3-butyric acid (2.30). The figures in parentheses are the $\log K_{ow}$ values determined in octanol-water.[28]

The standards and samples were chromatographed isocratically on a column (250 × 4.6 mm id) filled with a C_{18} stationary phase, particle size 5 μm (Nucleosil, Macherey und Nagel GmbH & Co. KG, Düren, Germany), equipped with a guard column (20 × 2.0 mm id) packed with 'porous' C_{18}-coated silica gel, particle size 35–70 μm (Alltech Associates Inc., Deerfield, Illinois, 60015, USA). The mobile phase was a 1:1 (v/v) mixture of A, 0.1 M aqueous HCOOH containing, per liter, 50 ml CH_3OH and B, CH_3OH. The flow rate was 0.9 mL/min. The analyses were carried out at 21 °C. The retention time (t_r) was the average of at least two measurements; experimental errors were less than 1%. The dead time (t_0) was determined from the retention time of KNO_3, which is not retained by the column.

The chromatographic retention of the solutes was expressed as the retention factor (k_{C18})

$$k_{C18} = (t_r - t_0)/t_0 \quad (4)$$

The $\log K_{ow}$ values of the examined indole-3-acetic acids were calculated from the regression equation,

$$\log K_{ow} = 1.316 + 2.228 \log k_{C18} \quad (5)$$

$$n = 7, \quad r^2 = 0.977, \quad s = 0.099.$$

obtained by regression of the published[28] $\log K_{ow}$ values of the reference compounds listed above against the corresponding experimental values of $\log k_{C18}$.

4.4. Determination of relative binding constants ($\log k_{HSA}$)

Chromatography was performed on a column of immobilized HSA (100 × 4 mm id; ChromTech, Cheshire, UK) connected to a guard column (10 × 4 mm id) from the same manufacturer, eluted with a mixture of 25 mM phosphate buffer (pH 7.0) and 2-propanol (75/25 v/v) at a flow rate of 0.9 mL/min and a column temperature of 5 °C (To reduce retention times and to improve the peak shapes of some strongly bound compounds we used a higher concentration of the organic modifier (2-propanol) than recommended by the supplier. It should, however, be pointed out that Valko et al.[29] applied even higher concentrations of 2-propanol in eluting indolocarbazole derivatives, without noticing any irreversible change in the binding properties of serum albumin). Determination of the dead time of the column and calculation of the retention factors (k_{HSA}) of the analyzed compounds were performed as in Section 4.3.

The retention times were the averages of at least two measurements. The average error for retention times was about 4%. This relatively high error was likely a consequence of progressive aging of the column.[30] The difference in retention times for two consecutive measurements never exceeded 1%.

4.5. Data analysis

The steric (Taft's E_s) and electronic (Hammett's σ) constants used in this study were taken from Refs. 28 and 31. $\log K_{ow}$ values for compounds **17**, **33**, **34**, and **35** (see Table 1) were calculated by ALOGPS 2.1 software.[32]

Multiple linear regression analysis was carried out by the commercial software MobyDigs.[33] In all regression equations n is the number of compounds used in the analysis, r^2 is the squared correlation coefficient, s is the standard deviation of the estimates, and F is the ratio of the regression and the residual variances. The degrees of freedom $(k, n-k-1)$ associated with F are specified in the superscript, where k is the number of independent variables in the equation. PRESS is the sum of squared prediction errors from the leave-one-out cross-validation analysis and q^2 is the cross-validated coefficient of determination.

Acknowledgments

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