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Characterization of an Important Drug Binding Area on Human Serum Albumin Including the High-Affinity Binding Sites of Warfarin and Azapropazone

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SUMMARY

This paper reports a variety of experimental observations which strongly support the assumption that the warfarin binding site, or site I of human serum albumin, is better described as the warfarin-azapropazone binding area, consisting of the overlapping binding sites for warfarin and azapropazone. In general, drugs interacting with one of the two sites will also displace drugs bound to the other site, although their displacing potencies for both sites may vary considerably. This is most pronounced in the case of glibenclamide, which strongly inhibits the binding of drugs to the azapropazone site with only minor effects on drugs bound to the warfarin site. The lone tryptophan residue of human serum albumin, previously shown to be part of the warfarin binding site, is obviously located in the not-overlapping part of the warfarin site, so that its modification affects only the binding of drugs to the warfarin and not to the azapropazone site of this large binding area. The observation of different but overlapping binding sites might explain the fact that the albumin binding of drugs which seem to be bound to similar sites because of their mutual displacement can be affected differently during several disease states.

INTRODUCTION

The interaction of drugs and other small organic molecules with HSA[5] has been intensively studied for different reasons. First, HSA is the most important carrier for drugs and endogenous substances in blood. Displacement reactions on the HSA surface seem to be responsible for at least some specific drug interactions observed *in vivo* (1–4). Several diseases of liver and kidneys can change the albumin binding of drugs, probably by means of a still-unknown endogenous inhibitor or by changing the secondary structure of the protein. As a consequence, distribution and elimination of such drugs can be seriously altered (5–9). Second, the albumin molecule represents a homogeneous, well-defined, and extensively studied protein sometimes with receptor-like properties (10, 11). Therefore, it is an excellent model with which to study molecular aspects of ligand-protein interactions.

During the last years several attempts have been made to systematize various observations made about the interaction of different drugs with HSA (12–14). Today, it is generally accepted that only a few specific binding sites exist on the HSA molecule, each of which binds drugs with quite different chemical structures. On the other hand, some of these binding sites have a fairly pronounced substrate specificity as indicated by their stereospecificity, and it is possible to change their properties dramatically by the chemical modification of only one amino acid residue of HSA (10, 14). The most important of these is the indole and benzodiazepine binding site and the warfarin binding site. Although the indole and benzodiazepine binding site is well characterized with regard to specific ligands (12–17), location within the primary structure of HSA, and amino acid residues involved (14, 18–22), much less is known about the warfarin binding site (14). Moreover, contradictory results exist regarding the participation of this site in the high-affinity binding of some drugs and regarding the function and location of the lone tryptophan residue of HSA within this site (14, 23–28). The aim of the present study was to clarify some of these conflicting data and to develop a model of this site which will explain how various drugs and other small molecules interact within this important ligand binding site of HSA. The data will show that this binding site is probably larger than originally thought.

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[5] The abbreviations used are: HSA, human serum albumin; HNB, 2-hydroxy-5-nitrobenzyl; NPS, *o*-nitrophenylsulfenyl; TNM, tetranitromethane.

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MATERIALS AND METHODS

Materials. HSA was obtained from Behringwerke, Marburg (electrophoretic purity 100%). Most of the radioactively labeled drugs were generous gifts of the manufacturers: [^{14}C]diazepam from Hoffmann-La Roche (Grenzach, Federal Republic of Germany); [^{14}C]azapropazone from Siegfried AG (Zofingen, Switzerland); [^{14}C]glibenclamide from Boehringer (Mannheim, Federal Republic of Germany); [^{14}C]phenylbutazone from Geigy (Wehr, Federal Republic of Germany). [^{14}C]Warfarin was obtained from Amersham Buchler (Braunschweig, Federal Republic of Germany). The radiochemical purity of the drugs used was higher than 99% as determined by thin-layer chromatography. All other drugs were obtained from the manufacturers or from commercial suppliers.

Preparation of HSA derivatives. Modification of the lone tryptophan residue of HSA by HNB bromide or NPS chloride has been reported in detail elsewhere (24, 29). HNB-HSA was prepared in 10 M urea (pH 4.4) at an 1100-fold molar excess of HNB. Another sample of HSA was treated the same way but without added reagent and was used as control (urea-HSA). NPS-HSA was prepared with a 22-fold molar excess of NPS in 20% acetic acid. The nitration of increasing amounts of tyrosine residues in HSA by TNM was described in detail earlier (20). Molar excesses (1.8-, 4-, and 15-fold) of TNM in 0.05 M Tris buffer (pH 8.0) were used to obtain derivatives with 0.9, 2.2, and 5.1 tyrosine residues modified per mole of HSA, termed TNM-HSA I, II, and III, respectively. After the modification, all HSA derivatives were purified by dialysis against water, lyophylized, and stored at 0°. The degree of tryptophan and tyrosine modification was determined spectrophotometrically and by amino acid analysis (24).

Equilibrium dialysis experiments. The binding of [^{14}C]azapropazone, [^{14}C]diazepam, [^{14}C]warfarin, [^{14}C]phenylbutazone, and [^{14}C]glibenclamide to HSA or to the modified HSA derivatives was determined using an HSA concentration of 36.2 μM and varying concentrations of the drugs. All solutions were prepared in 0.067 M phosphate buffer (pH 7.4). Aliquots (0.9 ml) of the HSA solutions were dialysed for 16 hr at 25° in the dark against 0.9 ml of buffer, using cellophane dialysis membranes (Union Carbide). No difference was observed whether the ^{14}C-labeled drugs were added to the protein or the buffer containing sites of the 1-ml dialysis cells.

RESULTS

The nonsteroidal antiinflammatory drug azapropazone is bound to HSA via one high-affinity binding site and two sites of considerably lower affinity (30, 31). Neither the modification of the highly reactive tyrosine residue of HSA (21) by TNM nor the modification of the lone tryptophan residue by HNB or NPS has a distinct effect on the interaction of azapropazone with its high-affinity binding site (Fig. 1), possibly indicating that this site is not identical with the indole and benzodiazepine binding site (14, 20) or the warfarin binding site (14, 24). Accordingly, the presence of 1 mole of diazepam per mole of HSA has almost no influence on the high-affinity binding of azapropazone, although the binding to secondary binding sites might be reduced as derived from the shape of the Scatchard plot (Fig. 1). In contrast, the presence of 1 mole of warfarin per mole of HSA is followed by a clear reduction of the binding of azapropazone to its high-affinity binding site (Fig. 1).

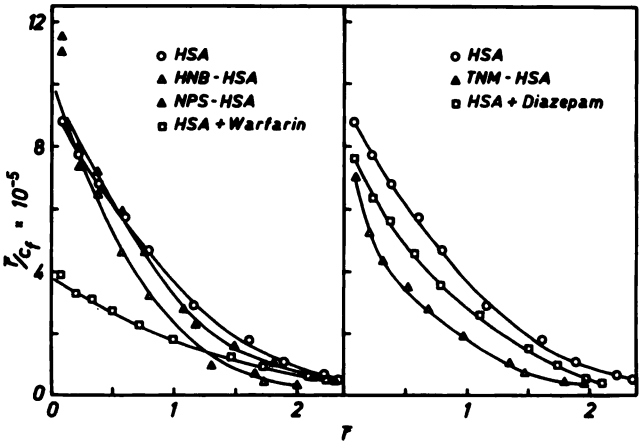

FIG. 1. *Scatchard plot of the binding of azapropazone to HSA, HNB-HSA, NPS-HSA, and TNM-HSA I (left) and of the binding of azapropazone to HSA in the presence of 1.1 mole of warfarin or diazepam per mole of HSA (right)*

Phenylbutazone, highly albumin-bound like azapropazone, is a potent inhibitor of the binding of warfarin to HSA (27, 28, 30, 32). Like azapropazone, phenylbutazone has only one high-affinity binding site on HSA and two sites of lower affinity (26, 28, 33) (Table 1). Also similar to azapropazone, the high-affinity binding site remains unaffected after modification of the tryptophan residue of HSA by NPS and HNB. The slightly reduced number of high-affinity binding sites found for HNB-HSA might be due to an uncomplete refolding of the protein during the preparation (29), since this observation was also made with urea-HSA (Table 1). The modification of up to five tyrosine residues of HSA has no influence on the high-affinity binding site of phenylbutazone (TNM-HSA II and III; Table 1).

Azapropazone and phenylbutazone have been repeatedly demonstrated to be potent inhibitors of the HSA binding of warfarin (3, 27, 28, 30–32). Furthermore, the presence of 1 mole of warfarin per mole of HSA increases the free fraction of azapropazone and phenylbutazone by 50% (Table 2). The mutual displacement of the three drugs at low drug to protein ratios indicates a competition

TABLE 1

Binding parameters of the interaction of phenylbutazone with HSA, Urea-HSA, HNB-HSA, NPS-HSA, and TNM-HSA II and III

The association constants k_1 and k_2 and the number of binding sites on the albumin molecule n_1 and n_2 were calculated for 21 equilibrium dialysis experiments according to Pennock (37).

Albumin	n_1	$10^{-4} \times k_1$	n_2	$10^{-4} \times k_2$
		M^{-1}		M^{-1}
HSA	1.02	82	2.00	2.2
Urea-HSA	0.92	84	1.98	1.9
HNB-HSA	0.77	79	1.98	1.8
NPS-HSA	1.01	80	1.96	4.6
TNM-HSA II	0.98	70	2.12	2.2
TNM-HSA III	0.99	68	1.98	2.2

TABLE 2

Free fraction of azapropazone and phenylbutazone alone and in the presence of 1 mole of warfarin per mole of HSA when bound to HSA and NPS-HSA

The HSA concentration was 36.2 μM, the concentration of the drugs 39.8 μM. Each value is the mean ± standard error of the mean of seven determinations.

Ligand	Free fraction of ligand		Increase in free fraction by warfarin
	Without warfarin	In presence of warfarin	
	%	%	%
Azapropazone			
HSA	9.64 ± 0.11	14.39 ± 0.09	49.3 ± 0.7
NPS-HSA	8.91 ± 0.17	12.19 ± 0.22	36.8 ± 2.4[a]
Phenylbutazone			
HSA	11.29 ± 0.17	17.25 ± 0.19	52.8 ± 1.6
NPS-HSA	8.35 ± 0.14	11.58 ± 0.25	38.7 ± 1.7[a]

[a] The increase is significantly ($p < 0.001$) lower for NPS-HSA compared with native HSA.

for the same high-affinity binding site, usually called warfarin binding site (11, 13) or site I (12). However, the lack of any effect of the tryptophan modification on the binding of azapropazone and phenylbutazone strongly opposes this conclusion, since the lone tryptophan residue of HSA has been reported to be an important part of the warfarin binding site (24). To integrate both conflicting observations the following hypothesis was worked out: There exist both an azapropazone and a warfarin binding site on the HSA molecule which overlap each other, thus forming a common binding area. The lone tryptophan residue would be located on one side of this area within the warfarin region. In agreement with this hypothesis is the observation of the significantly reduced potency of warfarin as an inhibitor of the binding of azapropazone and phenylbutazone after the selective modification of the tryptophan residue of HSA by NPS (Table 2) and by HNB (data not shown).

In order to characterize both regions of this binding area we used azapropazone and warfarin as selective marker ligands and studied their displacement by a number of drugs highly bound to HSA. In order to demonstrate the specificity of the binding to this area we compared these data with the displacing potency of the same drugs for diazepam, a drug which specifically labels a clearly different binding site of HSA (10–14).

The drugs investigated could be divided into three groups (Table 3): (a) drugs which displace diazepam and at a drug to albumin ratio of 1 but have almost no effect on the binding of azapropazone and warfarin (e.g., iopa-

TABLE 3

Displacement of azapropazone, diazepam, and warfarin by other drugs

The HSA concentration was 36.2 μM, the concentration of the marker ligands was 39.8 μM, and the concentrations of the displacing drugs were 39.8 and 119.4 μM, respectively. Under these conditions, 91.92 ± 0.14% azapropazone, 85.83 ± 0.20% diazepam, and 85.89 ± 0.14% warfarin were bound. Displacement is expressed as percentage decrease of the fraction bound and represents the mean of three to four experiments. A dash (—) indicates that the displacement is less than 1%. The last column represents the ratio between the displacement of warfarin and azapropazone at a drug to HSA ratio of 1.

Displacing drug	Azapropazone (D/P)		Diazepam (D/P)		Warfarin (D/P)		Warfarin/azapropazone (D/P), 1.1
	1.1	3.3	1.1	3.3	1.1	3.3	
Azapropazone	10.3	29.5	—	1.3	13.5	32.2	1.31
Diazepam	1.1	1.4	22.8	42.5	—	—	
Warfarin	5.8	10.4	—	—	11.7	29.4	2.02
Phenylbutazone	9.9	19.5	1.9	8.1	15.2	30.1	1.54
Oxyphenbutazone	6.4	15.4	—	2.5	10.9	24.7	1.70
Sulfinpyrazone	4.6	14.0	—	3.0	7.4	18.8	1.61
Acetylsalicylic acid	2.1	7.9	3.8	11.4	5.1	13.4	2.39
Ibuprofen	—	3.1	21.5	41.0	1.3	5.4	
Flurbiprofen	—	33.0	38.4	40.7	—	9.7	
Naproxen	1.8	5.9	47.9	67.6	3.3	17.3	1.82
Mefenamic acid	6.7	32.8	10.0	24.5	9.8	48.8	1.47
Indomethacin	1.3	4.2	6.6	21.8	2.9	11.4	2.23
Acenocoumarol	3.7	12.2	—	—	5.7	19.2	1.54
4-Hydroxycoumarin	3.1	9.7	—	—	5.4	14.6	1.73
Phenprocoumon	7.3	21.4	—	2.8	13.2	31.9	1.80
Iophenoxic acid	21.0	66.3	4.7	18.9	32.8	75.6	1.56
Iopanoic acid	2.1	23.2	23.6	35.1	3.9	33.1	1.87
Iodipamide	13.1	26.5	—	1.7	20.8	40.5	1.59
Cloxacillin	2.3	4.7	3.8	9.5	1.1	4.3	0.46
Flucloxacillin	1.6	3.7	8.9	18.3	—	2.6	
Sulfadimethoxin	6.8	22.1	—	2.9	10.4	31.6	1.54
Glibenclamide	6.0	20.1	—	7.6	1.9	12.0	0.32
Tolbutamide	3.5	12.3	1.3	6.6	5.8	15.6	1.63
Furosemide	4.4	12.6	—	2.5	7.2	20.4	1.66
Ethacrynic acid	3.5	16.5	14.3	59.9	2.2	15.2	0.62
Carbenoxolone	2.9	9.4	—	—	4.8	10.8	1.67
Sodium valproate	2.8	9.7	5.2	15.2	5.5	15.4	1.93
Clofibrinic acid	1.9	6.4	21.4	40.3	1.6	6.4	0.84
Dansylsarcosine	3.5	9.6	18.7	41.2	1.1	5.5	0.33

noic acid, naproxen, flurbiprofen, clofibrinic acid, and flucloxacillin); (b) drugs which displace the three marker ligands to a more or less similar degree (e.g., sodium valproinate, mefenamic acid, acetylsalicylic acid, and ethacrynic acid); and (c) drugs which at a molar drug to albumin ratio of 1 displace azapropazone and warfarin without affecting the binding of diazepam (e.g., iophenoxic acid, iodipamide, phenprocoumon, oxyphenbutazone, furosemide, and sulfadimethoxine.

Until now, we were unable to find drugs which reduce selectively the binding of either azapropazone or of warfarin (Table 3). However, the ratio between the observed displacing potencies for both marker ligands varies considerably (Table 3). Obviously, a certain degree of specificity exists for some of these drugs, either for the azapropazone or the warfarin region. For example, although acetylsalicylic acid and sodium valproinate displace warfarin more effectively than azapropazone, glibenclamide is nearly 3 times more potent as displacer of azapropazone than of warfarin (Table 3).

Because of this pronounced selectivity for the azapropazone region, the binding of glibenclamide to HSA was further investigated. Glibenclamide binds to the HSA molecule via sites of different affinity (Fig. 2). Compared with unmodified HSA, the binding characteristics of glibenclamide remain unchanged for NPS-HSA (Fig. 2) and for HNB-HSA (data not shown). The lack of any effect of the tryptophan modification is consistent with the preferential binding of glibenclamide to the azapropazone region of the hypothetical binding area, since this region does not include the tryptophan residue. Also in agreement with this model is the weak displacement of glibenclamide by warfarin and the marked displacement by azapropazone (Table 4). However, the strong displacement of glibenclamide by iodipamide might be contradictory to this model, since iodipamide binds preferentially to the warfarin region (34). Iodipamide represents a relatively large molecule consisting of two triiodobenzoic acid moieties connected by an aliphatic bridge. Thus, iodipamide might bind overlappingly to both regions of this binding area. Glibenclamide binds only weakly to the indole and benzodiazepine binding site, since it does not inhibit the binding of diazepam (Tables 3 and 4) and its binding is not affected by modification of the highly reactive tyrosine residue of HSA (Fig. 2) which is strongly involved in this site (20–22).

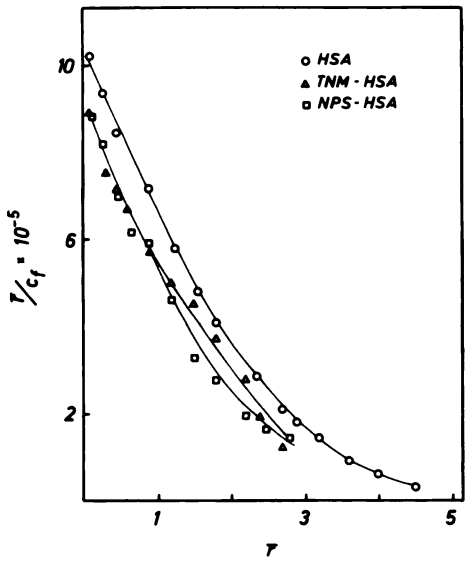

FIG. 2. *Scatchard plot of the binding of glibenclamide to HSA, TNM-HSA I, and NPS-HSA*

TABLE 4

Displacement of glibenclamide by different drugs

The HSA concentration was 36.2 μM, the concentration of glibenclamide 39.8 μM, and the concentrations displacing drugs were 39.8 and 119.4 μM, respectively. The values given represent the increase in the free fraction of glibenclamide in the presence of the displacing drugs compared with the free fraction of glibenclamide alone (3.33 ± 0.04%). Each value is the mean ± standard error of the mean of four to five experiments.

Displacing drug	Increase in free fraction of glibenclamide	
	D/P 1.1	D/P 3.3
	%	%
Glibenclamide	60 ± 5	270 ± 16
Azapropazone	39 ± 2	151 ± 2
Warfarin	4 ± 1	27 ± 3
Iodipamide	55 ± 6	190 ± 15
Diazepam	11 ± 2	26 ± 4

Bilirubin, the breakdown product of heme, is one of the most tightly HSA-bound substances known and interferes with the binding of many different drugs (35). In order to clarify the relationship between the high-affinity bilirubin binding site (36) and the azapropazone-warfarin binding area, we investigated the displacement of radioactively labeled marker ligands specific to this area by increasing concentrations of bilirubin (Fig. 3). As indicated by the shape of the displacement curves, the in-

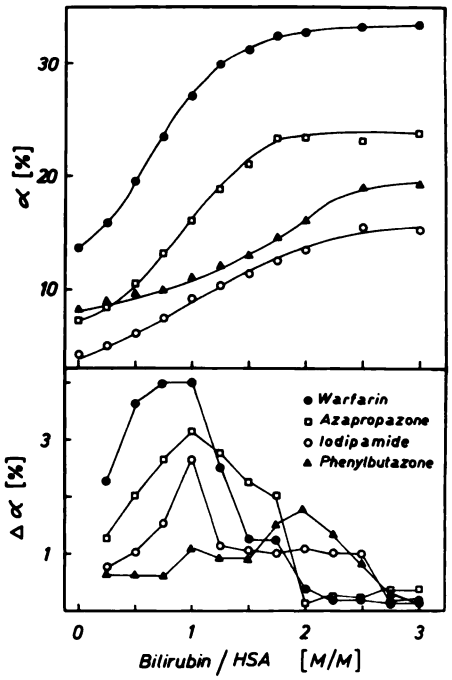

FIG. 3. *Displacement of azapropazone, warfarin, phenylbutazone, and iodipamide by increasing amounts of bilirubin*

The *upper part* gives the free fractions × 100 (2) of the four ligands at different bilirubin to HSA ratios. The *lower part* shows the increase in the free fraction × 100 per 0.25 mole of bilirubin per mole of HSA.

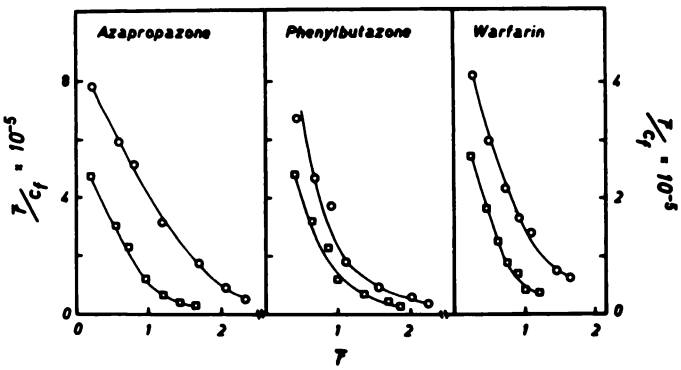

FIG. 4. *Scatchard plot of the binding of azapropazone, phenylbutazone, and warfarin to HSA (○) and to HSA in the presence of 1 mole of bilirubin per mole of HSA (□)*

crease in the free fractions of azapropazone, phenylbutazone, warfarin, and iodipamide is not linearly dependent on the bilirubin concentration as, for example, observed for the mutual interaction between warfarin, azapropazone, and phenylbutazone (30), but shows a sigmoid profile (Fig. 3, *upper*). Maximal displacement of all four ligands is reached at a bilirubin to HSA ratio of 2, whereas the maximal increase in displacement was observed at ratios between 1 and 2 (Fig. 3, *lower*) and not for bilirubin to HSA ratios between 0 and 1 as one has to assume if the primary bilirubin binding site is involved.

Scatchard presentations of the binding of azapropazone, phenylbutazone and warfarin to HSA in the presence of 1 mole of bilirubin are given in Fig. 4. The addition of 1 mole of bilirubin per mole of HSA shifts the Scatchard curves to the left without affecting their slopes, indicating unchanged binding constants—at least for the high-affinity sites. This observation sharply contrasts to the Scatchard plot of azapropazone in the presence of warfarin, where the high-affinity binding site of azapropazone was no longer demonstrable (Fig. 1). The graphic resolution of the Scatchard plots in Fig. 4 into single components according to Pennock (37) clearly indicated that the binding parameters for the primary binding site of all three ligands remain nearly unchanged in the presence of 1 mole of bilirubin per mole of HSA. However, there was a distinct effect on the secondary binding site (Fig. 4). Both findings together (Figs. 3 and 4) might suggest that the primary bilirubin binding site is not located within the azapropazone-warfarin binding area.

DISCUSSION

In agreement with the results of Sudlow *et al.* (12) and Sjöholm *et al.* (13), it is possible to demonstrate two clearly separate binding sites on the HSA molecule which can bind different drugs independently. The indole and benzodiazepine binding site can be labeled by specific marker ligands such as diazepam (13, 14), L-tryptophan (17), dansylsarcosine (12), iopanoic acid (34), and flurbiprofen (11, 31). Several amino acid residues of the HSA primary structure are involved in this specific drug binding site of HSA (14), as indicated in the scheme (Fig. 5). For example, the modification of the highly reactive tyrosine residue of HSA reduces the binding of several ligands specifically bound to this site (11, 20, 22).

Although the indole and benzodiazepine binding site of HSA is relatively well characterized, much less is known about the second specific drug binding site of HSA, termed site I (12) or warfarin binding site (11, 13). Moreover, contradictory data with respect to the binding of azapropazone to this site have been published (30, 31), since azapropazone binding to HSA is strongly inhibited by low concentrations of warfarin, but azapropazone binding to HSA is not altered by the modification of the lone tryptophan residue of HSA (30) which clearly participates in the warfarin binding site (24). These findings could be confirmed in the present study by equilibrium dialysis experiments. In addition, a quite similar binding pattern was found for the related drug phenylbutazone, whose high-affinity binding site is also not affected by tryptophan modification, although phenylbutazone is the "classical" displacer of HSA-bound warfarin (27, 28, 32).

Both conflicting observations can be explained by assuming an azapropazone-warfarin binding area as indi-

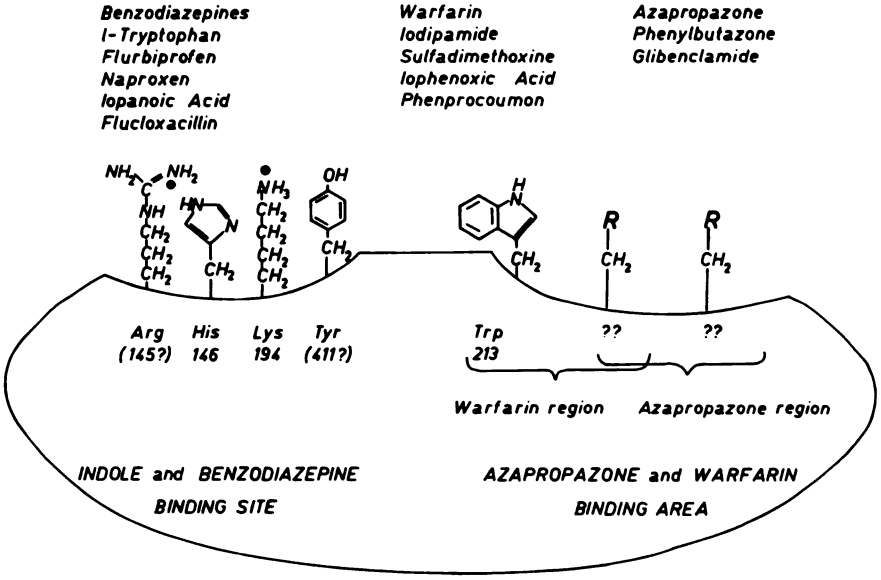

FIG. 5. *Model of the two most important drug binding sites on HSA, including typical ligands and the amino acid residues involved*

cated in Fig. 5. All of our consequent observations and most of the data reported in the literature fit surprisingly well into this model of the warfarin binding site. The basic assumption is that this area consists of the overlapping high-affinity binding sites of azapropazone and warfarin. Thus, when both drugs are added to the HSA molecule at the same time, a mutual displacement results. On the other hand, both sites are obviously not completely identical, since modification of the lone tryptophan residue reduces the binding of warfarin to this site (24) and similarly the binding of several other drugs specific for the warfarin region of this area (11, 14). However, modification of the tryptophan residue has no influence on the binding of azapropazone, phenylbutazone, and glibenclamide, although these drugs are potent displacers of warfarin and are displaced themselves by a number of drugs binding primarily to the warfarin region (3, 27, 28, 30–32). Thus, the lone tryptophan residue is obviously located within the non-overlapping part of the warfarin region (Fig. 5).

Some of the drugs investigated bind preferentially but not exclusively to one of both sites of the binding area, since none of the drugs displaces specifically only warfarin or azapropazone. On the other hand, the binding area (especially the non-overlapping part) seems to be large enough that in few cases two ligands can be bound nearly simultaneously and independently (e.g., warfarin and glibenclamide in the present study).

Because of its serious implications for intoxication, especially in newborn children, the HSA binding of bilirubin has been extensively characterized. Bilirubin is bound to the HSA molecule via one high-affinity binding site and at least one site of lower affinity (36). The high-affinity binding site is independent of the indole and benzodiazepine binding site (19). By contrast, the relationship to the warfarin site is not so clear, although it has been suggested that the high-affinity bilirubin and warfarin sites might be identical (13). While the data of the present study clearly support inhibition by bilirubin of the binding of drugs specifically for the azapropazone-warfarin binding area without any detectable preference for both subregions, our results strongly suggest that the high-affinity bilirubin binding site is not identical with the azapropazone-warfarin binding area but that the observed displacement of drugs such as azapropazone, warfarin, phenylbutazone, and iodipamide takes place at the secondary binding sites.

The location of the primary bilirubin binding site and of the azapropazone-warfarin binding area within different parts of the HSA structure is supported by the observation that the modification of the lone tryptophan residue is without any effect on the primary binding site of bilirubin (14). In addition, amino acid residues, which are possibly involved in this bilirubin binding site (14), are located in parts of the HSA structure quite different from the lone tryptophan residue as part of the warfarin region of the binding area.

In conclusion, the data reported are consistent with the hypothesis that the warfarin binding site (11, 13), or site I (12), is better characterized as the azapropazone-warfarin binding area. The assumption of distinct but overlapping binding regions within this area can also explain why, during various disease states of liver and kidneys, a pronounced increase occurs in the free fractions of azapropazone and phenylbutazone in human plasma, whereas the plasma protein binding of phenprocoumon (specific for the warfarin region) (10) is much less affected (6). Thus, the data presented in this report are not only of interest as an example of the nature of ligand binding sites on proteins, but also contribute to a better understanding of the pharmacokinetically important question of how drugs interact with HSA.

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