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A combined spectroscopic and crystallographic approach to probing drug–human serum albumin interactions

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ABSTRACT

The displacement of probes that bind selectively to subdomains IIA or IIIA on human serum albumin (HSA) by competing compounds has been followed using fluorescence spectroscopy, and has therefore been used to assign a primary binding site for these compounds in the presence and absence of fatty acids. The crystal structures have also been solved for three compounds: a matched pair of carboxylic acids whose binding strength to HSA unexpectedly decreased as the lipophilicity increased; and a highly bound sulphonamide that appeared not to displace the probes in the displacement assay. The crystallography results support the findings from the fluorescence displacement assay. The results indicate that drug binding to subdomain IB might also be important location for certain compounds.

1. Introduction

It is generally accepted that only the free fraction of drug in plasma is available to elicit a pharmacological effect, hence the determination of drug binding to plasma protein is a key part of the drug discovery process.[1] Drug binding can influence the therapeutic, pharmacodynamic and toxicological action of the drug, as well as being key to understanding the pharmacokinetic profiles as properties such as volume of distribution and clearance. Although there are numerous plasma proteins, the free fraction of drugs in plasma is predominantly determined by the extent of binding to human serum albumin (HSA), a 66 kDa protein typically present at concentrations of around 600 μM in human plasma.[2] The role of HSA within plasma is to transport various endogenous ligands such as fatty acids, bilirubin, heavy metals, hormones and vitamins as well as other metabolites, and as such is a very flexible protein containing numerous potential drug binding sites.[3] Two primary drug binding sites have been identified: site I, in subdomain IIA; and site II, in subdomain IIIA.[4–8] Site I is the primary binding site for drugs such as Warfarin and Azapropazone, and may be referred to in the literature as the Warfarin–Azapropazone site.[9] Site II, on the other hand, primarily binds drugs such as Diazepam and arylpropionic acids, and can also be referred to as the Indole–Benzodiazepine site.[10] It has been suggested that drugs which bind to site I are bulky heterocyclic molecules with a negative charge localised in the middle of the molecule and that site II drugs are aromatic carboxylic acids carrying a negative charge on the carboxyl group at one end of the molecule away from the hydrophobic centre.[9] Binding of ligands to these sites are selective to a certain extent, although some ligands can bind to both sites. It has also been noted that the presence of fatty acids, which can bind to numerous sites on HSA,[11] can impact the binding of drugs, particularly to site II.[8] Numerous secondary drug binding sites have also been indentified, distributed across the protein. Binding can also occur to alpha 1-acid glycoprotein (AGP), particularly with lipophilic basic drugs, although the concentration of this protein is only around 20 μM in plasma.[2]

There are numerous techniques available for measuring the extent of plasma protein binding, including ultrafiltration, capillary electrophoresis, immobilised albumin column methods, spectrofluorometry, however, equilibrium dialysis is often the method of choice due to the production of high quality binding data.[12] In this approach, protein containing and protein-free solutions are separated by a semi-permeable membrane through which drug molecules are allowed to freely diffuse. The concentration of drug is

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then measured from both compartments when the system is at equilibrium, and this allows the free fraction of drug to be calculated. The protein binding process is described by the equilibrium as below:

$$D + P \overset{K_1}{\rightleftharpoons} DP \quad (1)$$

where D represents the drug with an initial concentration typically in the region of 10 μM. P and K_1 represent, respectively, the protein (concentration ~600 μM) and the equilibrium binding constant (in units of M^{-1}). Advances in technology have recently led to an increased throughput for this traditionally labour-intensive approach which allows for significant numbers of compounds to be measured as part of the drug discovery process.[13]

In terms of drug design where modulation of plasma protein binding is required, efforts are often focussed on reducing lipophilicity, as this approach, in the main, increases the free fraction.[14] This can be justified by considering the lipophilic nature of the drug binding sites on HSA. More in-depth quantitative structure property relationships[15] can also be established for plasma protein binding however, there are occasional outliers, and in these cases additional experimental techniques need to be employed to understand the deviations and aid molecular design leading to drugs with improved free fractions. Clearly, crystallography can provide the most detail in terms of how compounds are bound to HSA, however relatively few compounds have been crystallised in part due to the flexible nature of the protein.[8] Due to the difficulties and costs associated with generating crystal structures, it is preferable that other techniques can be employed to probe the interactions of drugs with HSA. Fluorescence and NMR techniques have been shown to provide information regarding the protein residues involved in the drug binding,[16–19] and although these procedures are useful for exploring the interactions of individual compounds, or small numbers of compounds, they are not amenable to providing information on larger numbers of compounds in a medium throughput type of assay. Several other techniques which employ the use of probe molecules that are known to bind to specific sites on HSA, for example, Warfarin for site I and Diazepam for site II, can be employed to provide information about which site on albumin compounds bind. These techniques, although primarily developed as techniques to measure the binding strength of compounds, include surface plasmon resonance (SPR), scintillation proximity assay (SPA) and fluorescence spectroscopy. The probe molecules can then be displaced by a competing ligand and these changes detected using these various methods.

The SPR technique is based on the immobilisation of the target protein, in this case HSA, by chemical reaction onto a sensor chip surface and on probe binding the changes in the mass of the aqueous layer close to the sensor chip surface are assessed by measuring changes in the refractive index.[20] If the presence of a competing compound results in an increased signal, it indicates that the compound has bound elsewhere compared to the probe molecule. If the signal remains almost constant, it is indicative that the probe has been displaced by the competing ligand.[21] SPA, on the other hand, utilises albumin physically immobilised on yttrium silicate beads, which in the presence of tritiated Warfarin or Diazepam results in a scintillation count.[22] Only the radiolabelled probe that is bound to the HSA generates a measurable count. Therefore displacement of the probe by a competing ligand results in a reduced scintillation count. Although possible to infer the binding site on HSA by these methods, they rely on the assumption that immobilising the albumin does not impact the binding properties of the protein. Conversely, fluorescence spectroscopy can assess the binding sites on HSA in solution by employing probe molecules that when bound to the protein produce a characteristic fluorescence signal. For a test compound binding to the same sites as the probe molecule, this characteristic fluorescence signal would decrease upon the displacement of the probe molecule by the test compound. This approach was described by Epps et al. in order to measure the site-specific binding affinities of compounds, using Warfarin as probe for site I and Dansylsarcosine as a probe for site II.[23] In this work, we sought to extend the above approach to assess the HSA binding site identity of research compounds in a medium throughput manner. The present paper describes our initial attempt to evaluate this method with a variety of compounds that had known binding sites on HSA from the literature. This fluorescence method is then applied to study in-house compounds of interest. Finally, structural information obtained on three new HSA–drug complexes is described and illustrates how these findings substantiate the fluorescence spectroscopy site identification work and reveal why certain compounds possess interesting HSA binding characteristics.

2. Results and discussion

2.1. Binding site identification from fluorescence measurements

Initial work focussed on using the method of Epps et al. to examine if fluorescence spectroscopy could be employed to follow the displacement of the fluorescent probes Warfarin, for site I, and Dansylsarcosine, for site II.[23] Initial experiments employed 3 μM probe in the presence of 3 μM HSA, where each of these combinations was observed to produce characteristic fluorescence spectra. For these initial experiments, fatty acid-free HSA was employed to avoid complications arising from competition between the probe, the compound being assessed and the fatty acids for the various binding sites on albumin. The wavelength of the exciting light was 320 nm for the Warfarin–HSA complex, and 340 nm for the corresponding complex with Dansylsarcosine. Fluorescence emission was measured over the range 340–500 nm and 360–550 nm for the respective probes. To each combination, two competing compounds of known binding location, Phenylbutazone (site I) and Diazepam (site II), were then added in concentrations of up to 12 μM, and the changes in fluorescence spectra recorded. It was observed that Phenylbutazone resulted in a decrease of the Warfarin–HSA fluorescence intensity, but had no effect on the Dansylsarcosine–HSA complex. Conversely, Diazepam, which had no effect on the Warfarin–HSA complex, resulted in a reduction of the intensity of the Dansylsarcosine–HSA fluorescence spectrum. Control experiments were conducted, whereby no probe was added, to account for any inherent fluorescence of the competing drug molecules when bound to HSA. Figure 1 illustrates the effect of adding Diazepam on the fluorescence spectrum of the Dansylsarcosine complex with HSA, and Figure 2 illustrates the reduction in fluorescence intensity at the maximum emission wavelength ($\lambda_{em,max}$ = 386 nm for the HSA–Warfarin complex and 496 nm for the HSA–Dansylsarcosine complex) for both probe molecules with the two competing ligands. These initial results provided confirmation that the technique could be employed to investigate whether compounds bound to site I or site II on albumin.

Various experiments were then performed to determine optimum concentrations of HSA and of the probe compounds for these displacement studies, and from these it was decided to employ 3 μM HSA and 3 μM probe which gave the greatest change in fluorescence intensity and provided reproducible results. Once these conditions had been established, liquid handling protocols were then developed where automated liquid handling replaced the manual pipetting of solutions, and the range of emission wavelengths to be measured was reduced so that only changes in a narrow window close to the maximum fluorescence intensity were

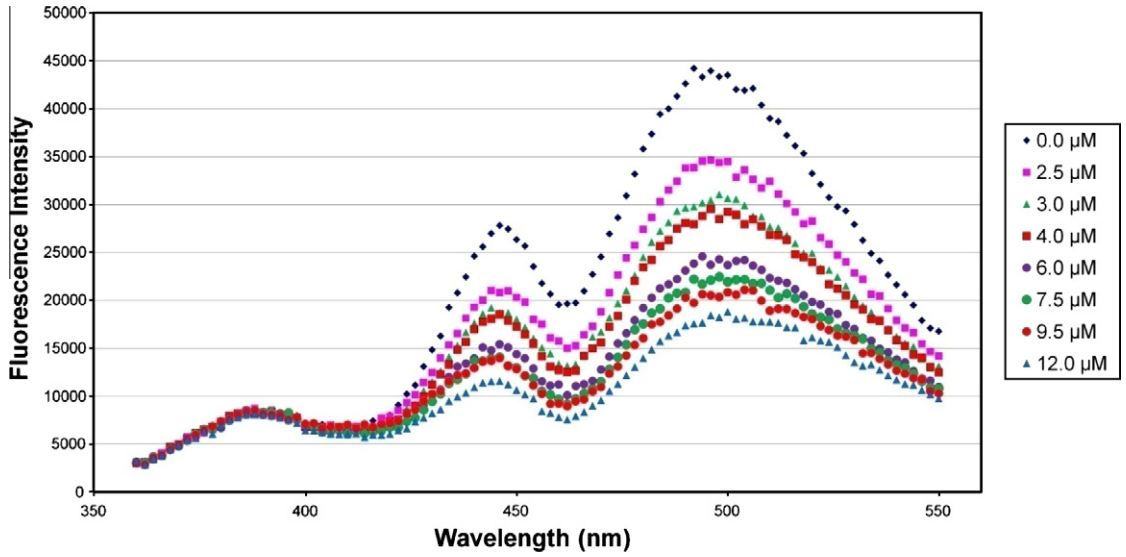


Figure 1. Changes in the fluorescence spectrum of the HSA complex of Dansylsarcosine on the addition of increasing amounts of Diazepam. [HSA] = 3 μM; [Dansylsarcosine] = 3 μM; [Diazepam] = up to 12 μM.

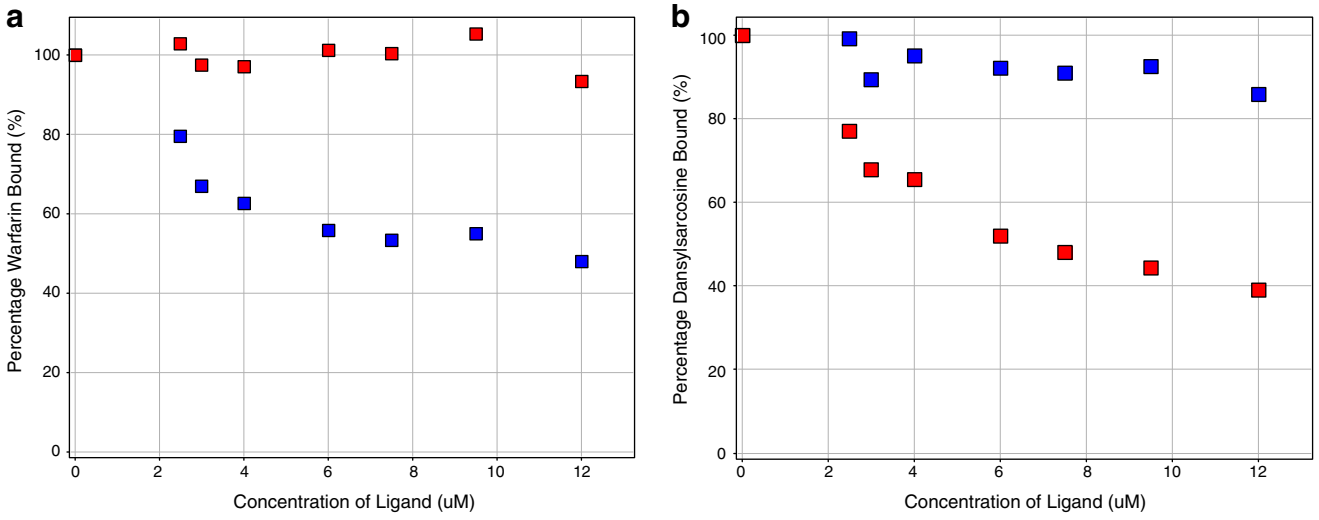


Figure 2. (a) The percentage of the site I specific fluorescent probe, Warfarin, which is bound to HSA in the presence of increasing concentration of Phenylbutazone (blue squares), and Diazepam (red squares), based on the decrease in fluorescence brought about by displacements of the probe. ([HSA] = 3 μM; [Warfarin] = 3 μM; [Diazepam], [Phenylbutazone] = up to 12 μM.) (b) The equivalent graph where the site II specific probe, Dansylsarcosine, is displaced by the same competing drugs. ([HSA] = 3 μM; [Dansylsarcosine] = 3 μM; [Diazepam], [Phenylbutazone] = up to 12 μM.)

assessed. The result of these changes was to produce a more medium throughput assay which was capable of assessing the binding site of several compounds in one experiment.

Given these concentrations of HSA and probe compound, numerous compounds for which their primary binding sites on HSA had been described in the literature,[24] were then assessed by this fluorescence displacement technique—see Table 1. Although there are slight differences in the binding strength of Warfarin and Dansylsarcosine to HSA ($\log K_1 = 5.13$ and 5.39, respectively), and therefore competing compounds will displace the probes to slightly different extents, it was found that a concentration of 12 μM for the competing compound was sufficient to displace enough of the probe compound to allow an assignment of the primary binding site on HSA. The change in fluorescence brought about by the competing drug was quantified by calculating the percentage displacement of the fluorescent probe by each compound, and these values are quoted as D_{12} values in Table 1, that is, the percentage displacement of probes from HSA brought about by the presence of 12 μM competing drug. Where a clear difference was observed for the displacement of the sites I and II probe molecules, a preferential binding site was assigned to the compounds. In some cases it became clear that the compounds were binding to both sites I and II.

It can be seen from Table 1, there is a reasonable correlation between the binding site quoted in the literature[5,8,24–38] for these compounds and the binding site deduced from the fluorescence displacement assay described here. Good agreement was seen for various compounds such as Clonazepam, Cloxacilline, Ketoprofen and Naproxen.[24] Agreement has also been shown for compounds such as Diazepam and Ibuprofen that have been studied in great detail and shown by crystallography to bind in site II, as has Phenylbutazone which binds in site I.[8] However, the displacement studies illustrated here are at odds with the crystallography data obtained for Diflusinal and Indomethacin. Diflusinal has been

Table 1
Assessment of the primary drug binding site on HSA from the displacement of Warfarin (D_{12} site I) or Dansylsarcosine (D_{12} site II), together with a comparison of the primary binding site from the literature

Compound	Literature binding site assignment[a]	D_{12} site I FAF	D_{12} site II FAF	Site assignment FAF	D_{12} site I FAC	D_{12} site II FAC	Site assignment FAC	Impact of FA on selectivity[b]
Acenocoumarin	I (5,26)	49.8	12.5	I/II	54.6	39	I/II	↓
Chlorothiazide	I (25)	28.9	8	I/II	16	18.8	II/I	↓, ↔
Chlorpropamide	I (27)	19.2	28.5	II	18.5	26.0	II	↑
Clonazepam	II (28)	0	16.2	II	3.8	19.5	II	=
Cloxacilline	II (25)	0	24.1	II	5.7	18.1	II	↓
Diazepam	II (8,25)	6.6	61	II	6	54.1	II	↓
Dicoumarol	I, II (25)	86.4	88.8	II/I	10.5	19.8	II/I	↑
Diflusinal	I (25,30); II (8,29,31)	59.7	33.2	I	77.0	82.7	II/I	↓, ↔
Fenbufen	II (31)	0	70	II	0	66.2	II	=
Flufenamic acid	II (32,33)	48.6	95.8	II/I	68.6	91.2	II/I	↓
Flurazepam	II (28)	0	4.3	II	6.3	2.7	I/II	↓, ↔
Furosemide	I (25)	36.2	19.2	I/II	47.3	49	II/I	↓, ↔
Glibenclamide	I, II (25)	28.6	33.9	II/I	50.7	62.2	II/I	↑
Ibuprofen	II (8,25)	0	80.3	II	0	70.3	II	=
Indomethacin	I, II (5,25,31); II (34); I (8)	25	64.8	II/I	63.2	67.4	II/I	↓
Isoxicam	I, II (35)	51.7	33.1	I/II	38	32.6	I/II	↓
Ketoprofen	II, I (25)	0	79.3	II	8.6	69.1	II	↓
Naproxen	II, I (25)	24.7	91.3	II/I	38.8	79.9	II/I	↓
Phenylbutazone	I (8,25,32)	53.5	25.3	I/II	63.5	45.0	I/II	↓
Phenytoin	I (25)	4	0	I	9.3	18.8	II/I	↓, ↔
Pindolol	I (25)	0	0.2	II	0	9.5	II	↑
Piroxicam	I, II (36)	6.7	45.5	II	53.5	44.8	I/II	↓, ↔
Salicylic acid	I (31)	15.9	24.9	II/I	7	24.8	II	↑
Sulfinpyrazone	I (25)	35.2	18.6	I/II	44.1	34.2	I/II	↓
Tamoxifen	I, II (25)	0	16.3	II	0	62.2	II	↑
Tenoxicam	I, II (35)	52.3	27.4	I/II	61.7	38.5	I/II	=
Tolbutamide	I, II (25,27)	0	9	II	5	8.5	II/I	↓
Tryptophan	II (25)	0	15.3	II	0	15.1	II	=

FAF = fatty acid-free HSA; FAC = fatty acid containing HSA. [HSA] = 3 μM; [Warfarin], [Dansylsarcosine] = 3 μM; [Compound] = 12 μM.
[a] References in parentheses.
[b] Impact of FA (fatty acids) on binding selectivity: ↓, less selective; ↑, more selective; =, no impact; ↔, change in primary binding site.

shown to bind to site II in crystallography studies both with full length HSA[8] and truncated sections of the protein.[29] In contrast to this, and more in agreement with our studies, Sjöholm et al.[25] and Davilas et al.[30] both claim that site I is the primary binding site for Diflusinal. Indomethacin, on the other hand, has been shown to bind to site I via crystallographic work,[8] whereas our studies are more in agreement with other work that indicates that there is significant binding at both sites I and II.[5,25,31] However, it should be noted that the crystallographic analysis with this compound was performed in the presence of fatty acids that would preclude any observation of possible binding to site II. The results obtained here for Chlorpropamide are also not in agreement with literature studies which indicate a preference for site I binding,[27] whereas results obtained here indicate a preference for site II. It is not clear at this time the exact reason for these differences, however possible causes could include the exact nature of the albumin employed in the study, as well as the purity profile of the protein such as fatty acid content (as discussed later), and how the experiments were performed. Immobilisation[25] of albumin may, in theory, give different results to solution based studies. Our results are also at odds with the literature claims[25] that Pindolol binds to site I, and Tamoxifen bind to both sites I and II. These conclusions were based on an increase in probe binding, something that we did not observe under our experimental conditions. It should also be noted that for Pindolol in plasma, the primary protein binding interaction occurs with a-1-acid glycoprotein and that the interaction with albumin is very weak,[37] and that it has been suggested that for Tamoxifen the binding on albumin occurs away from sites I and II.[38] Low levels of probe displacement were observed for several compounds such as Phenytoin, Flurezapam and Pindolol, despite being known as compounds that bind to site I or II. This was attributed to the fact that these compounds are known to be weak binders to HSA, with $\log K_1$ values of 4.09, 3.38 and 2.65, respectively, and therefore these compounds do not have the capacity to displace the probes. Increasing the concentration of the competing compound to around 100 μM did result in small amounts of probe displacement, however it was not significant enough to confidently assign a binding site to these compounds using this technique. Conversely, Flufenamic acid, which has a high binding strength ($\log K_1 = 6.68$) also led to displacement of the probe from the secondary binding site on albumin. Displacement experiments using 4 μM compound in place of the regular 12 μM resulted in 24% and 73% displacement of the site I and II probes, respectively, indicating a greater preference for site II binding compared to site I. It should also be noted that Glibenclamide and Tamoxifen, which possess aqueous solubility values at pH 7.4 of 11 μM and <0.7 μM, respectively, also were observed to precipitate during sample preparation, although it is evident that sufficient compound remained in solution to effect the displacement of the probes from HSA.

Our studies using fatty acid-free HSA indicated that a number of the compounds, whilst giving the correct assignment of the primary binding site, showed substantial secondary binding. This suggests that drug binding is not as specific as some literature may suggest and that most drugs will bind to one site preferentially, with some secondary binding to alternative sites as suggested by Curry and co-workers.[8] To examine the effects of fatty acids on the binding site preference of the compounds, the above experiments were repeated employing fatty acid containing HSA (Fraction V). It should be noted that the fatty acid nature or content of this albumin sample was not characterised and is therefore unknown. All experiments were however conducted using one batch of the albumin to ensure consistency between the findings. As before, D_{12} values were calculated for the displacement of both site-specific probes from Fraction V HSA and compared to those values obtained above using fatty acid-free HSA—see Table 1. It can be seen that the addition of fatty acid to the HSA does have an impact on the displacement of the probes for some compounds, and there are numerous compounds for which the preference for one binding site over the other becomes less selective. This is consistent with the work of Curry and co-workers,[8] who suggested that binding of fatty acids may remove (or neutralise) polar residues at the drug binding sites having an effect upon binding location. However, there are also some examples for which the presence of the fatty acids makes the binding more selective, for example, Tamoxifen. Subsequent analysis of drug binding constants in Fraction V and fatty acid-free HSA indicated that for most compounds there is little impact of the fatty acids on the binding strength (see Fig. 3), however it should be noted that the main outliers in this plot are compounds that had their primary binding site assigned as site II in the absence of the fatty acids. For these compounds it is clear that they are significantly more bound to fatty acid-free HSA compared to HSA in the presence of the fatty acids. Naproxen showed the biggest difference in binding constant ($\log K_1$ fatty acid-free HSA = 6.87; $\log K_1$ fatty acid containing HSA = 5.89); a significant decrease in binding strength when fatty acids are present. This is also in agreement with Curry's conclusion that drugs that bind to site II would be more susceptible to displacement by fatty acids than drugs that bind to site I, as the former site overlaps with one high affinity and one low affinity fatty acid binding site, whereas site I overlaps with a single low affinity fatty acid binding site.[11]

The displacement of fluorescent probes from HSA can be described mathematically by solving the mass balance equations of all species with initial concentrations of all species and the known binding constants of the probe and test compound (see Supplementary data). This model can be used to generate theoretical displacement and compare with those values obtained experimentally, as well as facilitate the future experimental design.

Theoretical dose–response curves for the displacement of each probe by its displacing ligand were constructed using a curve-fitting programme. Using the probe and HSA concentrations used in the experimental assay and protein binding data values measured via equilibrium dialysis, the theoretical model outlined above was examined for sites I and II binding using Warfarin and Dansylsarcosine as the probe molecules and Phenylbutazone and Diazepam as competing drug molecules. D_{12} values were obtained from the model in each case and compared to those obtained experimentally. Using the mathematical model, dose–response curves were plotted for the displacement of Warfarin by Phenylbutazone and

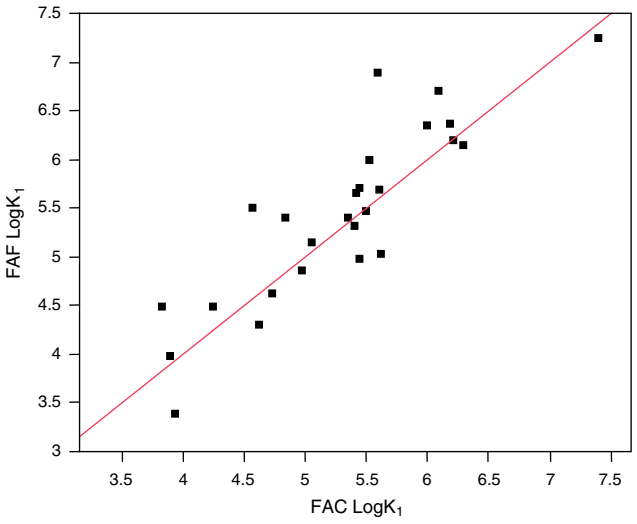


Figure 3. Comparison of binding constant ($\log K_1$) of drugs for fatty acid-free (FAF) HSA and for fatty acid containing (FAC) HSA. The straight line is the 1:1 fit, RMSE = 0.45.

the displacement of Dansylsarcosine by Diazepam. As can be seen from Figures 4 and 5 these were found to be in extremely good agreement with the experimentally derived values for the displacement of the probes.

2.2. Crystal structure determination

Crystallography is a powerful way to determine the location of compounds binding to HSA. However the generation of such information has not always proved to be straightforward presumably due to the flexible nature of albumin. However, a method has been developed at Proteros (see Section 4) using de-fatted HSA, and as part of this study into probing the binding sites, three compounds of interest were taken forward for their HSA structures to be determined. Compounds **1** and **2**, both carboxylic acids,[39] showed an interesting and surprising effect; when the alkyl linker length to the carboxylic acid was increased, the strength of binding to HSA decreased. This was despite an increase in the lipophilicity which usually translates to an increase in the binding strength. This phenomenon was also observed in rat plasma and rat serum albumin binding studies, and also when the nature of the heterocyclic amide was varied indicating that the effect on the binding could indeed be attributed to the change in the length of the alkyl linker. Compounds **1** and **2** were assessed in the fluorescence assay described above to determine their predominant binding sites on HSA in the absence of fatty acids, and it was found that both compounds primarily bound to site I. It was also found that there was very little difference in terms of the binding strength of these two compounds in both fatty acid free and fatty acid containing HSA. Compound **3**, an acidic sulphonamide,[40] was of interest as it was known that it was a strong binder to HSA, however, in the fluorescence displacement assay only minor amounts of both the site I and site II probes were displaced. D_{12} values for both sites were zero and less than 10% of the fluorescence of the probe molecules was displaced by the addition of up to 100 μM of compound **3**. The physical properties and fluorescence displacement details of all three compounds are collated in Table 2, and the collection and refinement details of the crystal structures are collated in Table 3. Figure 6 illustrates the binding of each of these compounds in HSA.[41]

Compound **1** was found to bind to two sites with higher occupancy in site I (subdomain IIA) and somewhat smaller occupancy in the IIA–IIB subdomain[42]—see Figure 7. The residues within a maximum distance of 3.9 Å to the ligand in the IIA pocket are Leu198, Lys199, Ser202, Phe211, Trp214, Arg218, Leu219, Arg222, Phe223, Leu238, His242 and Ile290. Compound **2** was also found to bind in site I (subdomain IIA)[43]—see Figure 7, with the residues within a maximum distance of 3.9 Å to the ligand in the IIA pocket being Tyr150, Lys199, Ser202, Phe211, Trp214, Arg218, Leu219, Arg222, Phe223, Leu238, His242, Arg257, Ile290 and Ala291. In both cases the core of the molecule is anchored by similar interactions such as the interaction of the indole ring with Trp214 and Ser202. Although there are some differences in the binding mode of the two ligands due to artefacts of the crystal structure, the structures provide insights into the interactions of the two ligands with HSA. In particular, it can be seen that whereas for compound **1** there is a close interaction of the carboxylate with Lys199 and His242, this is no longer possible in compound **2** due to the increased length of the spacer between the carboxylate and the core of the molecule. For compound **2** the key carboxylate contacts were observed to be Tyr150 and Arg257, with the Tyr150 interaction geometry being sub-optimal for the formation of a strong hydrogen bond interaction. This change of interactions perhaps indicates why there is a decrease in the binding strength in going from **1** to **2** despite the increase in lipophilicity. Both structures also confirm the assignment of the primary binding site from the fluorescence displacement experiments. Figure 8a shows compounds **1** and **2** overlayed with Warfarin,[8] the fluorescent probe used in this work. The indole group of both compounds occupies a distinct pocket formed by a re-orientation of Trp214 enabling a π-stacking interaction to be formed. This feature has previously been observed for Indomethacin and Iodipamide in HSA structures reported by Curry and co-workers.[8] The current results are further examples of the adaptive nature of the site I binding pocket.

In contrast to the assumption that most drug-like compounds bind to either site I (subdomain IIA) or site II (subdmain IIIA), compound **3** was found to bind to subdomain IB[44]—see Figure 7. The residues within a maximum distance of 3.9 Å to the ligand are Leu115, Val116, Arg117, Met123, Tyr138, Glu141, Ile142, Tyr161, Leu182, Leu185 and Arg186. The pyridyl nitrogen and the hydroxyl group of Tyr161, the sulphonamide oxygen atoms and the guanidino group of Arg117, are within hydrogen bonding distance. The finding that this compound binds in this region of HSA, and not to subdomain IIA or IIIA explains why the level of fluorescence displacement was so low when **3** was added to HSA complexes of Warfarin and Dansylsarcosine. This observation highlights that drug molecules can bind to alternative sites on albumin and not just the two main drug binding sites. This finding complements the work of Curry and co-workers who have shown that fusidic acid binds in subdomain IB, and who have also shown that this subdomain can act as a secondary binding site for compounds such

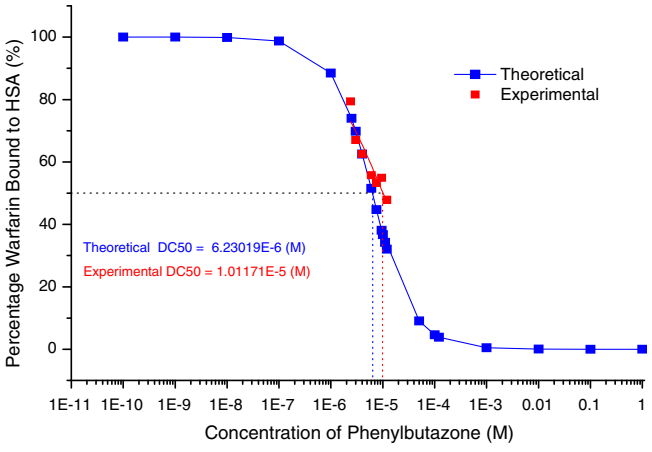


Figure 4. Dose–response curve for the displacement of Warfarin from HSA by Phenylbutazone using data obtained from the theoretical model and experimental data for comparison.

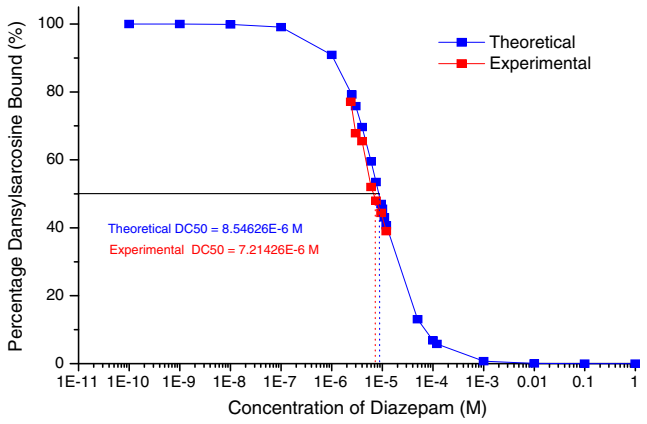
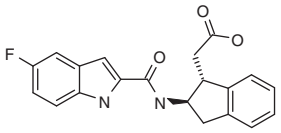
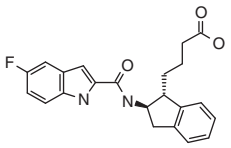


Figure 5. Dose–response curve for the displacement of Dansylsarcosine from HSA by Diazepam using data obtained from the theoretical model and experimental data for comparison.

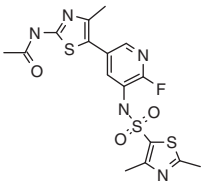
Table 2
Physical properties and probe displacement data for the compounds **1**–**3** selected for structural determination



1



2



3

Compound	Log *K* HSA fatty acid containing	Log *K* HSA fatty acid-free	Octanol log *D*	pK_a	Octanol log *P*	Aqueous solubility, pH 7.4 (μM)	D_{12} site I[a]	D_{12} site II[b]	Assignment of primary binding site in fatty acid-free HSA
1	5.97	6.11	1.26	3.95	4.71	1930	21	0	I
2	5.41	5.41	1.95	4.24	5.11	86	23	2	I
3	5.88	5.62	0.55	4.71	3.24	>1700	6[c]	7[c]	I/II

Assessment of the primary drug binding site on HSA from the displacement of [a] Warfarin (D_{12} site I) or [b]Dansylsarcosine (D_{12} site II): [HSA] = 3 μM; [Warfarin], [Dansylsarcosine] = 3 μM; [Compound] = 12 μM.
[c] [Compound] = 100 μM instead of 12 μM. No displacement of the probes was observed with compound concentrations of 12 μM.

as Azapropazone and Indomethacin.[8,45] It is also of note that Carter et al. suggest that site IB may in fact be a major drug binding region on HSA, although these claims are made in non-peer reviewed literature.[46,47] Figure 8b shows the superposition of compound **3** and fusidic acid in site IB. The acidic sulphonamide group of compound **3** is positioned at the entrance of the pocket in a similar region to the acetyloxy and carboxylate groups of fusidic acid. The compounds both form interactions with Tyr161 and Arg117, although the Arg117 residue is re-orientated between the two structures to enable appropriate interactions with the drug molecule to be formed. Interestingly, both compounds adopt an L-shaped conformation to bind to the site IB pocket.

3. Conclusions

The current work has clearly illustrated that the displacement of compounds having a known binding site on human serum albumin by competing ligands can be followed using fluorescence spectroscopy, and therefore a primary binding site on albumin for these ligands can be assigned. Warfarin as the probe for site I, and Dansylsarcosine as the probe for site II, have been shown to give results that are in reasonable agreement with the literature reports of the binding sites for various ligands. The results for a number of compounds are at odds with some of the literature findings, however, it is clear that for some compounds, for example, Diflusinal, the assignment of the primary binding site is not consistent across all studies. This assay has also been validated in terms of the magnitude of the displacement of these probe compounds by competing ligands with known HSA binding constants and assuming a 1:1 binding model. Although other techniques can be used to investigate the binding of compounds to specific sites on HSA, for example, fluorescence spectroscopy,[48,49] isothermal titration calorimetry,[50] high performance affinity chromatography,[51] flow injection analysis-quartz crystal microbalance systems[52] and circularly polarised luminescence spectroscopy,[53] these techniques are typically not readily adaptable to higher throughput assays. The approach described here has been adapted using liquid handling automation to increase the throughput and therefore allow numerous compounds to be assessed rapidly, and therefore the information obtained can be used to influence drug discovery projects. Future work will examine how this knowledge of the binding site for ligands can be applied, for example, in helping to understand outliers from quantitative structure property relationships and therefore building the potential for compounds to be designed that have improved plasma protein binding characteristics. Although potentially limited by the generation of HSA–ligand complexes that are inherently fluorescent, alternative probes for each site could be employed in this assay. Optimisation of λ_{ex} and λ_{em} for certain combinations of ligands and probes can also circumvent interference from the inherent fluorescence of ligand complexes with HSA. Results employing this technique with fatty acid free and fatty acid containing HSA have also shown the impact that these naturally occurring acids have on the binding site assignment, and the results presented here indicate that many compounds bind less selectively to albumin in the presence of fatty acids, compared to that in the absence of the fatty acids. However, there are compounds that bind more selectively stressing the need to examine each compound in detail under the various conditions. These results emphasise the flexible and variable nature of drug–albumin interactions.

The crystal structures have also been solved for three compounds of interest and these support the binding site assignment from the fluorescence displacement assay. For a pair of carboxylic acids, the structural information provided a rationale to explain why one ligand was less bound to albumin than would have been expected based on it's increased lipophilicity. Knowledge such as this within a project could encourage design of molecules in ways that would not have been previously expected. In the absence of widely applicable and reliable predictive models for plasma protein binding,[54–56] the combination of experimental results from the assays described here together with computational approaches[57,58] to model the interactions of drug molecules with albumin could also aid the drug discovery process when novel approaches are required to alter the extent of drug plasma protein binding. The finding that an acidic sulphonamide was primarily bound in subdomain IB, and not in either of the main drug binding sites I or II, as with Fusidic acid,[45] suggests that this alternative site might be an important location for drug compounds on HSA for certain compounds. The development of the fluorescence based assay with a probe for this site is currently underway.

4. Experimental

4.1. Chemicals

The fluorescent probes and all other drugs analysed using the fluorescence and SPR assay were purchased from Sigma Aldrich, Fluka or Anichem. Compounds 1, 2 and 3 were synthesised as re-

Table 3
Data collection, processing and refinement statistics for crystal structures of compounds **1**, **2** and **3** bound in human serum albumin

	Compound **1**	Compound **2**	Compound **3**
PDB entry	3LU6	3LU7	3LU8
Data collection and processing			
No. of crystals used	1	1	1
Wavelength (Å)	0.9999	0.98	0.92
Space group	*P* 1	*P* 1	*P* 1
Unit cell parameters			
a; *b*; *c* (Å)	58.56; 59.87; 95.57	58.54; 59.19; 95.6	58.78; 59.03; 96.18
α; β; γ (°)	74.5; 86.6; 74.5	75.3; 87.9; 75.5	75.4; 88.1; 76.9
Diffraction data			
Resolution (Å)	2.7 (2.85–2.70)	2.95 (3.13–2.95)	2.6 (2.74–2.6)
Unique reflections	31,188 (4594)[a]	27,472 (3793)	33,778 (5021)
$R(I)_{sym}$ (%)	8.8 (37.1)	10.0 (35.1)	6.0 (27.7)
Completeness (%)	94.2 (94.5)	92.9 (92.8)	90.1 (91.7)
Redundancy	1.9 (1.9)	2.1 (2.1)	1.8 (1.7)
$I/\sigma(I)$	10.3 (1.6)	9.1 (2.5)	11.1 (2.9)
Refinement			
Resolution range used in refinement (Å)	20.0–2.7	20.0–2.95	20.0–2.60
Reflections used in refinement (work/free)	29,708/1478	26,029/1392	32,431/1342
Final *R* values for all reflections (work/free) (%)	23.6/27.5	22.5/27.6	24.5/26.6
Total number of atoms:			
Protein	9204	9207	9185
Inhibitor	104	56	56
Water molecules	14	3	41
Phosphate	–	25	–
RMSDs			
Bonds (Å)	0.01	0.009	0.009
Angles (°)	1.15	1.12	1.17
Bonded B's (Å^2)	2.5	2.4	2.4
Ramachandran plot			
Most favoured (%)	88.2	89.4	90.6
Additionally allowed (%)	11	10	8.6
Generously allowed (%)	0.8	0.6	0.7
Disallowed regions (%)	–	–	0.1

[a] Numbers in parentheses characterise the highest resolution shell.

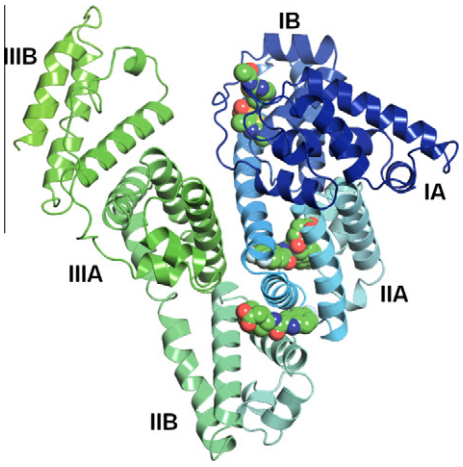


Figure 6. Summary of HSA structures containing compounds **1** and **2** bound in subdomain IIA, a secondary binding site for compound **1** between subdomains IIA and IIB, and compound **3** binding in subdomain IB. (Protein structure coloured by sub-domain.)

ported in Refs. 26,27. All other products used were of analytical grade (Fisher Scientific). Stock solutions of each were made up in HPLC grade dimethylsulphoxide (DMSO) from Fisher Scientific and diluted according to the procedures outlined. Albumins samples were purchased from Sigma Biochemicals; essentially fatty acid-free HSA (approx. 99%, lyophilised powder, A 3782), and fatty acid containing Fraction V HSA (97–99%, Lyophilised powder, A 9511) and used as received. Albumin stock solutions were made up in fresh phosphate buffer (0.013 M KH_2PO_4, 0.075 M NaCl, 0.054 M Na_2HPO_4 in water, pH 7.4) and diluted as required.

4.2. Protein binding strength via equilibrium dialysis

Dialysis membranes (Spectra/Por 2, 12–14 kDa molecular weight cut-off, 47 mm diameter, Spectrum Laboratories) were prepared for use by washing with distiled water and subsequent soaking in phosphate buffer (pH 7.4). Membranes were then blotted dry and placed between two 1 ml Teflon dialysis half-cells (Braun ScienceTec, Les Ulis, France). Each half-cell was filled individually with 1 ml of protein solution containing the compound of interest, while the corresponding half-cell was filled with 1 ml of isotonic phosphate buffer. Dialysis units were immersed in a 37 °C temperature-controlled water bath and rotated at 30 rpm for 18–19 h using a Dianorm apparatus (Braun ScienceTec). After this period, samples from both the half-cell containing buffer (protein free) and the half-cell containing protein were submitted for HPLC analysis using an Agilent 1100 series HPLC with a 110 binary pump and a UV diode ray detector. Acquisition and integration were carried out using Chemstation software (Agilent Technologies) version A.06.03 with relevant customised macro software. Integration of the subsequent chromatograms, are used to calculate the concentration of drug in the protein containing solution (D_p) and in the protein-free solutions (D_f), which are then used to derive the binding constant for the test compound (K_1) assuming a 1:1 binding model as shown in Eq. 1 where the compound can only bind to a single site on the protein molecule. This is expressed mathematically in Eq. 2 where D and D_f are the total and free drug concentrations, respectively, and Pr is the total protein concentration.

$$D = (D_f + D_p) = \frac{K_1 \cdot D_f \cdot \mathrm{Pr}}{1 + K_1 \cdot D_f} + D_f \quad (2)$$

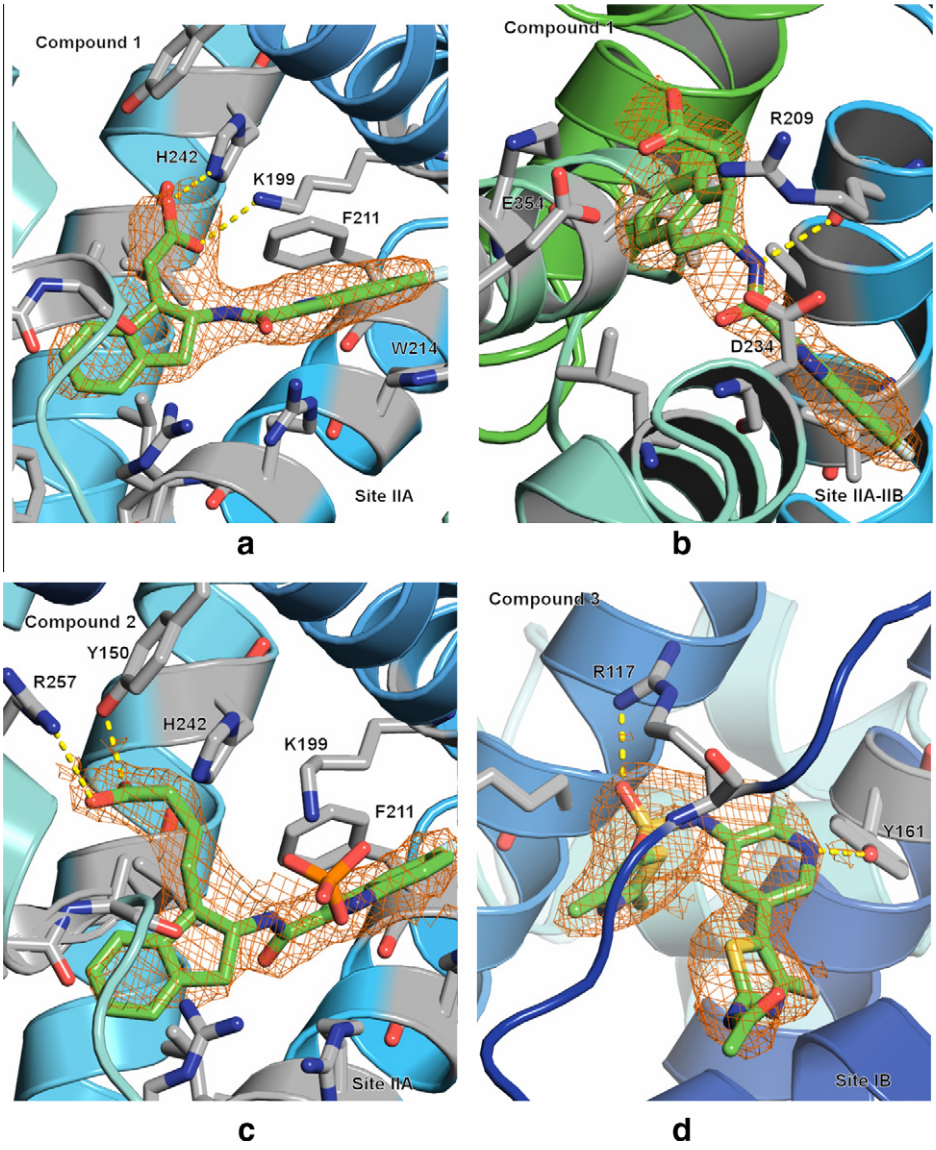


Figure 7. (a) Subdomain IIA binding pocket of HSA containing compound **1**. (b) Secondary binding site of compound **1** between subdomains IIA and IIB. (c) Subdomain IIA binding pocket of HSA containing compound **2**. (d) Subdmain IB binding pocket of HSA containing compound **3**. (Protein structure is colour-coded by subdomain as shown in Fig. 6.)

4.3. Physical property measurements

Log *D* measurements were made using a shake-flask method where the extent of partitioning between pH 7.4 buffer and octanol was measured. Compounds were dissolved in a known volume buffer, and following the addition of a known amount of octanol, the solutions were shaken for 30 min. Following centrifugation, analysis of the aqueous layer was performed by LC–UV to quantify the amount of compound in solution and then compared to analysis of the compound in solution before the addition of octanol to calculate the partitioning coefficient, *D*. The pK_a's of the compounds were measured using a spectrophotometric titration, and the log *P* for acidic compounds was calculated from the measured log *D* and pK_a using Eq. 3.

$$\log D = \log P - \log(1 + 10^{(\mathrm{pH}-\mathrm{p}K_a)}) \quad (3)$$

Assessments of aqueous solubility were made after an incubation of 24 h in pH 7.4 phosphate buffer. After centrifugation, analysis of the supernatant liquid was performed by LC–UV to quantify the amount of compound in solution.

4.4. Fluorescence measurements

The fluorescence of solutions containing a fluorescent probe and serum albumin was monitored in the presence of a competing drug at a particular drug concentration in pH 7.4 phosphate buffer. Warfarin was used as the fluorescent probe of albumin binding site I, and Dansylsarcosine as a probe of site II, unless stated otherwise. Liquid handling was conducted using manual pipettes or a Tecan Genesis Robotic Sample Processor RSP200. All solutions were placed in 96-well clear-bottomed fluorescent plates from Greiner Bio-one for use in the spectral acquisition. All fluorescence acquisition measurements were done using a Tecan Safire microplate reader. The solutions were excited at a wavelength of 320 nm for solutions containing Warfarin as probe, and 340 nm for solutions containing Dansylsarcosine. Fluorescence intensity was measured at 386 nm and 496 nm, respectively.

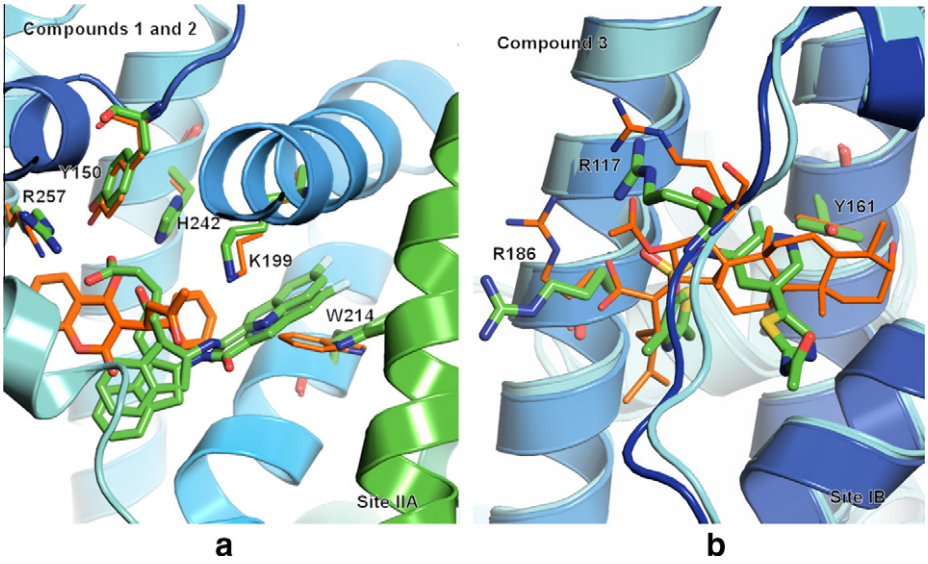


Figure 8. (a) Compounds **1** and **2** overlayed with Warfarin (orange representation—PDB ID:2BXD)[8] binding in subdomain IIA. (b) Subdomain IB binding pocket containing compound fusidic acid (orange representation—PDB ID:2VUF)[45] and compound **3**. (Protein structure is colour-coded by subdomain as shown in Fig. 6.)

4.5. Crystal structure determination

4.5.1. Purification and crystallization

Essentially fatty acid-free and globulin-free human serum albumin (HSA) was purchased from Sigma (A3782). A purification protocol was established and homogeneous protein was obtained in preparative amounts. Briefly, the lyophilised protein was dissolved in 50 mM potassium phosphate buffer pH 7.5, 150 mM NaCl. Several gel filtration chromatography steps using a Superdex S75 26/60 column were performed to obtain homogenous protein with a purity of more than 95% as judged from Coomassie-stained SDS–PAGE. The protein buffer was exchanged to 50 mM potassium phosphate, pH 7.5 and concentrated to 100 mg/ml. Similarly to protocols published previously,[8] crystals of the HSA–inhibitor complexes were obtained at 293 K after several days using the hanging drop vapour diffusion method under the crystallization condition of 24–34% (*m*/*V*) PEG3350 in 50 mM potassium phosphate buffer at pH 6.75–7.5 and a protein-reservoir ratio of 1:1. The HSA–inhibitor complexes were obtained by addition of an appropriate amount of inhibitor stock solution (DMSO) to a final concentration of 1–10 mM inhibitor and incubation for several hours prior to crystallization. In order to prevent interference of DMSO towards crystallisation, the DMSO concentrations in the protein drop did not exceed 2.5% (v/v).

4.5.2. Data collection

Crystals of appropriate size were optimised using the Free-Mounting-Technology.[59] Crystals were fished from the crystallisation drop using a cryo-loop, set into an air stream of 93.5% relative humidity and the adherent mother liquor was removed via Endo-Tech Premium PaperPoints. While keeping the relative humidity constant at 93.5%, the crystal was covered with Perfluoropolyether (PFO-X125/03) and flash-frozen in liquid nitrogen. Data were collected at 100 K at the Swiss Light Source, Villingen, Switzerland. Data processing and scaling were performed using the APRV package.[60]

4.5.3. Structure determination and refinement

Appropriate co-ordinates of HSA[8] were obtained from the Protein Data Bank (PDB) and used after removal of ligand and water atoms as search model for molecular replacement and initial rigid-body refinement in APRV. Five per cent of all data were used for R_{free} calculation. Amino acid side-chains were fitted into $2F_o - F_c$ and $F_o - F_c$ electron density maps using Coot.[61] Subsequent maximum likelihood refinement was performed using REFMAC 5.2.[62] After the first refinement cycle, water molecules and ligand were located in the electron density and added to the model. Refinement at later stages was performed after calculation of TLS[63] parameters, if a significant improvement of the model could be obtained. The final models were validated using PROCHECK.[64]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.052. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. Creasey, W. A. *Drug Deposition in Humans: The Basis of Clinical Pharmacology*; Oxford University Press: Oxford, UK, 1979.
2. Trainor, G. L. *Expert Opin. Drug Discov.* **2007**, *2*, 51.
3. Peters, T. *All About Albumin*; Academic Press: San Diego, 1996.
4. Fehske, K. J. *Biochem. Pharmacol.* **1981**, *30*, 687.
5. Fehske, K. J.; Schläfer, U.; Wollert, U.; Müller, W. E. *Mol. Pharmacol.* **1982**, *21*, 387.
6. Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1975**, *11*, 824.
7. He, X. M.; Carter, D. C. *Nature* **1992**, *358*, 209.
8. Ghuman, J.; Zunszain, P. A.; Petitpas, I.; Bhattacharya, A. A.; Otagiri, M.; Curry, S. *J. Mol. Biol.* **2005**, *353*, 38.
9. Wanwimolruk, S.; Birkett, D. J.; Brooks, P. M. *Mol. Pharmacol.* **1983**, *24*, 458.
10. Watanabe, H.; Tanase, S.; Nakajou, K.; Maruyama, T.; Kragh-Hansem, U.; Otagiri, M. *J. Biochem.* **2000**, *349*, 813.
11. Simard, J. R.; Zunszain, P. A.; Hamilton, J. A.; Curry, S. *J. Mol. Biol.* **2006**, *361*, 336.
12. Pacifici, G. M.; Viani, A. *Clin. Pharmacokinet.* **1992**, *23*, 449.
13. Wan, H.; Bergström, F. *J. Liq. Chromatorgr. Related Technol.* **2007**, *30*, 681.
14. Valko, K.; Nunhuck, S.; Bevan, M. H.; Abraham, M. H.; Reynolds, D. *J. Pharm. Sci.* **2003**, *92*, 2236.
15. Fessey, R. E.; Austin, R. P.; Barton, P.; Davis, A. M.; Wenlock, M. C. In *Pharmokinetic Profiling in Drug Research*; Testa, P., Krämer, S. D., Wunderli-Allenspach, H., Folkers, G., Eds.; Wiley-VCH: Zürich, 2006; pp 119–141.
16. Jenkins, B. G.; Lauffer, R. B. *Mol. Pharmacol.* **1989**, *37*, 111.

17. Delfini, M.; Bianchetti, C.; Di Cocco, M. E.; Pescosolido, N.; Porecelli, F.; Rosa, R.; Rugo, G. *Bioorg. Chem.* **2003**, *31*, 378.
18. Dufour, C.; Dangles, O. *Biochim. Biophys. Acta* **2005**, *1721*, 164.
19. Vayá, I.; Bueno, C. J.; Jiménez, M. C.; Miranda, M. A. *ChemMedChem* **2006**, *1*, 1015.
20. Chignell, C. F. *Ann. N.Y. Acad. Sci.* **1973**, *226*, 44.
21. Rich, R. L.; Day, Y. S.; Morton, T. A.; Myszka, D. G. *Anal. Biochem.* **2001**, *296*, 197.
22. Technical note TRK1093, Amersham Biosciences (GE Healthcare).
23. Epps, D. E.; Raub, T. J.; Kézdy, F. J. *Anal. Biochem.* **1995**, *227*, 342.
24. Muller, N.; Lapicque, F.; Drelon, E.; Netter, P. *J. Pharm. Pharmacol.* **1994**, *46*, 300.
25. Sjöholm, I.; Ekman, B.; Kober, A.; Ljungstedt-Pahlman, I.; Seiving, B.; Sjodin, T. *Mol. Pharmacol.* **1979**, *16*, 767.
26. Yamasaki, K.; Maruyama, T.; Kragh-Hansen, U.; Otagiri, M. *Biochim. Biophys. Acta* **1996**, *1295*, 147.
27. Brown, K. F.; Crooks, M. J. *Biochem. Pharmacol.* **1976**, *25*, 1175.
28. Müller, W. E.; Wollert, U. *Naunyn Schmiedebergs Arch. Pharmacol.* **1973**, *280*, 229.
29. Mao, H.; Hajduk, P. J.; Craig, R.; Bell, R.; Borre, T.; Fesik, S. W. *J. Am. Chem. Soc.* **2001**, *123*, 10429.
30. Davilas, A.; Koupparis, M.; Macheras, P.; Valsami, G. *J. Pharm. Pharmacol.* **2006**, *58*, 1467.
31. Honoré, B.; Brodersen, R. *Mol. Pharmacol.* **1984**, *25*, 137.
32. Sudlow, G.; Birkett, D. J.; Wade, D. N. *Mol. Pharmacol.* **1976**, *12*, 1052.
33. Kasai, S.; Horie, T.; Mizuma, T.; Awazu, S. *J. Pharm. Sci.* **1987**, *76*, 387.
34. Lui, E. M. K.; Farmer, P. S.; Dean, C. R. *J. Pharm. Sci.* **1977**, *66*, 950.
35. Bree, F.; Nguyen, P.; Albengres, E.; Uriem, S.; Riant, P.; Welling, P. G.; Tillement, J.-P. *Biochem. Pharmacol.* **1989**, *38*, 753.
36. El-Kemary, M.; Gil, M.; Douhal, A. *J. Med. Chem.* **2007**, *50*, 2896.
37. Lemaire, M.; Tillement, J. P. *Biochem. Pharmacol.* **1982**, *31*, 359.
38. Shah, I. G.; Parsons, D. L. *J. Pharm. Pharmacol.* **1991**, *43*, 790.
39. Birch, A. M.; Johnstone, C.; Plowright, A. T.; Simpson, I.; Whittamore, P. R. O. PCT Int. Appl., WO 2006082400.
40. Bengtsson, M.; Larsson, J.; Nikitidis, G.; Storm, P.; Bailey, J. P.; Griffen, E. J.; Arnould, J.-C.; Bird, T. G. C. PCT Int. Appl. WO2006051270.
41. All crystallographic figures were generated using PyMOL: DeLano, W.L. The PyMOL Molecular Graphics System (2002), DeLano Scientific, San Carlos, CA, USA, http://www.pymol.org.
42. PDB ID code 3LU6.
43. PDB ID code 3LU7.
44. PDB ID code 3LU8.
45. Zunszain, P. A.; Ghuman, J.; McDonagh, A. F.; Curry, S. *J. Mol. Biol.* **2008**, *381*, 394.
46. Carter, D. C.; Ho, J. X.; Wang, Z. International Patent Application WO2005/041895.
47. Carter, D. C.; Ho, J. X.; Wang, Z. International Patent Application WO2007/130950.
48. Bogdan, M.; Pirnau, A.; Floare, C.; Bugeac, C. *J. Pharm. Biomed. Anal.* **2008**, *47*, 981.
49. Mandeville, J.-S.; Froehlich, E.; Tajmir-Riahi, H. A. *J. Pharm. Biomed. Anal.* **2009**, *49*, 468.
50. Singh, S. K.; Kishore, N. *J. Pharm. Sci.* **2008**, *97*, 2362.
51. Joseph, K. S.; Moser, A. C.; Basiaga, S. B. G.; Schiel, J. E.; Hage, D. S. *J. Chromatogr., A* **2009**, *1216*, 3492.
52. Zhang, Q.; Huang, Y.; Zhao, R.; Chen, Y. *Biosens. Bioelectron.* **2008**, *24*, 48.
53. Montgomery, C. P.; New, E. J.; Parker, D.; Peacock, R. D. *Chem. Commun.* **2008**, 4221.
54. Kratochwil, N. A.; Huber, W.; Müller, F.; Kansy, M.; Gerber, P. R. *Biochem. Pharmacol.* **2002**, *64*, 1355.
55. Kratochwil, N. A.; Huber, W.; Müller, F.; Kansy, M.; Gerber, P. R. *Curr. Opin. Drug Discov. Dev.* **2004**, *7*, 507.
56. Votano, J. R.; Parham, M.; Hall, M.; Kier, L. B.; Oloff, S.; Tropsha, A. *J. Med. Chem.* **2006**, *49*, 7169.
57. Artali, R.; Bombieri, G.; Calabi, L.; Del Pra, A. *Il Farmaco* **2005**, *60*, 485.
58. Paal, K.; Shkarupin, A. *Bioorg. Med. Chem.* **2007**, *15*, 7568.
59. Kiefersauer, R.; Than, M.; Dobbek, H.; Gremer, L.; Melero, M.; Strobl, S.; Dias, J. M.; Soulimane, T.; Huber, R. *Appl. Cryst.* **2000**, *33*, 1223.
60. Kroemer, M.; Dreyer, M. K.; Wendt, K. U. *Acta Crystallogr., Sect. D* **2004**, *60*, 1679.
61. Emsley, P.; Kowtan, K. *Acta Crystallogr., Sect. D* **2004**, *60*, 2126.
62. Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta Crystallogr., Sect. D* **1997**, *53*, 240.
63. Winn, M.; Isupov, M.; Murshudov, G. N. *Acta Crystallogr., Sect. D* **2001**, *57*, 122.
64. Laskowski, R.; MacArthur, M.; Moss, D.; Thornton, J. *J. Appl. Crystallogr.* **1993**, *26*, 283.