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Human serum albumin binding assay based on displacement of a non selective fluorescent inhibitor

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Abstract—In this paper, we describe a fluorescent antibacterial analog, **6**, with utility as a competition probe to determine affinities of other antibacterial analogs for human serum albumin (HSA). Analog **6** bound to HSA with an affinity of 400 ± 100 nM and the fluorescence was environmentally sensitive. With 370 nm excitation, environmental sensitivity was indicated by a quenching of the 530 nm emission when the probe bound to HSA. Displacement of dansylsarcosine from HSA by **6** indicated it competed with compounds that bound at site II (ibuprofen binding site) on HSA. Analog **6** also shifted the NMR peaks of an HSA bound oleic acid molecule that itself was affected by compounds that bound at site II. In addition to binding at site II, **6** interacted at site I (warfarin binding site) as indicated by displacement of dansylamide and the shifting of NMR peaks of an HSA bound oleic acid molecule affected by warfarin site binding. Additional evidence for multiple site interaction was discovered when a percentage of **6** could be displaced by either ibuprofen or phenylbutazone. A competition assay was established using **6** to determine relative affinities of other antibacterial inhibitors for HSA.

We recently reported on the discovery of a novel class of antibacterial agents, the anthranilates, which as a class display potent broad-spectrum antibacterial activity.[1] Extensive results from biological assays indicated that high affinity to human serum albumin (HSA) is the main reason for lack of in vivo activity for this series of compounds.[2] HSA is a 66.5 kDa monomeric protein composed of three structurally similar α-helical domains, I through III.[3] These subdomains are further divided into subdomain A containing six α-helices and subdomain B containing four α-helices. Pioneering work on determining the binding sites of drugs indicated that high affinity HSA binding of many drugs occurred at either of two sites referred to as Sudlow sites I (warfarin site) and II (ibuprofen site).[4] These studies involved competitive displacement of fluorescent probes from HSA by various drugs and subsequent binding site classification based on which probes were displaced. Two fluorescent probes were particularly useful for site I and II classification. Dansylsulfonamide (DNSA) bound to HSA with high affinity at site I, K_{d1} = 5.6 μM, and to another site with much lower affinity K_{d2} = 143 μM. Dansylsarcosine bound to just site II with high affinity, K_d = 6 μM. Many of the molecules investigated in the literature preferentially bound at one of the two sites but iopanoic acid bound to both sites I and II. Sudlow's binding data also indicated that probenecid, amitriptyline, and debrisoquine did not bind to either site I or II and they therefore concluded there must be a third high affinity HSA binding site.

We evaluated numerous ways to measure the affinity of our anthranilate analogs to HSA such as chromatographic, spectroscopic, and the displacement of a high affinity ligand from HSA.[5] In addition we utilized the addition of serum to the biological assay.[2] The difference of activity in the presence and absence of serum can be related to HSA affinity.[6] Of these techniques only the addition of serum to our antibacterial assay and the use of the Hummel–Dryer chromatography[7] provided us with data useful for guidance of the medicinal chemistry effort. The characterization of binding of **1** and **2** (Fig. 1) to HSA illustrated that the compounds bound

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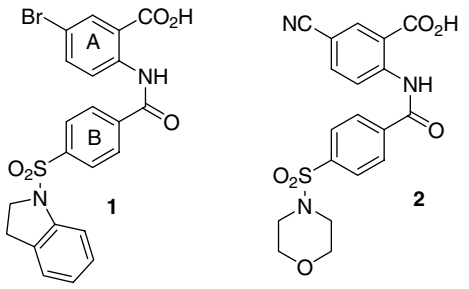


Figure 1. Examples of characterized leads.

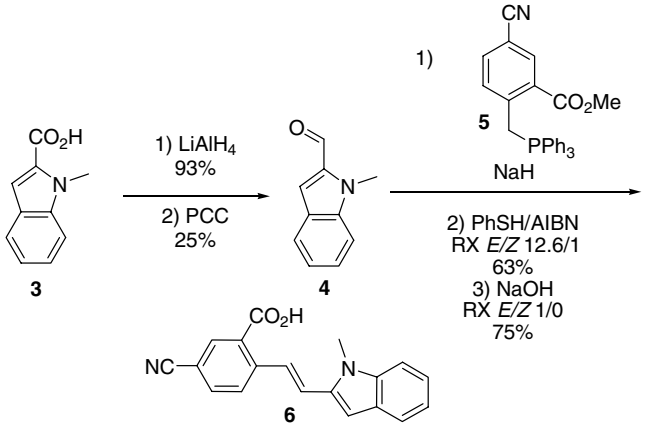
to multiple sites of HSA with high affinity. It was therefore of high desire to develop an HSA affinity assay that could measure overall affinity of our compounds in a rapid reliable manner.[8] In this letter, we describe the discovery of a fluorescent probe, its HSA binding profile, and its utilization in a preliminary 96-well displacement assay.

Identification of fluorescent probe. In a recent disclosure, we discovered that the amide connecting the A and the B rings could be replaced with a styrene linkage.[9] This provided the avenue to prepare an extended conjugated system. We prepared a series of heterocycles in this styrene series both as potential antibacterial agents and as potential fluorescent probes for HSA. The incorporation of indole provided us with an analog **6**, that was visibly fluorescent during preparation and purification. The conversion of indole acid **3** to the aldehyde was easily accomplished (Scheme 1).[10] A screen of numerous reagents to stereospecifically prepare the *E*-alkene was unsuccessful. The use of ylide **5** provided good yield of the styrene but with no preference of olefin geometry. Due to the inability to obtain selective olefination we turned our attention to olefin isomerization utilizing thiophenol and AIBN.[11] The isomerization resulted in ca 12/1 (*E*/*Z*) mixture of olefins and now the desired isomer **6** could easily be recrystallized from MeOH in high yield (Scheme 1).

HSA binding profile of **6**. Ideally, a probe would have a high quantum yield, an excitation wavelength higher than the absorption bands of most other translation inhibitors, and an emission wavelength in a range with little interference. Figure 2 shows the excitation (Fig. 2a) and emission spectra (Fig. 2b) for **6**. The excitation maximum was at 370 nm and the emission maximum was at 533 nm. A linear response in intensity versus concentration of **6** was detected over the range examined.

Titration of 0.5 μM **6** with HSA (fraction V, Calbiochem catalog No. 126658) indicated the fluorescence emission intensity of **6** was environmentally sensitive. The fluorescence intensity of **6** at 533 nm decreased with increasing concentration of HSA and there was a corresponding increase in intensity at 470 nm (data not shown). Fractional saturation of the HSA binding site(s) for **6** can therefore be determined by following the change in fluorescence of the probe as it binds or is released from HSA. Figure 3 shows the average binding isotherm obtained for the interaction of **6** with HSA. The data represent the average isotherm from triplicate titrations of 110 nM **6** with HSA in PBS at 23. °C. These data were well fit using an equation for a single site binding isotherm resulting in an equilibrium dissociation constant, K_d, of 0.39 μM. Although the data fit a single site model, **6** could be displaced by compounds known to bind at either site I or site II on HSA. Ibuprofen, warfarin, diclofenac, and phenylbutazone were each able to displace **6** from HSA. Ibuprofen has an affinity at site II of ~1 μM.[3] Warfarin and phenylbutazone bind at site I with K_d's of 3 and 1.4 μM, respectively. High affinity binding (K_d = 2 μM) of diclofenac was suggested to occur at the benzodiazepine site with the second site (K_d = 17 μM) shared with warfarin.[12]

In addition to the single concentration displacement experiments, displacement of **6** from HSA was examined



Scheme 1.

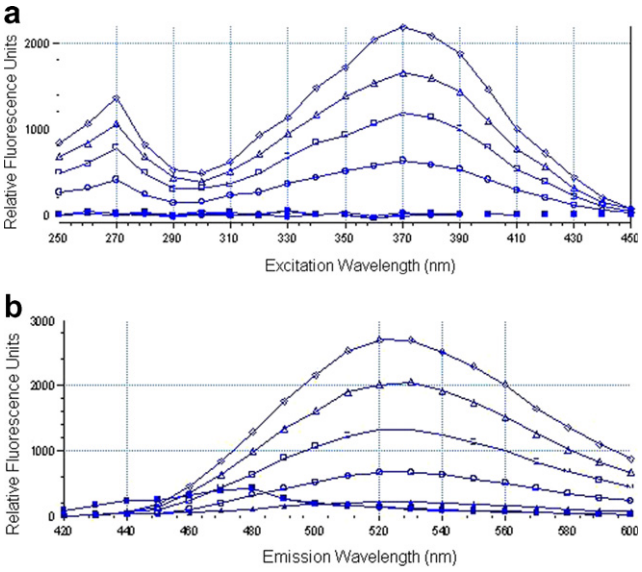


Figure 2. Excitation (a) and emission (b) spectra of **6** at 2.0 μM (diamonds), 1.5 μM (triangles), 1.0 μM (squares), and 0.5 μM (circles) in PBS. Additional spectra shown in each panel are baseline scan of PBS.

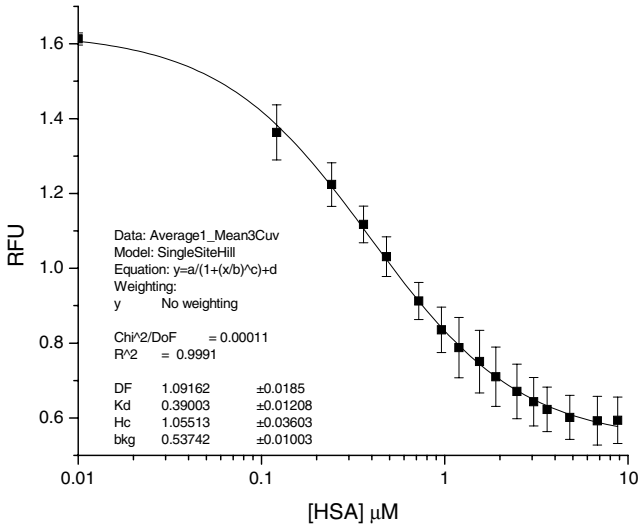


Figure 3. Data points are the average binding isotherm from triplicate titrations of 110 nM **6** with HSA(V) in PBS, pH 7.0, error bars indicate the standard deviations. Excitation was 370 nm with emission monitored at 530 nm. The solid line resulted from least-squares curve fitting of the data using the indicated equation for a single site binding isotherm.

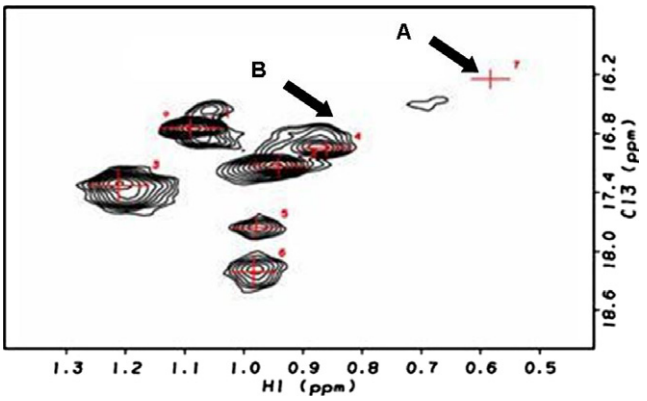


Figure 4. HSQC spectra of ^{13}C-methyl labeled oleic acid complexed with 0.5 mM HSA in 50 mM phosphate solution, pH 7.0. Mole ratio of HSA: Oleic acid:6 = 1:4.2:1.0. Arrow A indicates the disappearance of the resonance for oleic acid bound at the ibuprofen site. Arrow B indicates the resonance for oleic acid bound at the warfarin site. This resonance is altered when the mole ratio of analog **6** is increased to 2.0.

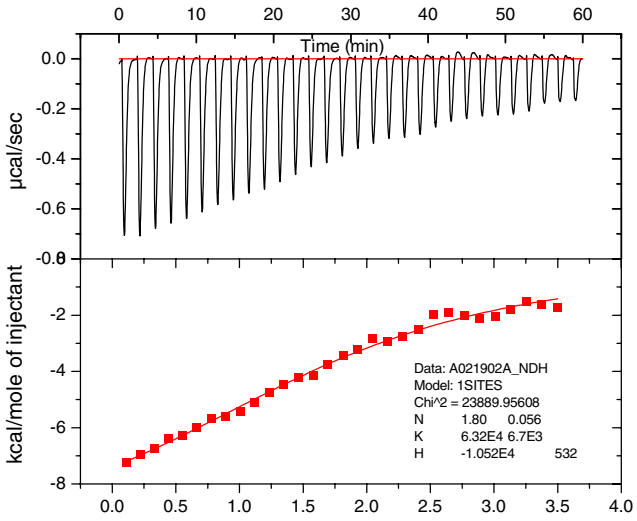


Figure 5. Isothermal titrating calorimetry results for **6** interaction with HSA (V) in PBS. The top plot shows the enthalpy change per 6 μL injection of a 0.5 mM solution of **6** into a 20 μM solution of HSA (V) in PBS at 35 °C. Bottom panel, data points are the integrated enthalpies for each injection and the solid line results from non linear least-squares fitting of the data to the equation for a single site binding model.[14]

by titrating a solution of **6** bound to HSA with ibuprofen or phenylbutazone. The experiment had the starting concentration of **6** at 0.5 μM with 19 μM HSA in PBS. The large molar excess of HSA should ensure binding of **6** at only the high affinity sites on HSA. First, phenylbutazone was titrated into the **6**/HSA solution until little change in fluorescence was detected. Then, ibuprofen was titrated into that solution until the effect was saturated. Ibuprofen was able to displace about 75 to 80% of **6** bound to HSA and about 10% was displaced by phenylbutazone.

Characterization of **6** *binding to HSA by NMR*. A recent report from these laboratories has described an NMR assay for characterization of ligand binding with HSA.[13] The binding of **6** to HSA was also characterized by this NMR technique that uses the displacement of oleic acid. In the presence of **6** at a molar ratio of 1.0:1.0 (HSA: **6**), an oleic acid resonance from the ibuprofen site was displaced (Figure 4, arrow A). While the oleic acid resonance from the warfarin site was shifted at a molar ratio of 1.0:2.0–2.0 (HSA: **6**), oleic acid resonance at arrow B was altered. Therefore, the displacement of oleic acid experiment suggests that **6** can effect binding of oleic acids at both the ibuprofen and warfarin sites.

Characterization of **6** *binding to HSA by ITC*. Figure 5 shows the binding isotherm for the interaction of **6** with HSA as measured by isothermal titrating calorimetry (ITC). The top panel shows the enthalpy change per 6 μL injection of **6** into 20 μM HSA. The heat of dilution of **6** into buffer was negligible. The bottom panel of Figure 5 shows the integrated enthalpy change for each injection and the best fit line using a single site model. The agreement between the model and the experimental data was good and indicated approximately 2 molecules of **6** bound per HSA. The enthalpy change for the interaction was −10.4 kcal/M with an average affinity of 15 μM.

A difference in the K_d determined by ITC compared to the fluorescence method was not surprising given the complexity of the binding interaction. The ITC technique was performed by titrating **6** into HSA until an excess of **6** saturated the binding interaction, while the fluorescence method titrated **6** with HSA until excess HSA saturated the effect. With ITC, the response is due to binding of **6** to HSA at a high affinity site or sites. As these become saturated, lower affinity sites start to fill and the enthalpy change for these interactions is also

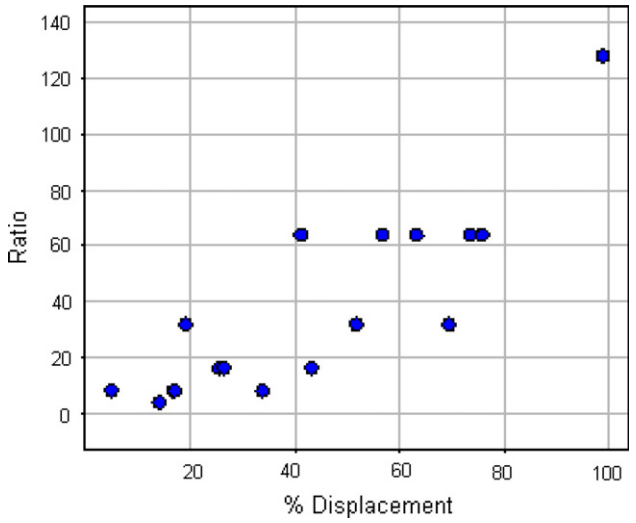


Figure 6. The graph shows the ratio of the antibacterial activity of the series of thioether compounds utilizing the MIC values (vs *S. aureus* 9218) for each compound with and without 10% serum in relation to the displacement of that compound by **6** from HSA.

detected. If binding enthalpies for the various sites are not sufficiently different, the measured enthalpy change is a convolution of enthalpy at each site and no sharp break is detected in the isotherm at set stoichiometries. Such was the case for **6**. With the fluorescence method, HSA is in excess and **6** binding occurs mainly at high affinity sites.

Affinity determined by competitive displacement of **6**. Initially, several translation inhibitors with thioether appendages were examined using the competitive displacement assay.[8] The percentage displacement of 0.5 μM **6** from 1.4 μM HSA for each of the compounds was tested at 12 μM. Larger percentage displacement indicates tighter relative HSA affinity for the compound. K_d's assuming a single site binding model for **6** to HSA were calculated but were considered approximate since **6** binds to more than one site. This was compared to our alternative way of measuring protein binding which was addition of serum to the antibacterial assay, Figure 6. There appears to be a reasonable relationship between the two assay formats but with this new assay the benefit is the direct measurement of HSA affinity irrespective of the antibacterial activity of the compound. This assay was subsequently utilized to screen hundreds of compounds guiding the medicinal chemistry effort going forward.

Conclusion. An HSA affinity assay was developed using a fluorescent antibacterial analog **6**. Compounds that bound at site I or II on HSA competed with **6** for HSA binding. Analog **6** was useful for determining affinities of other antibacterial analogs and helped guide syntheses of compounds with reduced HSA affinity. Good correlations between HSA affinity determined using **6** and the effect of serum on minimum inhibitor concentrations (MICs) were found for several sub-classes of antibacterial compounds. The assay performed in 96-well plates has relatively good sample throughput, ~100 compounds a day in our laboratory and could easily be expanded to a higher density format.

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