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Controlling Plasma Protein Binding: Structural Correlates of Interactions of Hydrophobic Polyamine Endotoxin Sequestrants with Human Serum Albumin

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Abstract: Hydrophobically substituted polyamine compounds, particularly *N*-acyl or *N*-alkyl derivatives of homospermine, are potent endotoxin (lipopolysaccharide) sequestrants. Despite their polycationic nature, the aqueous solubilites are limited owing to the considerable overall hydrophobicity contributed by the long-chain aliphatic substituent, but solubilization is readily achieved in the presence of human serum albumin (HSA). We desired first to delineate the structural basis of lipopolyamine−albumin interactions and, second, to explore possible structure−activity correlates in a well-defined, congeneric series of *N*-alkyl and -acyl homospermine lead compounds. Fluorescence spectroscopic and isothermal titration calorimetry (ITC) results indicate that these compounds appear to bind to HSA via occupancy of the fatty-acid binding sites on the protein. The acyl and carbamate compounds bind HSA the strongest; the ureido and *N*-alkyl analogues are significantly weaker, and the branched alkyl compound is weaker still. ITC-derived dissociation constants are weighted almost in their entirety by enthalpic ΔH terms, which is suggestive that the polarizability of the carbonyl groups facilitate, at least in large part, their interactions with HSA. The relative affinities of these lipopolyamines toward HSA is reflected in discernible differences in apparent potencies of LPS-sequestering activity under experimental conditions requiring physiological concentrations of HSA, and also of in vivo pharmacodynamic behavior. These results are likely to be useful in designing analogues with varying pharmacokinetic profiles.

Keywords: Albumin; plasma protein binding; lipopolyamines; endotoxin sequestrants

Introduction

Lipopolysaccharide (LPS), otherwise termed "endotoxin" (signifying that the toxic is intrinsic to the bacterium, and not externally secreted or elaborated), is the major constituent of the outer leaflet of the outer membrane of all Gram-negative bacteria.[1−3] Lipopolysaccharides, as the name suggests, consist of a highly variable and biologically inert polysaccharide portion and a structurally conserved, and toxically active lipid called lipid A.[4,5] The presence of LPS in blood sets off a cascade of exaggerated systemic inflam-

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matory host response[6] that ultimately manifests clinically in the frequently fatal shock syndrome characterized by endothelial damage, coagulopathy, loss of vascular tone, myocardial dysfunction, tissue hypoperfusion, and multiple-system organ failure.[7−9] Underlying the overwhelming inflammatory response is the activation of the innate immune system,[10,11] which results in the production of a plethora of proinflammatory mediators, important among which are the cytokines tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and IL-6, secreted mainly by monocytes and macrophages (Mϕ).[12−15]

We have been exploring the feasibility of targeting lipopolysaccharide by agents that would bind to the lipid A moiety and sequester it,[16−26] thereby preventing its recognition by LPS receptors[27−34] on the monocyte/macrophage and other effector cells. Our efforts have converged on simple hydrophobically substituted polyamine compounds, particu-

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larly *N*-acyl or alkyl derivatives of homospermine.[16,17,23–25] Despite their polycationic nature, the aqueous solubilites of even the trifluoroacetate or hydrochloride salts of these compounds are limited owing to the considerable overall hydrophobicity contributed by the long-chain acyl or alkyl substituent;[23–25] however, solubilization is readily achieved in the presence of human serum albumin (HSA), which allowed us to characterize in substantial detail the in vitro and in vivo activities of these compounds.[23–25]

While it is not altogether remarkable that albumin, with its extraordinary ligand-binding properties,[35,36] acts as a convenient drug carrier[37] for our LPS-sequestering molecules, given that plasma protein binding significantly impacts on both pharmacokinetic (plasma half-life and volume of distribution[38−41]) and pharmacodynamic (altered efficacy due to varying free drug concentrations[42]) outcomes, we desired first to delineate the structural basis of "drug"−albumin interactions and, second, to explore possible structure−activity correlates in a defined set of compounds. We have focused our studies on a congeneric series of *N*-alkyl and -acyl homospermine leads which have been shown to be active in sequestering LPS in vitro, and conferring dose-dependent protection against lethal endotoxemia in a murine model of sepsis.[23–25] Our results indicate that these compounds appear to bind to HSA via sequential occupancy of the fatty-acid binding sites, and that the affinity for HSA of *N*-acyl compounds is stronger than that of *N*-alkyl compounds. This is reflected in discernible differences in apparent potencies of LPS-sequestering activity under experimental conditions requiring physiological concentrations of HSA. These results are likely to be useful in designing analogues with varying pharmacokinetic profiles.

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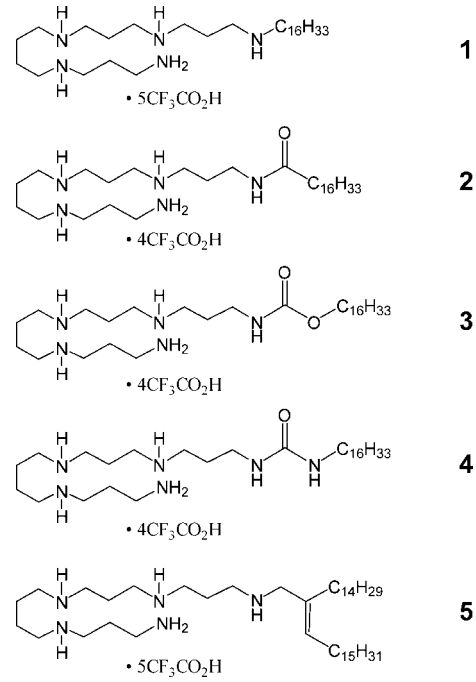


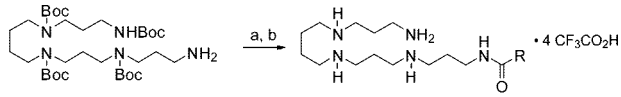
Figure 1

Materials and Methods

Syntheses of Polyamine Derivatives. The syntheses and characterization of **1**, **2**, and **5** have been published (Figure 1).[23–25] All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture or air sensitive reactions were conducted under argon atmosphere in oven-dried (120 °C) glass apparatus. THF was distilled from sodium benzophenone ketyl, while dichloromethane was distilled over calcium hydride, prior to use. Solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using silica gel 60 (230−400 mesh), while thin layer chromatography (TLC) was carried out on silica gel HLF, precoated glass plates. All yields reported refer to isolated material judged to be homogeneous by TLC and NMR spectroscopy. Unless noted otherwise, NMR spectra were recorded with the chemical shifts (δ) reported in ppm relative to Me_4Si (for ^1H) and $CDCl_3$ (for ^{13}C) or DMSO-d_6 (for ^{13}C) as internal standards respectively.

Compound 3. To the strategically Boc-protected homospermine precursor[23] (203 mg, 0.31 mmol) in EtOAc (4 mL) and saturated aqueous $NaHCO_3$ solution (4 mL) was added cetylchloroformate (0.5 g, 1.5 mmol) dissolved in ethyl acetate (2 mL), and the mixture was stirred overnight at room temperature (Scheme 1). The mixture was extracted with ethyl acetate (3 × 25 mL). The combined extract was washed with brine and dried (anhydrous Na_2SO_4) and solvent removed under vacuum. The residual liquid thus obtained was treated with CF_3CO_2H (15 mL) and the solution stirred at room temperature overnight. Excess solvent was removed in a rotary evaporator and the residue dried in high vacuum. The viscous residue obtained was triturated with CH_2Cl_2 to yield the pure carbamate derivative **3** as a white solid (176 mg, 58%): ^1H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, J =

Scheme 1



N^1,N^2,N^3,N^4-*tetra*-Boc-homospermine

3, R = $OC_{16}H_{33}$ (58%, two steps)
4, R = $NHC_{16}H_{33}$ (72%, two steps)

Reagents: a. $H_{33}C_{16}OCOCl$, EtOAc, aq. $NaHCO_3$ (for **3**), or, $H_{33}C_{16}NCO$, THF (for **4**). **b**. CF_3CO_2H (excess, room temperature).

6.6 Hz, 3H), 1.23 (s, 28H), 1.51−1.71 (m, 5H), 1.89 (br s, 4H), 2.85−3.03 (br m, 16H), 3.91 (t, $J = 6.6$ Hz, 3H) 8.7(br m, 4H); ^{13}C NMR (100.6 MHz, DMSO-d_6) 13.9, 22.0, 25.3, 28.6, 28.7, 28.9, 29.0, 31.2, 36.1, 39.0, 43,8, 46.1, 63.7; MS (FAB) calcd for $C_{30}H_{65}N_5O_2$ (free amine) *m/z* 527.8, found 528.5 $(MH)^+$.

Compound 4. To a solution of the amine **1** (104 mg, 0.16 mmol) in anhydrous THF (3 mL) was added hexadecylisocyanate (55 mg, 0.2 mmol) dissolved in anhydrous THF (2 mL). The resulting solution was stirred at room temperature for 5 h. The reaction was quenched by the addition of water (10 mL) and the mixture extracted with ethyl acetate (3 × 25 mL). The combined extract was washed with brine and dried (anhydrous Na_2SO_4) and solvent removed under vacuum. The residual liquid thus obtained was treated with CF_3CO_2H (8 mL) and the solution stirred at room temperature overnight. Excess solvent was removed and the residue dried in high vacuum. The viscous solid thus obtained was triturated with CH_2Cl_2 to yield the urea derivative **4** as a white solid (113 mg, 72%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.83 (t, $J = 6.8$ Hz, 3H), 1.23 (s, 28H), 1.63 (br s, 6H), 1.89−1.91(m, 4H), 2.86−3.05 (br m, 18H); ^{13}C NMR (100.6 MHz, DMSO-d_6) 14.3, 22.4, 24.1, 26.1, 29.0, 29.2, 29.3, 29.4, 31.6, 31.4, 36.5, 39.3, 44.2, 46.5; MS (FAB) calcd for $C_{30}H_{66}N_6O$ (free amine) *m/z* 526.8, found 527.6 $(MH)^+$.

Reagents. Defatted human serum albumin (HSA), warfarin, dansylsarcosine (DS), and *cis*-parinaric acid were procured from Sigma-Aldrich, Inc. (St. Louis, MO). NBD-labeled dodecanoic acid (NBD-DD) was from Molecular Probes (Eugene, OR).

Fluorescence Spectroscopy. Dansylsarcosine displacement assays to quantify the affinities of binding of compounds to HSA have been described earlier.[43] This assay was adapted to a microtiter plate format wherein the first column of a 384-well plate, containing replicate wells of 80 μL (1 mM stock in DMSO) of the polyamine compounds, was serially 2-fold diluted across the remaining 23 columns. 40 μL of a mixture of HSA (50 μM) and DS (100 μM) in 50 mM Tris buffer, pH 7.4, was then added to each well and allowed to equilibrate for 15 min. All liquid handling was performed on a Precision 2000 automated microplate pipetting system (Bio-Tek Instruments Inc., VT). Fluorescence measurements were made at 25 °C on a Fluoromax-3 with Micromax Microwell 384-well plate reader, using DataMax software (Jobin Yvon Inc, NJ). The excitation and emission wavelengths for the DS experiments were 350 and 475 nm, respectively, with both emission and excitation monochromator bandpasses set at 5 nm. Effective displacements (ED_{50}) were computed at the midpoint of the fluorescence signal versus compound concentration displacement curve, determined using an automated four-parameter sigmoidal fit utility of the Origin plotting software (Origin Laboratory Corp., MA). NBD-DD displacement experiments were similarly performed in Tris buffer with 20 μM HSA and 250 μM of the fluorescent probe with excitation/emission wavelengths of 455/538 nm.

Isothermal Calorimetry (ITC). ITC experiments were performed using a VP-ITC microcalorimeter (Microcal Inc., MA) as described earlier.[23,44] A typical titration experiment involved 35 consecutive injections at 360 s intervals consisting of 3 μL injections of the polyamine derivatives as 3 mM stocks in DMSO into the sample cell (cell volume: 1.4119 mL) containing 0.025 mM HSA, in Tris buffer (pH 7.4, 50 mM), at 37 °C. The titration cell was stirred continuously at 310 rpm. Appropriate control experiments (DMSO alone injected into buffer containing HSA) were performed for each experiment to correct for heats of dilution of DMSO. The resulting data were then analyzed using Microcal's ITC data analysis package, VP Viewer 2000, which uses the scientific plotting software, Origin 7 (Origin Laboratory. Corp., MA).

Inhibition of LPS-Induced NF-κB Induction. The inhibition of induction of NF-κB (a key transcriptional activator of the innate immune system) was quantified using human embryonic kidney 293 cells cotransfected with TLR4 (LPS receptor), CD14 and MD2 (coreceptors), available from InvivoGen, Inc. (HEK-Blue, San Diego, CA), as described elsewhere. Stable expression of secreted alkaline phosphatase (seAP) under control of NF-κB/AP-1 promoters is inducible by LPS, and extracellular seAP in the supernatant is proportional to NF-κB induction. HEK-4 cells were incubated at a density of 10^5 cells/mL in a volume of 80 μL/well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently stimulated with 10 ng/mL LPS. Concurrent with LPS stimulation, serially diluted concentrations of test compounds were added to the cell medium using a rapid-throughput, automated protocol employing a Bio-Tek P2000 liquid handler as described above, and left to incubate overnight. Polymyxin B was used as reference compound in each plate. Positive (LPS stimulation only) and negative controls (HEK-detection medium only) were included in each experiment. seAP was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 620 nm.

TNF-α Release Assay ex Vivo in Human Blood. 100 μL aliquots of fresh whole blood, anticoagulated with EDTA, obtained by venipuncture from healthy human volunteers

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with informed consent and as per guidelines approved by the Human Subjects Experimentation Committee, was exposed to an equal volume of 20 ng/mL of *Escherichia coli* 0111:B4 LPS, with graded concentrations of test compounds diluted in saline for 4 h in a 96-well microtiter plate. The effect of the compounds on modulating TNF-α production was examined using a FACSArray multiplexed flow-cytometric bead array (CBA) system (Becton-Dickinson-Pharmingen, San Jose, CA) as described elsewhere.[23−25]

Results and Discussion

The highly conserved multidomain structure of mammalian albumins enables these molecules to serve as carrier proteins for a wide range of chemically dissimilar molecules, including otherwise insoluble ligands such as fatty acids, drugs, metals, and bilirubin.[45] Warfarin and dansylsarcosine (DS) have long been employed as fluorescent probes which report on two important ligand binding sites,[46,47] originally termed Sites I and II, respectively, which have been assigned to domains II-A and III-A, based on the high-resolution crystal structure of HSA.[45] Fluorimetric titrations of the polyamine derivatives with HSA precomplexed with warfarin showed little changes (data not shown), while a clear concentration-dependent decrease in HSA-associated DS intensity was observed (Figure 2A). We further verified that the decrease in fluorescence emission intensity was a consequence of true displacement of HSA-bound DS by demonstrating that (i) indirect excitation of DS (Förster resonance energy transfer) via the lone tryptophan residue (W214)[43] of HSA also resulted in similar concentration-dependent quenching; (ii) this was accompanied by a commensurate decrease in the steady-state polarization and anisotropy values of DS emission; (iii) incremental increases in DS/HSA ratios resulted in progressive shifts to the right of the quench curves as would be expected due to mass action-driven Schild effects[48] (data not shown).

Although these data are unequivocally indicative of true complexation of the polyamine derivatives with HSA, we were not clear whether DS displacement was due to bona fide occupancy of domain III-A by the polyamine derivatives, or due to occupancy at a different site with consequent allosteric changes that secondarily displace DS as has been

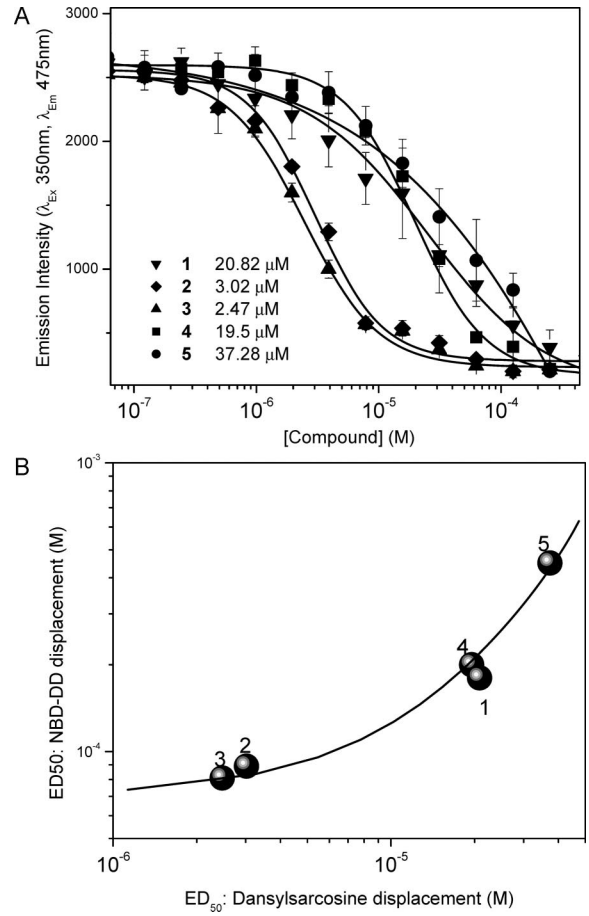

Figure 2

observed, for instance, with fatty acids[49] and lipid A.[43] Our original doubts were heightened by the fact that Horovitz−Levitzki computations[50] of the displacement data shown in Figure 2A yielded an apparent polyamine/HSA stoichiometry of ∼5:1, which was inconsistent with domain III-A occupancy, but rather suggested that the interaction could be mediated via the fatty acid binding sites since the crystal structure of HSA complexed with long chain fatty acids (myristic acid) has revealed five binding sites distributed asymmetrically throughout the protein, each of which is characterized by a long hydrophobic pocket capped with polar side chains at the portal, many of which are basic.[51]

We therefore wished to examine if the interactions of the polyamine derivatives with HSA could be experimentally verified both in terms of the binding site per se, as well as the stoichiometry of the resultant complex. First, we used

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fluorescently labeled dodecanoic acid (NBD-DD)[52,53] which is known to bind to the fatty acid site in HSA. We found that there was indeed a correlation between DS and NBD-DD displacement potencies (Figure 2B); the relationship was quasilinear, as would be expected due to the nonequivalence of the microscopic association constants of the individual sites.[43] Similar results were also obtained with *cis*-parinaric acid, a conjugated polyene probe for the fatty acid binding site[54,55] (data not shown). While these data strongly implicated the involvement of the fatty acid binding sites, we were keen to obtain confirmatory evidence using an independent technique. We elected to use isothermal titration calorimetry (ITC) which is not prone to the artifacts that fluorescence spectroscopy is occasionally beset by, and the technique has been used in the past to characterize fatty-acid−albumin interactions.[56−60] As shown in Figure 3A as a representative example, **2** binds HSA with a K_a of 5.05×10^5 (K_d: 1.98 μM) and a stoichiometry of 5 relative to HSA; the binding sites are apparently equivalent since the isotherm could be fit cleanly with a monotonic sigmoidal function. Notably, the binding is enthalpically driven (ΔH: -1.128×10^4 cal/mol), which is possibly a consequence of significant electrostatic or dipole−dipole interactions[61] of the amide functionality with polar residues lining the entrance to the hydrophobic cavity in HSA. Previously observed calorimetric parameters for free fatty acid:HSA complexes indicate an even higher enthalpic component; for instance, ΔH for palmitic acid binding to albumin has been reported to be

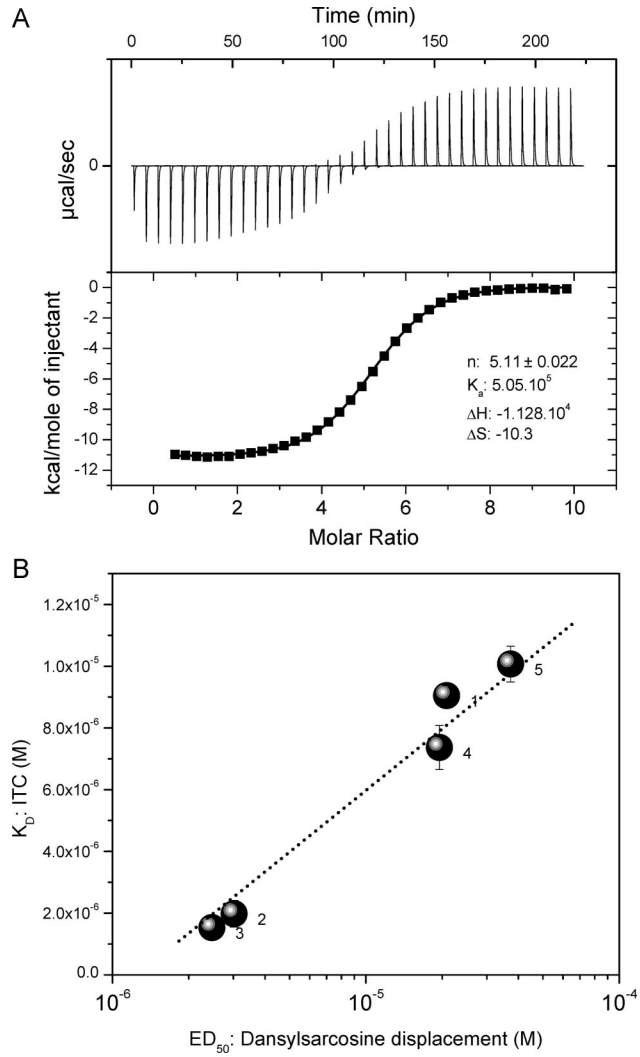

Figure 3

38.9 ± 2.6 kJ/mol (9.3 ± 0.62 kcal/mol).[57] Measures of hydrophobic effects will have to await ΔC_p measurements. ITC data were obtained on all five compounds which showed an excellent correlation with both DS (Figure 3B) as well as NBD-DD displacement data (data not shown).

An examination of the rank-order of the affinity toward HSA (Figures 2 and 3) indicates that the acyl and carbamate compounds (**2** and **3**, respectively) bind HSA the strongest; the ureido (**4**) and *N*-alkyl (**1**) analogues are significantly weaker, and the branched alkyl compound (**5**) is weaker still. ITC-derived dissociation constants are weighted heavily by enthalpic ΔH terms, which is suggestive that the polarizability (or the absence) of the carbonyl groups facilitates, at least in large part, their interactions with HSA. Consistent with this hypothesis is the observation that the affinity of the carbamate is significantly higher than that of the urea compound. The more extensive delocalization in the latter would be expected to render the ureido carbonyl much less polarizable.

It was of interest to examine if differential interactions with HSA would be contributory to their biological activity *in vitro*. We have established in considerable detail and depth

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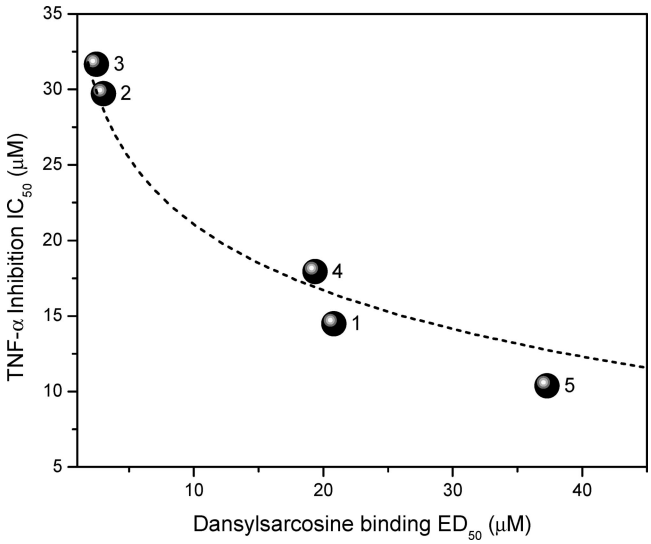

Figure 4

that the free compound effectively sequesters LPS by binding to the lipid A moiety.[21–25,62] We therefore compared the LPS neutralization potencies of these compounds in assays employing whole human blood ex vivo wherein physiological concentrations of albumin (∼5 g/dL) are maintained throughout the experiment. A distinct correlation between HSA binding (DS displacement IC_{50} value) and the potency in inhibiting TNF-α was observed, with the acyl and carbamate compounds being the least potent (Figure 4).

Strong interactions with HSA would be predictive of a longer plasma $t_{1/2}$ and lower volume of distribution, the *N*-acyl insulin Detemir being an excellent case in point.[63,64] These data, collectively, would predict that compounds **2** and **3** would exert a significantly longer duration of action in vivo than **1** or **5**. Although pharmacokinetic data (in rodents) is available as yet only for **1**,[65] a comparison of time-course of protection (pharmacodynamic) data in a murine model of lethal endotoxemia is indeed suggestive that **3**[23] is longer-lived than **1**[25] or **5**.[24] The application of these results may be helpful in the development of analogues with controlled PK/PD parameters.

These studies may be applicable to drug discovery and development in general in that appending appropriately positioned long-chain acyl groups to highly polar or water-soluble compounds exhibiting rapid clearance may result in significant increases in intravascular retention and improved pharmacokinetic profiles by virtue of enhanced plasma protein binding. These experiments have also been instructive for us in that it has served to emphasize and reinforce what ought to be axiomatic: that binding affinity (often obtained via homogeneous assays in buffers) alone is often not an adequate determinant of biological activity, and relatively simple, but frequently overlooked parameters such as measurements of protein binding may be valuable in guiding the process of exploring structure−activity relationships.

Abbreviations Used

DS, dansylsarcosine [2-(1-(dimethylamino)-*N*-methylnaphthalene-5-sulfonamido)acetic acid]; HSA, human serum albumin; ITC, isothermal titration calorimetry; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; NBD-DD, 12-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoic acid; PBS, phosphate-buffered saline; PMB, polymyxin B; seAP, secreted alkaline phosphatase; TNF-α, tumor necrosis factor-alpha.

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