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Rational Design of Diflunisal Analogues with Reduced Affinity for Human Serum Albumin

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Abstract: Many lead compounds bind to serum albumin and exhibit markedly reduced efficacy in vivo as compared to their potency in vitro. To aid in the design of compounds with reduced albumin binding, we performed nuclear magnetic resonance (NMR) structural and binding studies on the complex between domain III of human serum albumin (HSA-III) and diflunisal, a cyclooxygenase inhibitor with antiinflammatory activity. The structural studies indicate that the aromatic rings of diflunisal are involved in extensive and specific interactions with hydrophobic residues that comprise the binding pocket in subdomain IIIA. The carboxylic acid of diflunisal forms electrostatic interactions with the protein similar to those observed in the X-ray structure of HSA complexed to myristic acid. In addition to the structural studies, NMR-derived binding constants were obtained for diflunisal and closely related analogues to develop a structure-affinity relationship for binding to subdomain IIIA. On the basis of the structural and binding data, compounds were synthesized that exhibit more than a 100-fold reduction in binding to domain III of HSA, and nearly a 10-fold reduction in affinity for full length albumin. Significantly, several of these compounds maintain activity against cyclooxygenase-2. These results suggest a rational strategy for designing out albumin binding in potential drug molecules by using structure-based design in conjunction with NMR-based screening.

Introduction

Human serum albumin (HSA) is the most abundant protein in serum plasma and is capable of binding to a diverse range of endogenous and exogenous compounds.[1,2] These properties of HSA can markedly affect a compound's in vivo distribution and efficacy and hinder many potential pharmaceutical agents from reaching their target site. Among the three homologous domains in HSA (I, II, and III), domain III harbors the primary binding site for medium-long chain fatty acids,[3−5] and has high affinity for small anionic aromatic compounds[2,6,7] that often represent pharmaceutical agents or drug leads. Thus, efforts to reduce the albumin binding of potential drug candidates must address the binding properties of HSA-III.

With the advent of high-resolution NMR spectroscopy and X-ray crystallography, the structure-based design of compounds with increased affinity for a molecular target is now com-

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monplace in the pharmaceutical industry.[8−11] Guided by detailed structural information on protein−ligand complexes, structural modifications can be designed to maximize the contacts between a protein and a small molecule or, alternatively, alter the chemical properties (e.g. solubility) without disrupting binding. In principle, this same approach can be used to design compounds with *decreased affinity* for HSA-III. To accomplish this, structural information is required to determine how a particular compound class binds to HSA-III along with knowledge of the structure-affinity relationships to understand the individual interactions that stabilize complex formation. However, the rational design of compounds with reduced albumin binding has been limited due to the lack of binding data to individual sites on albumin and the lack of structural information on HSA/ligand complexes. In fact, the only structures of HSA/ligand complexes that are publicly accessible are the HSA/myristate structure[4] and the recently described crystal structures of HSA complexed to anesthetics.[12]

To define how small anionic compounds bind to domain III of HSA with the aim of designing molecules that have reduced binding, the structures of HSA/ligand complexes and the binding affinities of compounds for the individual sites on HSA are needed. Here we illustrate how NMR can be used to provide this information. As an example, we have studied the interaction of diflunisal with domain III of HSA and have measured the binding affinities of diflunisal analogues using NMR. Diflunisal is an aspirin-like nonsteriodal antiinflammatory drug that inhibits cyclooxygenase-2 (COX-2), an enzyme involved in prostaglandin synthesis.[13] It is superior to aspirin as an antiinflammatory agent because of its higher potency, better in vivo tolerance, and longer duration of action.[14] However, it is 99% protein-bound in plasma, necessitating a regimen of at least 250 mg twice daily or more for efficacy.[15] In addition to cost, such high dosing levels can lead to undesirable side effects, such as gastrointestinal irritation during chronic therapy. Thus, compounds with reduced affinity for serum albumin may result in drugs with significantly lower dosing levels and improved in vivo tolerance. In this paper, we delineate the specific interactions that stabilize the HSA-III/diflunisal complex, and use this information to design compounds that have significantly reduced affinity for both domain III of HSA and full-length human serum albumin.

Results and Discussion

Location of the Diflunisal Binding Site. By using a standard suite of ^{2}H-decoupled triple resonance experiments (see the Materials and Methods section), greater than 95% of the backbone assignments for free and diflunisal-bound HSA-III were obtained. The secondary structure predicted for the HSA-III/diflunisal complex based on C_α and C′ chemical shifts and $H_N(i)$-$H_N(i+1)$ NOEs (Figure 1) agrees well with that of the crystal structure of HSA/myristic acid complex.[4] Helix α1, which shows the most significant difference, is a continuous helix connecting domains II and III in the crystal structure of the full-length protein, but only part of the helical conformation is observed for HSA-III as a result of cloning truncation. Overall, these data indicate that the solution structure of HSA-III is very similar to that observed in the crystal structure of full-length albumin.

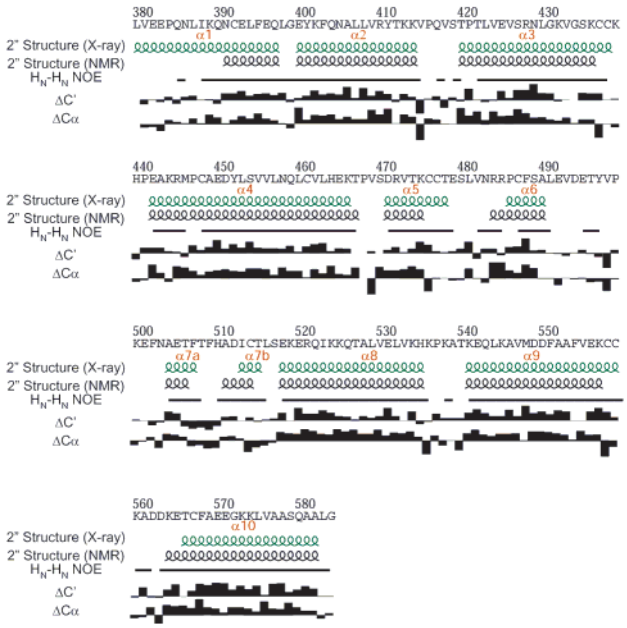

Figure 1. Summary of NMR sequential $H_N(i)$-$H_N(i+1)$ NOEs and C′ and C_α chemical shift indexes (CSIs) for diflunisal-bound HSA-III. The CSIs are derived from the comparisons to the random coil values published by Wishart et al.[28] The secondary structure predicted the H_N-(i)-$H_N(i+1)$ NOEs and chemical shift indexes are shown in black. The secondary structure observed in the crystal structure of myristate-bound HSA[4] is in green.

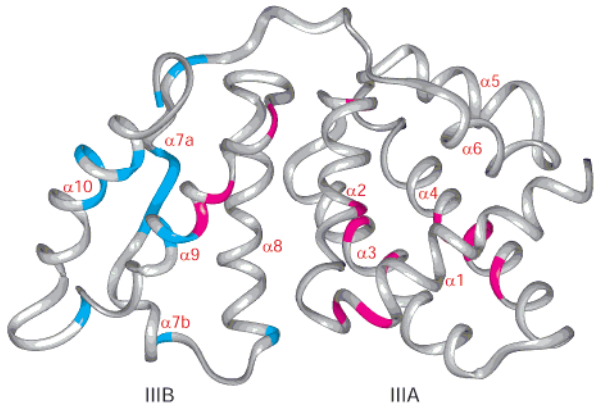

Figure 2. Chemical shift perturbations observed in HSA-III upon binding to diflunisal. Residues whose backbone ^{15}N and ^{1}H chemical shifts are affected by high-affinity ligand binding are highlighted in magenta. Those affected by weak-affinity binding are in cyan. The crystal structure shown is that of apo HSA.[6]

To identify the diflunisal binding sites on HSA-III, perturbations in the ^{15}N/^{1}H HSQC spectra of the protein were monitored as a function of added diflunisal. The pattern of chemical shift changes indicates that there are at least two diflunisal-binding sites on HSA-III: a high affinity site located in subdomain IIIA ($K_D = 17\ \mu$M) and a weaker binding site located in subdomain IIIB ($K_D \geq 1000\ \mu$M) (Figure 2). The high-affinity site in subdomain IIIA is the same region of HSA that is occupied by two myristic acid molecules (Myr 3 and Myr 4) in the crystal

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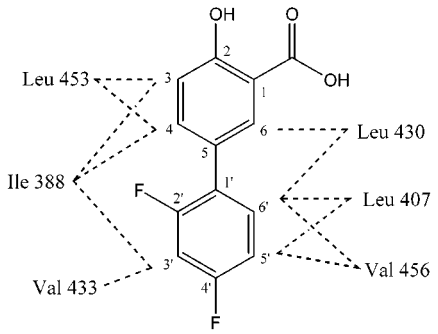


Figure 3. NOEs identified between diflunisal and methyl groups of valine, leucine, and isoleucine of HSA-III.

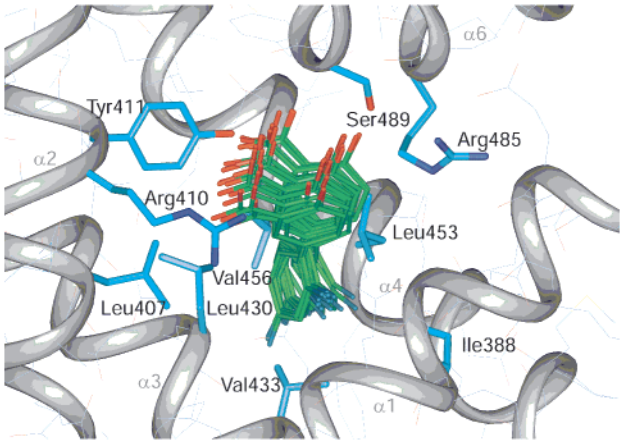


Figure 4. Superposition of 18 low-energy structures of diflunisal (green carbon atoms) complexed to HSA-III (gray ribbons). The selected side chains (cyan carbon atoms) of the averaged minimized HSA-III structure are also shown.

structure of the HSA/myristic acid complex, while the low-affinity site in subdomain IIIB contains a single myristic acid (Myr 5).[4]

Structural Characterization of HSA-III/Diflunisal Complex. Examination of the crystal structure of HSA shows that subdomain IIIA is populated with valine, leucine, and isoleucine residues, whose methyl groups may potentially make contacts with diflunisal. Thus, to rapidly identify the intermolecular contacts between diflunisal and HSA-III, we used a sample of perdeuterated HSA-III in which the valine, leucine, and isoleucine (δ1 only) residues contained ^{13}C-labeled methyl groups.[16] A total of 19 intermolecular NOEs were assigned between the aromatic protons of diflunisal and the methyl protons of valine, leucine, and isoleucine (Figure 3). By using these NOEs, diflunisal was docked into the crystal structure of HSA-III. The NOE data unambiguously orient diflunisal in subdomain IIIA. A superposition of 18 low-energy structures of diflunisal bound to HSA-III is shown in Figure 4. The pairwise root-mean-square deviation (rmsd) of diflunisal heavy atoms is approximately 1.3 Å, and the rmsd from the mean structure is 0.9 Å.

Diflunisal binds to the hydrophobic cavity formed by five helices (α1, α2, α3, α4, and α6) within subdomain IIIA (Figure 4). The carboxyl and hydroxyl groups of diflunisal point toward the hydrophilic entrance while the two aromatic rings penetrate into a deep hydrophobic cavity. The carboxyl group of diflunisal is in close proximity to Arg 410 and Tyr 411 from helix α2 and can potentially interact with these residues through electrostatic contacts and hydrogen bonds. The hydroxyl group of diflunisal is also within hydrogen bonding distance to Ser 489 from helix α6. Diflunisal is stabilized by extensive hydrophobic interactions in the binding cavity. The aromatic rings of diflunisal are packed against hydrophobic residues Leu 387 and Ile 388 in helix α1, Leu 407 in helix α2, Leu 430 and Val 433 in helix α3, and Leu 453 and Val 456 in helix α4.

Comparison to the Crystal Structure. When compared to the crystal structure of the HSA/myristic acid complex, similarities can be drawn between the binding of diflunisal and the two myristic acids in subdomain IIIA (Figure 5). In the crystal structure, the carboxyl group of Myr 4 makes electrostatic and hydrogen bonding contacts with Arg 410 and Tyr 411 from helix α2 and Ser 489 from helix α6.[4] Comparable interactions are available to the salicylic acid moiety of diflunisal. Arg 410, Tyr 411, and Ser 489 are highly conserved in the sequences of nine mammalian serum albumins,[5] indicating the importance of electrostatic and hydrogen bonding interactions at this location. In addition, the salicylic ring of diflunisal partially

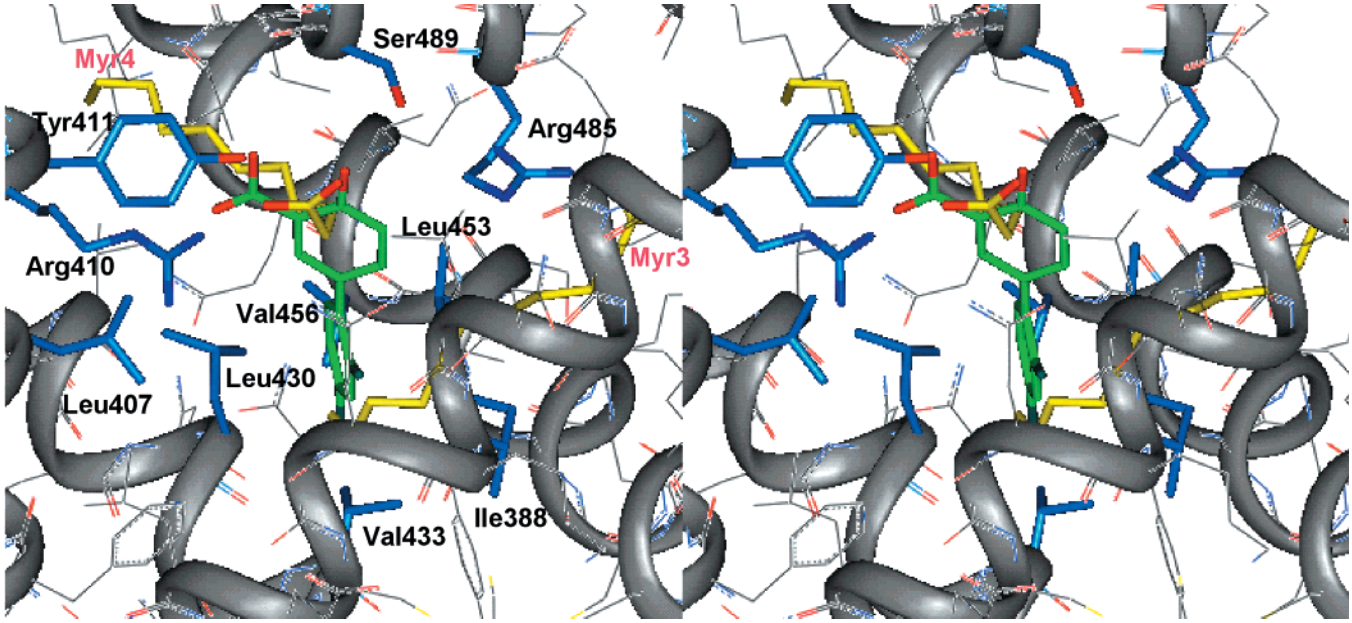
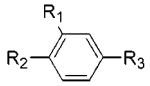


Figure 5. Comparison of the binding between diflunisal and two fatty acids in subdomain IIIA. The coloring schemes are similar to Figure 4, and the carbon atoms of the myristic acids are in yellow.

Table 1. NMR-Derived Binding Constants for Diflunisal Analogs to HSA-IIIA



No.	R_1	R_2	R_3	NMR K_D (μM)
1	CO_2H	OH		17 ± 4
2	CO_2H	OH		10 ± 2
3	CO_2H	OH	H	171 ± 51
4	CO_2H	OH		38 ± 17
5	CO_2H	OH		49 ± 14
6	CO_2H	OH	Cl	47 ± 17
7	CO_2H	OH	NH_2	> 1000
8	H	OH		105 ± 30
9	NH_2	OH		> 1000
10	CO_2H	H		4 ± 3
11	H	CO_2H		5 ± 1
12	CO_2H	H		161 ± 27
13	CO_2H	H		128 ± 11
14	H	CO_2H		> 1000
15	H	CO_2H		> 1000
16	H	CO_2H		> 1000
17	CO_2H	H		172 ± 23
18	CO_2H	H		324 ± 83
19	CO_2H	H		42 ± 2

occupies the position of the aliphatic side chain of Myr 4 and the difluorophenyl group forms interactions similar to the aliphatic tail of Myr 3. The aromatic rings of diflunisal and the aliphatic chains of the myristates both interact extensively with the hydrophobic residues in the binding pocket.

Structure-Affinity Studies of Diflunisal Analogues. From the structural model of the HSA-III-diflunisal complex, both electrostatic and hydrophobic interactions appear to be important for diflunisal binding to subdomain IIIA. To delineate the relative contributions of these interactions, NMR derived dissociation constants were obtained for a set of diflunisal analogues (Table 1). Diflunisal exhibits a dissociation constant of 17 μM for HSA-IIIA. Removal of the fluoro groups (**2**) did not significantly affect the dissociation constant, indicating that they play little role in binding to HSA-IIIA. However, complete removal of the phenyl ring resulted in nearly a 20-fold reduction in albumin binding (e.g., compare **2** with **3**), supporting the important role of the hydrophobic interactions observed in the structure. The nature of these interactions is not limited to aromatic rings, since compounds with an aliphatic (**4** and **5**) or a chloro group (**6**) at this position also bind tightly to HSA-IIIA. In contrast, substitution of an amino group severely reduces binding to this site (e.g., compare **2** with **7**).

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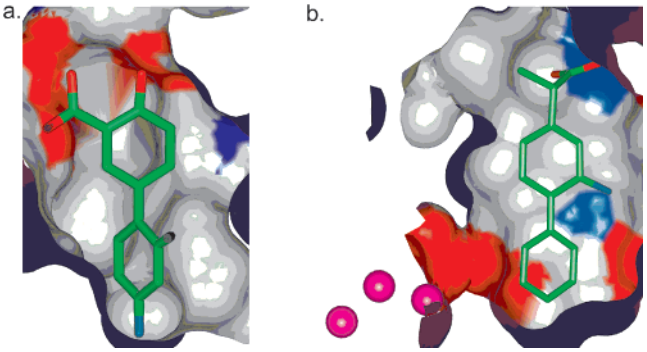


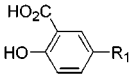
Figure 6. Surface representations of the (a) diflunisal binding pocket in HSA-IIIA and the (b) flurbiprofen binding pocket in cyclooxygenase (PDB accession number 1CQE). Diflunisal and flurbiprofen are shown in sticks. The surface is colored by atom type (carbon in gray, oxygen in red, and nitrogen in blue). Three water molecules near the flurbiprofen binding site are shown as magenta balls.

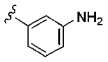
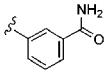
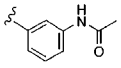
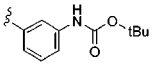
Electrostatic interactions also appear to be important in stabilizing the HSA-III-diflunisal complex. Removal of the carboxyl group results in a 10-fold decrease in binding affinity (e.g., compare **2** with **8**). Furthermore, the aniline analogue exhibits a more than 100-fold reduction in affinity (e.g., compare **2** with **9**). This supports the critical role of the electrostatic interaction between the carboxyl group and Arg 410 observed in the structure. Unlike the carboxyl group, the hydroxyl group is not as important for complex formation (e.g., compare **2** with **10**). In addition, the position of the carboxyl group does not appear to be important, since the meta (**10**) and para (**11**) substituted biphenyl acids have comparable activity.

In addition to the removal of the carboxylic acid and difluorophenyl ring of diflunisal, other modifications appeared to reduce binding to HSA-IIIA. For example, biphenyl acid compounds that contain either anilines (e.g., compare **10** with **12** and **13**), amides (e.g., compare **11** with **14**), or charged amines (e.g., compare **11** with **15** and **16**) on the bottom ring exhibited 50−200-fold reductions in affinity for HSA-III. This is supported by the structure of the diflunisal/HSA-III complex in which the difluorophenyl ring sits deep within a hydrophobic cavity. In addition to polar substitutions, the hydrophobic packing of diflunisal can also be disrupted to reduce albumin binding. For example, the structural data indicate that the C6, C5′, and C6′ positions of diflunisal are tightly packed against the protein (Figure 6a) and binding may be sensitive to substitution at these positions. Indeed, compounds with a 3′,5′ substitution pattern (e.g., **17** and **18**) bind to HSA-III 4−8-fold more weakly than those with a 2′,4′ substitution (e.g., compare **17** with **19**).

Rational Design of Diflunisal Analogues. The NMR structural data on the HSA-III/diflunisal complex and the analogue binding data provide useful information for designing compounds with reduced albumin binding. However, modifications to diflunisal that not only reduce albumin binding but also significantly reduce binding to its therapeutic target, COX-2, should be avoided. Thus, the obvious routes to reducing albumin binding (e.g., removal of carboxylic acid or difluorophenyl group) are not appropriate, since these groups are critical for therapeutic efficacy.[14] The SAR of diflunisal for HSA-III and the structure of the HSA-III/diflunisal complex suggest that the introduction of polar groups on the bottom ring of diflunisal could significantly reduce binding to domain III of albumin. The effects of these substitution patterns on diflunisal's cyclooxygenase activity have not been previously investigated. However, the crystal structure of cyclooxygenase complexed

Table 2. HSA-IIIA Binding, Albumin Binding, and COX-2 Inhibition Data for Rationally Designed Diflunisal Analogs



No.	R_1	HSA-III K_D (μM)[a]	% Albumin-bound[b]	Albumin K_D (μM)[c]	Cox-2 IC_{50} (μM)[d]
1		17	99.7	3	0.1
2		10	99.6	4	1
20		360	98.2	18	100
21		1000	97.8	23	100
22		>1000	96.2	40	30
23		>1000	99.2	8	10

[a] Determined by NMR titrations against domain III of albumin. [b] Determined by ultracentracentrifugation with full length albumin. [c] Dissociation constant for full length albumin estimated from the ultracentrifugation data with use of a single binding site model. [d] Inhibition of cyclooxygenase-2.

with flurbiprofen, a biphenyl propionic acid that is highly similar to diflunisal, suggests that the environment of the bottom aromatic ring may indeed tolerate polar substitutions. In fact, the crystal structure indicates that the region of the active site proximal to the 5′ position of flurbiprofen is occupied by three water molecules (Figure 6b). Furthermore, there appears to be no steric hindrance to accessing this space from the 5′ position of flurbiprofen. Thus, if diflunisal binds to cyclooxygenase in a manner similar to flubiprofen, diflunisal analogues with polar substituents at the 5′ position would be predicted to have reduced binding to HSA-III and may maintain inhibitory activity against cyclooxygenase.

On the basis of this analysis, four compounds (**20**−**23**) were prepared which contain polar substituents at the 5′ position of a parent diflunisal analogue (**2**). Binding to HSA-III and full-length albumin was measured for a total of six compounds (**1**, **2**, **20**−**23**), and the results are given in Table 2. For compounds **1**, **2,** and **20**−**22**, there is a good agreement between the reduction in affinity for domain III of HSA and reduced binding to full-length albumin. However, the magnitude of the reduction in affinity for this series of compounds is more than 100-fold for HSA-III, while it is only about 10-fold for full-length albumin. These results suggest that the compounds retain some of their affinity for full-length albumin by binding to sites other than domain III. To determine whether these compounds still inhibit cyclooxygenase, a cell-based assay was performed to determine COX-2 inhibitory activity. The acetanilide analogue (**22**) exhibited an IC_{50} value of 30 μM, while the *tert*-butyl carbanilate analogue (**23**) exhibited an IC_{50} value of 10 μM: only a 10-fold loss compared to the parent compound **2**. It is important to note that compounds **2** and **20**−**23** do not contain the fluoro groups that are present in diflunisal. These groups significantly increase COX-2 activity (10-fold) but do not seem to affect albumin binding (e.g., compare **1** with **2**). Thus, the design of next-generation compounds would include the incorporation of fluoro groups to increase inhibitory activity against COX-2 while retaining the reduced albumin affinity afforded by the polar substituents.

Concluding Remarks

Binding to human serum albumin is a ubiquitous problem in drug discovery. Traditionally, approaches for overcoming this problem by using rational drug design have been limited. This is due to the lack of structural information on albumin/ligand complexes and the difficulty in developing structure-affinity relationships for the individual subsites on albumin. Here we have shown that NMR can be used to obtain specific structure-affinity relationships for a series of biphenyl acid compounds that bind to subdomain IIIA of HSA. In addition, NMR-derived structural information on diflunisal complexed to HSA-III revealed that the binding of this small organic compound to subdomain IIIA is mediated by specific electrostatic and hydrophobic interactions. The binding data and structural studies, together with information on how flurbiprofen binds to cyclooxygenase-2, suggested a strategy for reducing albumin binding while maintaining COX-2 inhibitory activity. All of the compounds that were synthesized based on this hypothesis exhibited reduced binding to HSA-III as well as full-length albumin, and one of these compounds retained significant inhibition of COX-2 activity. These results suggest that the strategy described here has broad applications in the pharmaceutical industry. In addition to conventional structure-based drug design to improve the potency of lead compounds, the rational design of compounds with reduced albumin binding should become a powerful tool in the iterative drug design process.

Materials and Methods

Sample Preparation. Unless otherwise noted, all compounds were obtained from commercially available sources, and were dissolved in dimethyl sulfoxide-d_6 as 100 mM stock solutions.

Isotopically labeled HSA-III was prepared from bacteria and purified as previously described.[17] The selective methyl labeling of valine, leucine, and isoleucine was achieved based on the method published by Goto et al.[16] by growing the *Escherichia coli* in 99% deuterated M9 media containing $^{15}NH_4Cl$, ^{13}C-glucose, [3-^2H] ^{13}C α-ketoisovalerate, and [3,3-$^2H_2$] ^{13}C α-ketobutyrate. The purified protein samples were dissolved in 50 mM sodium phosphate buffer (pH 6.5) containing 150 mM NaCl, 0.02% NaN_3, and 0.1 mM EDTA with H_2O/D_2O (95/5).

NMR Spectroscopy. NMR spectra were acquired at 303 K on Bruker DRX500, DRX600, and DRX800 spectrometers with *xyz*-shielded gradient triple resonance probes. Data for sequential specific assignments of the protein backbone and methyl group resonances were recorded on a 1.0 mM $^{15}N/^{13}C/^2H$ labeled HSA-III with selective Val, Leu, Ile (δ1) methyl-protonation. Backbone assignments were obtained by using 2D $^{15}N/^1H$ HSQC[18] and 3D CT-HNCA and HN(CO)CA,[19] HNCO[20] and HN(CA)CO,[21] and HN(CA)CB[20] spectra. Side chain methyl groups were assigned by using 2D $^{13}C/^1H$ HSQC and 3D (H)−C−(C−CO)NH TOCSY and H−(CC−CO)HN TOCSY[22] spectra. All

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assignments were confirmed by using $^{15}N/^{1}H/^{1}H$ NOESY[23] and $^{15}N/^{1}H/^{15}N$ NOESY[24] spectra. Data were recorded for both the free HSA-III and the HSA-III/diflunisal complex (1.0/0.8 ratio, respectively, to avoid occupancy of the low-affinity diflunisal binding site located in subdomain IIIB). Assignments were obtained for more than 95% of the backbone residues for both free and diflunisal-bound HSA-III. In the free form of the protein, the signals for residues Gln 385 to Lys 389, Met 446, and Val 467 to Asp 471 were too weak to observe in the NMR spectra. However, upon binding to diflunisal, the signals corresponding to residues Val 467 to Asp 471 were observed, indicating that the N-terminal helix is stabilized by diflunisal binding to the high-affinity site in subdomain IIIA. NOEs between the ligand and the protein were identified by using a 3D $^{13}C/^{1}H$ NOESY[25] spectrum with a mixing time of 150 ms.

Ligands were tested for binding by acquiring sensitivity-enhanced $^{15}N/^{1}H$ HSQC spectra on 50−100 μM samples of ^{15}N-labeled protein in the absence and presence of test compound. Binding was monitored through the chemical shift changes of the backbone amides as a function of ligand concentration over the range of 0 to 2 mM. The data were fitted by a least-squares grid search, assuming a single-site binding model. The reported dissociation constants are the average values of three to five residues. For compounds causing no observable chemical shift changes in the $^{15}N/^{1}H$ HSQC at concentrations of 0.5 mM, their dissociation constants were estimated to be greater than 1.0 mM. To make sure that the ligands bind to the same site as diflunisal, the chemical shift changes of a set of residues (i.e. Leu 430, Gly 431, Leu 453, Val 456, and Val 455) within the high-affinity diflunisal-binding site were monitored.

Structural Analysis. By using the NOE data, diflunisal was docked into the crystal structure of the HSA-myristate complex with use of the X-PLOR program.[26] During docking, the coordinates of the protein backbone were fixed. Diflunisal was rotated to a random orientation with respect to the protein and docked into HSA-III by intermolecular NOEs. The system was initially minimized in the absence of van der Waals interactions. Subsequent minimizations were performed by increasing the F_{repel} function from 0.5 to 0.7, followed by a 1.5 ps molecular dynamics simulation at 300 K. A final minimization was performed with the repel function adjusted to 0.75. All final structures had no NOE violations greater than 0.05 Å and no van der Waals clashes.

COX-2 Inhibition Assay. Cyclooxygenase-2 activity was measured by using previously published methods.[27] Briefly, WISH cells were stimulated with IL-1 (10 ng/mL) for 18 h to induce COX-2 expression and prostaglandin production. Supernatants from the cell incubations were assayed for PGE_2 by enzyme immunassay.

Protein-Binding Measurements. Protein binding was determined by ultracentrifugation. To a solution of human serum albumin (Sigma, 40 mg/mL in 50 mM phosphate buffered saline, pH 7.4), compounds were added to a final concentration of 20 μg/mL in 1 mL total volume. The mixtures were allowed to equilibrate for 1 h at room temperature and were then centrifuged at 86 000 × g for 18 h. The resulting gradient was fractionated into 5 × 200 μL samples and extracted with acetonitrile in preparation for analysis by reverse-phase high-performance liquid chromatography. A mobile phase of 55:45 CH_3CN/0.1% trifluoroacetic acid at a flow rate of 1 mL/min with a Luna C18 (100 × 4.6 mm) column and ultraviolet detection at 210 nm were used for compound detection. To calculate the fraction of free compound (χ_{free}), the area of the chromatographic compound peak for the upper protein-free fraction was divided by the total peak area for the entire gradient. Protein binding is expressed as 100 × (1 − χ_{free}). The reported values are the average of two measurements.

Chemistry. (a) General. ^{1}H NMR spectra were recorded on a GE QE300 spectrometer, and chemical shifts are reported in parts per million (ppm, δ) relative to tetramethylsilane as an internal standard. Mass spectra were obtained on a Kratos MS-50 instrument. Silica gel 60 (E. Merck, 230−400 mesh) was used for preparative column chromatography. Reverse-phase HPLC was performed on a YMC (75 × 30 mm) preparative column with 0.1% aqueous trifluoroacetic acid and acetonitrile. All solvents were HPLC grade. Reagents were obtained commercially and were used without further purification. Analytical TLC with Merck F254 commercial plates was used to follow the course of reactions.

(b) Methyl 5-Bromo-2-hydroxybenzoate. A solution of 5-bromosalicylic acid (10.0 g, 46.1 mmol) in methanol (200 mL) was cooled to 0 °C with an ice bath, followed by the bubbling of anhydrous HCl gas through it for 5 min. The solution was refluxed for 4 h and cooled to ambient temperature, and the solvents were removed under reduced pressure. The residue was dissolved in hot ethyl acetate and cooled, and the formed precipitate was collected by filtration to provide the titled compound (9.11 g, 86%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.88 (s, 3H), 6.97 (d, J = 8 Hz, 1H), 7.66 (dd, J = 3, 9 Hz, 1H), 7.84 (d, J = 2 Hz, 1H), 10.50 (s, 1H). MS (ESI$^+$) m/z 230 $(M + H)^+$ (bromine pattern).

(c) Methyl 3′-Amino-4-hydroxy[1,1′-biphenyl]-3-carboxylate. A solution of methyl 5-bromo-2-hydroxybenzoate (0.57 g, 2.5 mmol), 3-aminophenylboronic acid monohydrate (0.21 g, 2.6 mmol), 1,1′-bis-(diphenylphosphino) ferrocene-palladium dichloride:methylene chloride complex (1:1) (0.20 g, 0.24 mmol), and aqueous sodium carbonate (2.47 mL of 2 M, 4.95 mmol) in dioxane (25 mL) was heated to 100 °C under an atmosphere of nitrogen for 16 h. The solvents were removed under reduced pressure and the resulting residue taken up in methylene chloride and sonicated for 15 min. The mixture was filtered and the supernatant was reduced in volume and purified on silica eluting with 30% ethyl acetate in hexanes to provide the titled compound (0.13 g, 22%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.92 (s, 3H), 5.17 (s, 2H), 6.53 (d, J = 12 Hz, 1H), 6.72 (d, J = 11 Hz, 1H), 6.80 (m, 1H), 7.05 (m, 2H), 7.73 (dd, J = 2, 9 Hz, 1H), 7.94 (d, J = 3 Hz, 1H), 10.48 (s, 1H). MS (ESI$^+$) m/z 244 $(M + H)^+$.

(d) 3′-Amino-4-hydroxy[1,1′-biphenyl]-3-carboxylic Acid. (20). A solution of 3′-amino-4-hydroxy[1,1′-biphenyl]-3-carboxylic acid (0.13 g, 0.53 mmol) and lithium hydroxide monohydrate (0.112 g, 2.67 mmol) in aqueous dioxane (10 mL, 1:1) was stirred under an atmosphere of nitrogen for 16 h. The solvents were removed under reduced pressure and the residue purified by reverse-phase HPLC eluting with 0.1% aqueous trifluoroacetic acid and acetonitrile to provide the titled compound (0.057 g, 48%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.54 (d, J = 8 Hz, 1H), 6.75 (d, J = 9 Hz, 1H), 7.84 (m, 1H), 7.02 (d, J = 9 Hz, 1H), 7.09 (t, J = 8 Hz, 1H), 7.72 (dd, J = 2, 8 Hz, 1H), 7.97 (d, J = 3 Hz, 1H). MS (ESI$^-$) m/z 228 $(M - H)^-$.

(e) 3′-(Acetylamino)-4-hydroxy[1,1′-biphenyl]-3-carboxylic Acid (22). To a solution of methyl 3′-amino-4-hydroxy[1,1′-biphenyl]-3-carboxylate (0.221 g, 0.79 mmol) and *N*-methylmorpholine (0.174 mL, 1.58 mmol) in dichloromethane (20 mL) at 0 °C under an atmosphere of nitrogen was slowly added acetyl chloride (0.068 mL, 0.95 mmol). The mixture was slowly allowed to come to ambient temperature and stirred for 6 h. The mixture was diluted with dichloromethane (20 mL), washed with water (1 × 15 mL) and brine (1 × 20 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure to provide methyl 3′-(acetylamino)-4-hydroxy[1,1′-biphenyl]-3-carboxylate (0.215 g, 95%). MS (ESI$^+$) m/z 286 $(M + H)^+$. The obtained material was taken up and stirred with lithium hydroxide monohydrate (0.21 g, 5 mmol) in aqueous dioxane (5 mL, 1:1) under an atmosphere of nitrogen for 12 h. The mixture was then acidified to a pH of 5 with aqueous HCl (0.5 M) and filtered to provide the titled compound (0.19 g, 95%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.06 (s, 3H), 7.06 (d, J = 9 Hz, 1H), 7.27 (d, J = 8 Hz, 1H), 7.56 (t, J = 3 Hz, 1H), 7.56 (m, 1H), 7.77 (dd, J = 2, 8 Hz, 1H), 7.84 (m, 1H), 8.01 (m, 1H). MS (ESI$^-$) m/z 270 $(M - H)^-$.

(f) 4′-Hydroxy-3′-(methoxycarbonyl)[1,1′-biphenyl]-3-carboxylic Acid. A solution of methyl 5-bromo-2-hydroxybenzoate (1.07 g, 4.65 mmol), 3-carboxyphenyl boronic acid (0.771 g, 4.65 mmol), 1,1′-bis-

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(diphenylphosphino) ferrocene-palladium dichloride:methylene chloride complex (1:1) (0.38 g, 0.465 mmol), and aqueous sodium carbonate (4.6 mL of 2 M, 9.30 mmol) in dioxane (50 mL) was heated to 85 °C under an atmosphere of nitrogen for 16 h. The solution was cooled to ambient temperature, slowly added to aqueous HCl (75 mL, 0.5 M), and extracted with ethyl acetate (3 × 50 mL). The combined organic layer was washed with brine (1 × 50 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified on silica with 5% methanol in dichloromethane to provide the titled compound (0.57 g, 45%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.93 (s, 3H), 7.13 (d, J = 8 Hz, 1H), 7.59 (m, 1H), 7.90 (m, 3H), 8.03 (d, J = 3 Hz, 1H), 8.13 (m, 1H), 10.59 (s, 1H). MS (ESI$^-$) m/z 271 (M − H)$^-$.

(g) Methyl 3′-{[(2,4-Dimethoxybenzyl)amino]carbonyl}-4-hydroxy-[1,1′-biphenyl]-3-carboxylate. To a solution of 4′-hydroxy-3′-(methoxycarbonyl)[1,1′-biphenyl]-3-carboxylic acid (0.057 g, 0.21 mmol), *N*-hydroxybenzotriazole (0.034 g, 0.25 mmol), and 2,4-dimethoxybenzylamine (0.038 g, 0.25 mmol) in dichloromethane (10 mL) at 0 °C under an atmosphere of nitrogen was added 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (0.049 g, 0.254 mmol). The solution was allowed to come to ambient temperature, stirred for 12 h, diluted with additional dichloromethane (40 mL), washed with water (2 × 20 mL) and brine (1 × 20 mL), dried (Na_sSO_4), filtered, and concentrated under reduced pressure. The residue was purified on silica with ethyl acetate−pentane to provide the titled compound (0.051 g, 57%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.75 (s, 3H), 3.82 (s, 3H), 3.93 (s, 3H), 6.49 (m, 1H), 6.58 (m, 1H), 7.13 (d, J = 8 Hz, 1H), 7.55 (t, J = 4 Hz, 1H), 7.78 (m, 1H), 7.85 (1H), 7.92 (m, 1H), 8.09 (m, 1H), 8.12 (m, 1H), 8.90 (m, 1H), 10.56 (s, 1H). MS (APCI$^+$) m/z 422 (M + H)$^+$.

(h) 3′-(Aminocarbonyl)-4-hydroxy[1,1′-biphenyl]-3-carboxylic Acid (21). Methyl 3′-{[(2,4-dimethoxybenzyl)amino]carbonyl}-4-hydroxy-[1,1′-biphenyl]-3-carboxylate (0.051 g, 0.12 mmol) was dissolved in trifluoracetic acid (5 mL) and heated to 50 °C under an atmosphere of nitrogen for 10 h. The mixture was concentrated under reduced pressure and partitioned between water (20 mL) and ethyl acetate (20 mL). The layers were separated and the aqueous layer extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed with aqueous sodium bicarbonate (2 × 20 mL) and brine (1 × 20 mL), dried (Na_s-SO_4), filtered, and concentrated under reduced pressure to provide methyl 3′-(aminocarbonyl)-4-hydroxy[1,1′-biphenyl]-3-carboxylate (0.03 g, 0.12 mmol) which was used without further purification. MS (ESI$^+$) m/z 272 (M + H)$^+$.

Methyl 3′-(aminocarbonyl)-4-hydroxy[1,1′-biphenyl]-3-carboxylate was stirred with lithium hydroxide monhydrate (0.036 g, 0.086 mmol) in aqueous dioxane (5 mL, 1:1) under an atmosphere of nitrogen for 6 h. The solvents were removed under reduced pressure and the residue purified on reverse-phase HPLC to provide the titled compound (0.17 g, 55%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.08 (m, 1H), 7.42 (m, 1H), 7.52 (t, J = 4 Hz, 1H), 7.78 (m, 1H), 7.82 (m, 1H), 7.90 (dd, J = 3, 8 Hz, 1H), 8.12 (d, J = 3 Hz, 1H). MS (ESI$^-$) m/z 256 (M − H)$^-$.

(i) Methyl 3′-[(*tert*-Butoxycarbonyl)amino]-4-hydroxy[1,1′-biphenyl]-3-carboxylate. A solution of 3-aminophenylboronic acid (0.78 g, 5.0 mmol), di(*tert*-butyl) carbonate (1.27 mL, 5.54 mmol), and 4-(dimethylamino)pyridine (0.01 g) in acetonitrile (50 mL) under an atmosphere of nitrogen was stirred for 16 h. The mixture was concentrated under reduced pressure, taken up in ethyl acetate (75 mL), washed with aqueous HCl (3 × 25 mL of 0.5 M) and brine (1 × 25 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure to provide 3-[(*tert*-butoxycarbonyl)amino]phenylboronic acid (0.5 g, 43%).

A solution of 3-[(*tert*-butoxycarbonyl)amino]phenylboronic acid (0.5 g, 2.13 mmol), methyl 5-bromo-2-hydroxybenzoate (0.491 g, 2.13 mmol), 1,1′-bis(diphenylphosphino) ferrocene-palladium dichloride: methylene chloride complex (1:1) (0.174 g, 0.213 mmol), and aqueous sodium carbonate (2.13 mL of 2 M) in dioxane (30 mL) was heated to 60 °C under an atmosphere of nitrogen for 16 h. The mixture was concentrated under reduced pressure, taken up in THF (30 mL), sonicated for 15 min, and filtered. The supernatant was chromatographed on silica with methanol−dichlormethane to provide the titled compound (0.347 g, 47%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.49 (s, 9H), 3.93 (s, 3H), 7.10 (d, J = 9 Hz, 1H), 7.20 (m, 1H), 7.32 (t, J = 8 Hz, 1H), 7.40 (m, 1H), 7.75 (m, 2H), 7.95 (d, J = 3 Hz, 1H), 10.52 (s, 1H). MS (ESI$^-$) m/z 342 (M − H)$^-$.

(j) 3′-[(*tert*-Butoxycarbonyl)amino]-4-hydroxy[1,1′-biphenyl]-3-carboxylic Acid (23). A solution of methyl 3′-[(*tert*-butoxycarbonyl)-amino]-4-hydroxy[1,1′-biphenyl]-3-carboxylate (0.063 g, 0.18 mmol) and lithium hydroxide monhydrate (0.038 g, 0.92 mmol) in aqueous dioxane (5 mL, 1:1) was stirred under an atmosphere of nitrogen for 12 h. The mixture was concentrated under reduced pressure, diluted with water (10 mL), acidified with aqueous HCl (0.5 M), and extracted with ethyl acetate (3 × 20 mL). The combined organic solutions were washed with brine (1 × 10 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by HPLC to provide the titled compound (0.034 g, 56%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.49 (s, 9H), 7.06 (d, J = 9 Hz, 1H), 7.22 (d, J = 8 Hz, 1H), 7.32 (t, J = 8 Hz, 1H), 7.40 (m, 1H), 7.76 (m, 2H), 7.98 (d, J = 3 Hz, 1H), 9.40 (s, 1H). MS (ESI$^-$) m/z 328 (M − H)$^-$.

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Supporting Information Available: A list of the 19 intermolecular NOEs assigned between HSA-III and diflunisal (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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