The Interaction of Lipophilic Drugs with Intestinal Fatty Acid-binding Protein*

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Intestinal fatty acid-binding protein (I-FABP) is a small protein that binds long-chain dietary fatty acids in the cytosol of the columnar absorptive epithelial cells (enterocytes) of the intestine. The binding cavity of I-FABP is much larger than is necessary to bind a fatty acid molecule, which suggests that the protein may be able to bind other hydrophobic and amphipathic ligands such as lipophilic drugs. Herein we describe the binding of three structurally diverse lipophilic drugs, bezafibrate, ibuprofen (both R- and S-isomers) and nitrazepam to I-FABP. The rank order of affinity for I-FABP determined for these compounds was found to be R-ibuprofen ≈ bezafibrate > S-ibuprofen ≈ nitrazepam. The binding affinities were not directly related to aqueous solubility or partition coefficient of the compounds; however, the freely water-soluble drug diltiazem showed no affinity for I-FABP. Drug-I-FABP interaction interfaces were defined by analysis of chemical shift perturbations in NMR spectra, which revealed that the drugs bound within the central fatty acid binding cavity. Each drug participated in a different set of interactions within the cavity; however, a number of common contacts were observed with residues also involved in fatty acid binding. These data suggest that the binding of non-fatty acid lipophilic drugs to I-FABP may increase the cytosolic solubility of these compounds and thereby facilitate drug transport from the intestinal lumen across the enterocyte to sites of distribution and metabolism.

A major limiting factor in the development of clinically useful drugs is poor and variable drug absorption. With few exceptions drug candidates that are not absorbed after oral administration fail during development because of the low patient acceptability of other routes of administration. Thus, there has been considerable interest in the elucidation of the mechanisms that mediate drug transport across the enterocyte. The bulk of the research efforts in the field of drug absorption has been centered on the transport of drug molecules across the apical membrane of the enterocyte (1–3). In contrast, the study of drug transport across the cellular cytoplasm to the basolateral membrane of the enterocyte has been almost entirely neglected. In this respect the barriers faced by lipophilic drug compounds are similar to those faced by endogenous lipophilic molecules such as fatty acids, where cytoplasmic solubilization is mediated by a family of low molecular mass proteins (12–15 kDa) broadly classed as intracellular lipid-binding proteins (4). Members of this family include retinol-binding proteins, sterol carrier proteins, and cytosolic fatty acid-binding proteins (FABPs)1 (4–6). Two such binding proteins are expressed at high levels in the enterocyte, intestinal (I)-FABP and liver (L)-FABP. The classification of FABPs is based on the tissue in which they were first identified (4, 7–9). Together, I-FABP and L-FABP constitute 3–6% of the total cytosolic protein mass in the enterocyte (10).

FABPs display a common tertiary structure. Their consensus topology consists of two β-sheets, each composed of five anti-parallel β-strands capped by a helix-turn-helix motif (4). The 10 anti-parallel β-strands are organized into a β-barrel, which forms a “clam-like” structure that encloses a large solvated cavity. FABPs function by binding their lipid targets within the water-filled cavity (4). Available evidence suggests that the α-helical region acts as a “dynamic portal,” which opens to allow ligand entry then interacts with the bound lipid to cap the cavity opening (11, 12). I-FABP binds fatty acid in a 1:1 stoichiometric ratio, whereas L-FABP binds two fatty acids in an interdependent manner (13–15). In both cases, the carboxylate head group of one fatty acid chain interacts with the positively charged guanidinium side chain of an arginine residue. In the case of L-FABP, the second fatty acid binds “tail first” in the binding cavity leaving the carboxylic acid region fully exposed (15). The capacity of L-FABP to bind fatty acid in the absence of a charge interaction led to the speculation that L-FABP may possess specificity for ligands other than fatty acids. Indeed, it has been demonstrated that certain FABPs, in particular L-FABP, are able to bind to a range of bulky hydrophobic ligands (16–22). Since the concentration of L-FABP in the enterocyte is far greater than the concentration of free fatty acid, it is possible that interactions with other hydrophobic ligands, even those with lower affinity than fatty acids, may be biologically significant.

In addition to their role in cytosolic solubilization of endogenous lipophilic molecules, FABPs appear to act as transport proteins. It has been demonstrated that FABPs are capable of transporting fatty acid from the apical membrane to the endo-

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1 The abbreviations and trivial names used are: FABP, fatty acid-binding protein; ANS, 1-anilino-8-naphthalene sulfonic acid; I-FABP, intestinal fatty acid-binding protein; L-FABP, liver-fatty acid-binding protein; Me₆SO, dimethyl sulfoxide; HSA, human serum albumin; HSQC, heteronuclear single-quantum correlation; CPK, Corey, Pauling, Koltun.
plasmic reticulum or the nucleus (23–25). Furthermore, it has been proposed that the high levels of expression of both I- and L-FABP are required for the optimal transport of fatty acids through the enterocyte (26).

In view of the available information regarding the broad binding specificity of L-FABP and the abundance of both I- and L-FABP in the enterocyte, it is possible that lipophilic drugs are solubilized and transported in the cytoplasm in a manner similar to endogenous lipophilic molecules. Since the optimal transport of fatty acids across the cytoplasm involves both I- and L-FABP types, it is likely that the solubilization and transport of exogenous hydrophobic ligands also involves both FABP types. This suggests that I-FABP has the capacity to bind a diverse range of lipophilic ligands. Despite the extensive investigations into the binding selectivity of L-FABP, currently there is little information regarding the binding of non-fatty acid hydrophobic ligands to I-FABP. In fact, other than one early report of the interaction of I-FABP with a range of chemical pollutants (18), the binding of I-FABP to exogenous ligands has been largely ignored.

In this article we provide evidence that non-fatty acid lipophilic drug molecules are capable of binding specifically to rat I-FABP. We have employed competitive displacement fluorescence measurements in combination with NMR techniques to characterize the interaction between I-FABP and the lipophilic compounds bezafibrate, ibuprofen, nitrazepam, and 1-anilinonaphthalene-8-sulfonic acid (ANS) (Fig. 1). These data give substance to the potential role of FABPs as a mode of cytosolic transport for lipophilic drugs across the enterocyte. Knowledge of the structural determinants of drug binding to I-FABP provides the first step in defining the role of FABPs in drug absorption and transport. Such information could potentially lead to an improved understanding of the determinants of both oral bioavailability and intestinal metabolism and may prove to be a useful tool in pharmaceutical development.

**EXPERIMENTAL PROCEDURES**

**Materials**—Isotope-labeled ammonium chloride ($^{15}$NH$_4$Cl, >98% $^{15}$N) and deuterated water ($^2$H$_2$O, 99.9% $^2$H) were purchased from Cambridge Isotope Laboratories (Andover, MA). Isopropyl β-thiogalactopyranoside was purchased from BioVectra (Prince Edward Island, Canada). Bezafrate, ibuprofen, nitrazepam, diltiazem, ANS, myristate, and palmitate were obtained from Sigma. E. coli strain BL21 Codon Plus (DE3)-RIL was purchased from Stratagene (La Jolla, CA). All other reagents were of the highest purity available commercially.

**Expression and Purification of Rat I-FABP**—The cDNA of rat I-FABP was isolated and ligated into the pTrc99 expression vector, and the recombinant protein was expressed in E. coli BL21 cells. Uniformly $^{15}$N-labeled I-FABP was biosynthesized in E. coli BL21 cells grown in M9 medium with $^{15}$NH$_4$Cl as the sole nitrogen source according to the method of Marley (27). Rat I-FABP was purified as described previously (28, 29), with minor modifications to the protocol. Cells were lysed and nucleic acids removed by the addition of 0.1% (w/v) protamine sulfate. Following ammonium sulfate precipitation (60% saturation) the soluble fraction was desalted, and buffer was exchanged into 10 mM Tris-HCl, pH 8.0, 150 mM NaCl. This crude protein solution was applied to a MonoQ HR 10/10 column (Amersham Biosciences) in the same buffer, and I-FABP was eluted in the unbound fraction. The eluted fraction was concentrated, diafiltered into 10 mM Tris-HCl, pH 8.0, 150 mM NaCl. This crude protein solution was applied to a MonoQ HR 10/10 column (Amersham Biosciences) in the same buffer, and I-FABP was eluted in the unbound fraction. The eluted fraction was concentrated, diafiltered into 10 mM Tris-HCl, pH 8.0, 150 mM NaCl. This crude protein solution was applied to a MonoQ HR 10/10 column (Amersham Biosciences) in the same buffer, and I-FABP was eluted in the unbound fraction. The eluted fraction was concentrated, diafiltered into 10 mM Tris-HCl, pH 8.0, 150 mM NaCl. This crude protein solution was applied to a MonoQ HR 10/10 column (Amersham Biosciences) in the same buffer, and I-FABP was eluted in the unbound fraction.

**Fluorescence Measurements**—Steady-state fluorescence spectra were measured on a Cary Eclipse fluorescence spectrophotometer (Varian, Mulgrave, Victoria, Australia). The binding of ANS to I-FABP was monitored by measuring the fluorescence signal between 420 and 600 nm following excitation at 400 nm. Slit widths were set to 5 and 10 nm for the excitation and emission monochromators, respectively. For the measurement of the ratio of FABP interaction, I-FABP (1 μM) in buffer B (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA) in a final volume of 1 ml was titrated with ANS (0–50.0 μM). The concentration of ANS was quantified by UV-visible spectroscopy at 350 nm using a molar extinction coefficient of 16,900 (cm M$^{-1}$).

**Fig. 1.** Chemical structures of palmitate, myristate, bezafibrate, ANS, ibuprofen, nitrazepam, and diltiazem.
\[ \Delta F = \Delta F_{\text{max}} [\text{L}] / K_d + [\text{L}] \]  
(Eq. 1)

\[ I_{50} = \frac{F_{\text{min}} + (F_{\text{max}} - F_{\text{min}}) / (1 + 10^{\log IC_50 - \log[L] / \text{RHSlope}})} \]
(Eq. 2)

\[ K_{\text{diss}} = \frac{I_{50}}{[\text{L}] + [\text{drug}]/K_{\text{diss}} / \text{ANS}} \]
(Eq. 3)

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(Eq. 3)

**RESULTS**

**Displacement of ANS by Non-fatty Acid Lipophilic Drugs**

The effect of titrating 1 \( \mu \text{M} \) I-FABP with increasing concentrations of ANS was examined (data not shown). Nonlinear regression analysis of the binding data demonstrated that ANS bound to I-FABP with an apparent dissociation constant (\( K_{\text{diss}} \)) of 7.4 ± 0.25 \( \mu \text{M} \) at 20 °C. Scatchard analysis yielded a stoichiometry of one ANS molecule per I-FABP in the complex. These findings are in agreement with previously reported values (36–39).

The affinity and specificity of I-FABP for the lipophilic drug molecules ibuprofen, bezafibrate, and nitrazepam were examined by employing a fluorimetric assay that utilized the displacement of the fluorescent probe ANS from the fatty acid binding cavity by a competing ligand. None of the displacing ligands absorbed at the emission or excitation wavelengths employed in the fluorescence assay, and therefore, no correction for inner filter effects was required. The assay was used to determine binding affinities for myristate and palmitate. The effect of titrating 1 \( \mu \text{M} \) I-FABP with increasing concentrations of ANS was examined (data not shown). Nonlinear regression analysis of the binding data demonstrated that ANS bound to I-FABP with an apparent dissociation constant (\( K_{\text{diss}} \)) of 7.4 ± 0.25 \( \mu \text{M} \) at 20 °C. Scatchard analysis yielded a stoichiometry of one ANS molecule per I-FABP in the complex. These findings are in agreement with previously reported values (36–39).

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The results of the competitive displacement experiments with each of the drugs are shown in Fig. 2, and the apparent inhibition constants (\( K_i \)) derived from these experiments are documented in Table I. Ibuprofen was examined both as a racemic mixture and as the two separate enantiomers. I-FABP displayed a higher affinity for the \( R \)-enantiomer of ibuprofen, and the \( K_i \) for the racemic mixture was an average of the \( K_i \) values derived for the individual enantiomers. These data reflect the fact that the assay measures displacement of ANS from the I-FABP binding cavity. When titrating two ligands that both displace ANS, the measured \( K_i \) value for the mixture reflects contributions from the separate \( K_i \) values of the individual ligands. Bezafibrate displayed an affinity similar to

| Ligand      | \( K_i \) (\( \mu \text{M} \)) |
|-------------|-----------------|
| ANS         | 7.4 ± 0.25      |
| Palmitate   | 0.033 ± 0.008   |
| Myristate   | 0.065 ± 0.0032  |
| Ibuprofen   | 96 ± 1.8        |
| R-Ibuprofen | 63 ± 1.4        |
| S-Ibuprofen | 135 ± 4.0       |
| Bezafibrate | 60 ± 1.7        |
| Nitrazepam  | 2300 ± 85       |
| Diltiazem   | NB²             |

² T. D. Goddard and D. G. Kneller, unpublished work.
The rank order of I-FABP binding affinity for these ligands in the \(^1\)\(^\text{H}-\)\(^{15}\text{N} HSQC spectra provides a mechanism for identifying the location of ligand binding to I-FABP. The binding site for each drug on I-FABP was mapped by recording a series of two-dimensional \(^1\)\(^\text{H}-\)\(^{15}\text{N} HSQC spectra of uniformly \(^{15}\text{N}\)-labeled I-FABP with the addition of the different drugs from a concentrated stock. An overlay of the 2D \(^1\)\(^\text{H}-\)\(^{15}\text{N} HSQC spectra of \(^{15}\text{N}\)-labeled I-FABP, both free and in the presence of increasing concentrations of nitrazepam, is shown as an example (Fig. 3). Significant changes in chemical shift (>0.5 ppm in the \(^{15}\text{N} \) dimension and >0.1 ppm in the \(^{1}\text{H} \) dimension) are observed only for a subset of residues, indicating that there was no major change in the overall structure of the protein upon addition of each ligand. For those residues that moved significantly during the titration, a continuous change was observed as a function of added ligand, indicating that the binding of all four compounds was in the fast exchange limit on the NMR time scale. No significant chemical shift changes were observed upon addition of up to 10% (v/v) Me\(_2\)SO, indicating that the observed changes were due to the test compounds as distinct from simple solvent effects. Furthermore, titration of ibuprofen from a stock solution prepared in Me\(_2\)SO produced the same pattern of changes in chemical shift as the titration of ibuprofen in buffer C. Mapping the perturbed resonances onto the three-dimensional structure of I-FABP derived from the myristate-I-FABP co-crystal complex indicated that all of the ligands specifically bind in the internal cavity of I-FABP (Fig. 4). In all cases, the resonances with the most significant perturbations were concentrated either within the binding cavity or the portal region. However, specific differences in the patterns of residues that titrated in the two-dimensional \(^1\)\(^\text{H}-\)\(^{15}\text{N} HSQC spectra of ANS and the respective drugs were evident, suggesting different binding locations.

The individual enantiomers of ibuprofen produced perturbation maps distinct from the racemic mixture, although the sum of the perturbed residues of both enantiomers was consistent with the perturbation map of the racemic mixture. In comparison, the other ligands appeared to interact with slightly different binding locations within the cavity. The residues that titrated in the two-dimensional \(^1\)\(^\text{H}-\)\(^{15}\text{N} HSQC spectra can be categorized into two groups. The first group contains residues that form part of the fatty acid binding cavity and are all within 5 Å of the documented position of bound fatty acid in all reported fatty acid-I-FABP structural complexes. A number of these fatty acid-binding residues were found to have significant chemical shift perturbations in all of the drug titrations and are presumed to be common contact subsites for all of the ligands examined (Fig. 5, A and B). The second group comprises perturbed residues distal from the fatty acid binding cavity; these residues are localized within the dynamic portal region (Fig. 5C).

Relative Binding Affinities Determined by NMR Spectroscopy—The dissociation constants for each interaction were determined by quantitative analysis of the change in amide chemical shift observed in the two-dimensional \(^1\)\(^\text{H}-\)\(^{15}\text{N} HSQC spectra. The peaks corresponding to the backbone amides of Ile\(_{66}\), Phe\(_{88}\), Phe\(_{33}\), Ala\(_{104}\), and Tyr\(_{117}\) were chosen for analysis because these backbone resonances lie in a well resolved region of the two-dimensional \(^1\)\(^\text{H}-\)\(^{15}\text{N} HSQC spectra, are significantly perturbed in all of the drug titrations, and are localized in the fatty acid binding cavity of I-FABP. Titrations of diltiazem produced no perturbations in the two-dimensional \(^1\)\(^\text{H}-\)\(^{15}\text{N} HSQC spectra, which is consistent with the fluorescence data and demonstrates the specificity of the NMR binding assay. Fig. 6 shows representative titration curves for bezafibrate binding. The \(K_d\) value for each drug-I-FABP interaction was calculated; these results are presented in Table II.
determined by NMR spectroscopy was in accordance with the fluorescence data. The titration range for nitrazepam was limited by the low aqueous solubility of this drug. Consequently, it was not possible to saturate the binding to I-FABP. This results in a less accurate estimation of $\Delta \delta_{\text{max}}$ and likely accounts for the discrepancy between the results of the fluorescence and NMR assays.

It is widely acknowledged that the high protein concentrations required for NMR experiments dictate that titrations must be performed in the region where ligand depletion occurs, which makes determination of accurate $K_d$ values problematic. Nevertheless, the NMR titrations revealed a parallel affinity profile to that observed for the ANS displacement experiments, and the associated errors were within the limits expected for such NMR data (42).

Molecular Docking—To provide a more detailed picture of the drug-I-FABP interactions, molecular docking models were generated based on the high resolution (1.5 Å) crystal structure of the I-FABP-myristate complex (43). Initially, myristate was docked into the binding cavity of the I-FABP crystal structure from the myristate co-crystal complex. A single cluster of results was produced with all solutions having a root mean square deviation of <2 Å from the myristate orientation in the crystalline complex. In all cases, the carboxylate head group of the fatty acid formed a salt bridge with Arg106 and a hydrogen bond between one of the carboxylate oxygens and the indole proton of Trp62 (Fig. 4F), as seen in the crystalline complex. Thus, the docking protocol produced data consistent with the high resolution crystal structure of the FA-I-FABP complex.

When the drug molecules were docked into I-FABP using the same protocol, more than one solution was obtained in each case. This again appears to be consistent with the available x-ray crystal structural data (44). A crystalline complex of ANS with adipocyte lipid-binding protein revealed two distinct binding orientations in the asymmetric unit cell, suggesting that small ligands may bind in more than one orientation inside the large binding cavity of FABPs. Structures of the drug I-FABP-complexes obtained from the docking are presented in Fig. 4 and described below. The docking of ANS resulted in the ligand binding in one of two general regions. In the first binding orientation, the sulfonate of ANS ion paired with the guanidinium side chain amine group of Arg106 (Fig. 4A). The aromatic rings of ANS formed numerous interactions with hydrophobic and aromatic side chains in the I-FABP cavity. In the second binding orientation, the sulfonate group paired with Arg126 (Fig. 4B). Once again the aromatic portion of ANS interacted with hydrophobic and aromatic side chains in the cavity. Neither of the individual docking modes accounted for all of the observed chemical shift perturbations; however, taken together, these docking solutions were in good agreement with the chemical shift perturbation map from the NMR data.

The docking results for bezafibrate also yielded two binding orientations. In the first, the carboxylate of bezafibrate ion paired with Arg106 (Fig. 4C), whereas in the second it ion paired with Arg106 (Fig. 4D). Numerous interactions including hydrogen bonding, aromatic stacking, and hydrophobic interactions were observed between the remainder of the bezafibrate molecule and side chains in the I-FABP cavity. In both orientations, stabilizing interactions occurred between cavity side chains and the central benzene ring, 4-chloro-phenyl, ethylene amide linker and the carbonyl oxygen moieties of bezafibrate. Taken together, these two orientations were in good agreement with the observed NMR data.

In the case of ibuprofen, the R-enantiomer produced one cluster of results (Fig. 4E), whereas S-ibuprofen produced two clusters (Fig. 4F). In all of the solutions the carboxylate group of the ligand was found to form an ion pair with Arg106. In line with this result, Arg106 displayed a significant chemical shift perturbation with each addition of ibuprofen to the protein. The remainder of the ibuprofen structure interacted with residues that are involved in stabilizing the hydrophobic tail of the bound myristate in the crystalline complex. Significant chemical shift perturbations were observed for these residues in the ibuprofen titrations. Although a number of different orientations were observed for nitrazepam binding, the cluster with the highest docking
score was most consistent with the NMR data (Fig. 4G). In this model, the nitrogens of the benzodiazepin-2-one ring formed hydrogen bonds with Asp34 and Arg56, respectively, the ketone oxygen formed a hydrogen bond to the side chain hydroxyl of Ser53, and the nitrate group formed a hydrogen bond to the hydroxyl of the Tyr14 side chain. A number of hydrophobic and aromatic stacking interactions were also observed. In this binding orientation, nitrazepam occupied a location similar to that of bezafibrate in the cavity of I-FABP. This is consistent with the NMR data in which bezafibrate and nitrazepam produced similar patterns of chemical shift perturbations. In all cases the molecular docking models are consistent with the chemical shift perturbation maps and provide a structural representation for each drug-protein interaction.
**DISCUSSION**

It is generally accepted that one of the physiological roles played by I-FABP is in the intracellular trafficking, processing, and compartmentalization of dietary fatty acid after absorption across the apical membrane of enterocytes (45). Despite the considerable volume of evidence in support of this general hypothesis, however, the precise intracellular functions of FABPs remain unclear, and their role in the intracellular transport of non-fatty acid molecules is poorly described.

Although FABPs share a common functionality, namely the ability to bind fatty acid, the central cavities of different FABPs vary in volume between 450 and 670 Å³, which is at least twice the volume required to accommodate a fatty acid ligand (4, 45). It is possible, therefore, that FABPs may have more diverse cellular functions than their role in fatty acid trafficking.

Many poorly water-soluble lipophilic drugs are metabolized in the enterocyte (46). This process commonly takes place within the endoplasmic reticulum, which the drug can access only from the cytoplasm. This suggests that at least a proportion of the absorbed dose of a lipophilic drug enters the cytoplasmic compartment of the enterocyte as opposed to simply diffusing around the apical and basolateral membranes (2). In the largely aqueous environment of the bloodstream, many of these drugs are bound to transport proteins such as human serum albumin (HSA) (47, 48). It seems likely, therefore, that in the largely aqueous environment of the enterocyte cytoplasm, they are also solubilized by binding to carrier proteins such as FABPs. Despite the wealth of structural data describing fatty acid-I-FABP interactions, no structural data defining the binding of non-fatty acid molecules to I-FABP has been published. Elucidation of the molecular determinants responsible for the ligand binding specificity of I-FABP is integral to our understanding of the putative cellular roles for this member of the FABP family, specifically with respect to drug absorption and cellular disposition profiles. Given the broad ligand-binding specificity of FABPs and their abundance in the enterocyte, the central hypothesis examined in this work was that FABPs may facilitate the cytosolic solubilization of nonfatty acid lipophilic drugs and thereby effect their transport across the enterocyte cytoplasm. Three structurally diverse lipophilic drugs, bezafibrate, ibuprofen, and nitrazepam, were validated as fatty acid-binding site ligands by a combination of two-dimensional 1H-15N HSQC NMR spectroscopy chemical shift perturbation experiments and ANS displacement fluorimetric assays. NMR experiments were employed to define the relative contributions of individual residues in the fatty acid binding cavity of I-FABP to the binding of each compound. Mapping the nuclei that undergo chemical shift changes upon ligand titration is a sensitive indicator of the positions that are either adjacent to the binding location or at which structural changes occur upon complex formation (49). The observed chemical shift perturbations were mapped into the crystal structure of I-FABP in order to define the protein-drug interface. It was apparent that the binding of all of the ligands was site-specific and localized to the fatty acid binding cavity. Dissociation constants were determined for each drug interaction both from the NMR data and fluorescence spectroscopy, and a similar affinity profile was observed with both assay methods. The order of affinity obtained with both methods was found to be: R-ibuprofen ≈ bezafibrate > S-ibuprofen ≫ nitrazepam. Each of the drugs had a considerably lower affinity for I-FABP than FA. However, the cytosolic concentration of FABPs is 0.2–0.4 mM in the intestine (10, 50), which is far in excess of the total fatty acid concentration, leaving an excess of FABP available for binding lower affinity ligands. Consequently, it is possible that FABP plays a role in the transport of these low affinity ligands.

This situation is analogous to that in the bloodstream, where many therapeutic drugs bind HSA with a relatively low affinity (K_d ≈ 10 μM), and yet the % of the total plasma drug concentration bound to HSA is often significant both from a physiological and clinical perspective (47, 48). For example, the antibiotic rifampicin binds to HSA with a low affinity (K_d = 218 μM), and yet it has been reported to be 73.3% bound to HSA. As such, HSA-mediated transport is a significant factor in its in vivo distribution profile (51).

The chemical shift perturbation experiments were complemented by molecular docking of each drug into the crystal structure of I-FABP, which provided an insight into the mode of binding for the different drugs. In each case many of the residues that displayed significant chemical shift perturbations in the NMR data were clustered around the docked ligand in the models, suggesting that the models are representative of the binding of the drugs. Although each of the drugs bound in the FA binding cavity of I-FABP, there were subtle differences in the observed pattern of changes in the NMR titrations, and these are reflected in differences in the orientations of the different drugs in the molecular modeling data. In the binding cavity of I-FABP, there are two positively charged residues: Arg^106 and Arg^108. For ligands with anionic functionalities, including FA, ANS, ibuprofen, and bezafibrate, it is evident that one of these residues is involved in an ionic interaction with the ligand. However, in the case of nitrazepam, which contains no anionic functional group, there is no possibility of forming ionic interactions. Nevertheless, nitrazepam is observed to bind in a similar location in the cavity, albeit at lower affinity.

The combined NMR, fluorimetric and docking data clearly illustrate that each compound binds in a slightly different orientation. Consequently, the large binding cavity of I-FABP allows it to accommodate a range of structurally diverse ligands; however, selectivity is displayed for different structures.

In summary, the structural data obtained from the NMR chemical shift perturbation and molecular docking experiments have defined the binding sites for a range of lipophilic drugs on I-FABP and enabled the relative affinities of each compound to be determined. These data provide a detailed account of the binding determinants of xenobiotics in the I-FABP binding cavity and yield further insight into the specificity determinants of this class of intracellular transporters. This information may provide an insight into the mechanisms by which poorly water-soluble drugs are absorbed and transported through the enterocyte.

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