Characterization of the binding of drugs to human intestinal fatty acid binding protein (IFABP): Potential role of IFABP as an alternative to albumin for in vitro – in vivo extrapolation of drug kinetic parameters

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Abbreviations:

ANS, 1-anilino-8-naphthalene sulfonate; AZT, zidovudine; BSA, bovine serum albumin; CL_{H}, hepatic clearance; CL_{int}, intrinsic clearance; CYP, cytochrome P450;

FABP, fatty acid binding protein; HLM, human liver microsomes; HSA-FAF,
essentially fatty acid free human serum albumin; IFABP, intestinal fatty acid binding protein; IV-IVE, in vitro - in vivo extrapolation; LFABP, liver fatty acid binding protein; UGT, UDP-glucuronosyltransferase.
Abstract

This work characterized for the first time the binding of acidic, neutral and basic drugs to human intestinal fatty acid binding protein (IFABP) and, for comparison, to BSA, and investigated whether IFABP can substitute for BSA as a constituent in incubations of human liver microsomes (HLM) in IV-IVE studies. Each molecule of purified IFABP bound a single molecule of the fluorescent probe ANS or arachidonic acid with $K_d$ values similar to those reported for rat IFABP. Basic drugs bound negligibly to IFABP. Based on fraction unbound at a protein concentration of 0.5% (w/v), binding of acidic and neutral drugs ranged from minor ($fu > 0.8$) to moderate ($fu 0.5 – 0.8$). Of the compounds screened, highest binding to IFABP was observed for sulfinpyrazone (an acid) and $\beta$-estradiol (a neutral compound). However, binding to IFABP was lower than to BSA for all drugs investigated. To determine the potential suitability of IFABP as an alternative to BSA for enhancing the prediction accuracy of IV-IVE based on human liver microsomal kinetic data, the kinetics of zidovudine (AZT) glucuronidation by HLM were characterized in the absence and presence of BSA and IFABP (0.5 – 2.5%, w/v). Each protein reduced the $K_m$ for AZT glucuronidation in a concentration dependent manner, although a higher content of IFABP in incubations (2.5% vs. 1 – 1.5% for BSA) was necessary for a 10-fold reduction in this parameter. The results indicate that IFABP is likely to have advantages over BSA in microsomal kinetic studies with drugs that bind extensively to albumin.
Introduction

Numerous proteins mediate the intra- and extra-cellular binding and transport of fatty acids, which both facilitates the physiological functions of fatty acids while minimizing cellular exposure to high (and potentially toxic) concentrations of these compounds. Albumin is the most abundant fatty acid binding protein, and is also the best characterized in terms of structure and function (Hamilton, 2004). Apart from functioning as the primary plasma transporter of unesterified fatty acids, serum albumin also binds a myriad of endogenous and exogenous compounds, including drugs with acidic or electronegative features.

Like albumin, members of the intracellular fatty acid binding family of proteins (FABPs), which are amongst the most abundantly expressed cytosolic proteins, bind saturated and unsaturated long-chain fatty acids with high affinity (Veerkamp and Maatman, 1995; Zimmermann and Veerkamp, 2002; Chumurzynska, 2006). Within the cell, FABPs solubilize and transport fatty acids, and function more generally as lipid chaperones that coordinate the biological actions of fatty acids (Furuhashi and Hotamisligil, 2008). To date, nine human FABPs, each with a characteristic tissue distribution, have been identified (Chumurzynska, 2006; Furuhashi and Hotamisligil, 2008). The intestinal FABP (IFABP) is expressed along the entire length of the small intestine, although most abundantly in the medial segment, and accounts for approximately 3% of enterocyte cytoplasmic protein (Vassileva et al., 2000; Agellon et al., 2002). Rat IFABP binds a single molecule of fatty acid in a slightly bent conformation (Sacchatteni et al., 1989; Zhang et al., 1997). The carboxylate head group of the fatty acid is deeply buried in the ligand binding site, where it hydrogen-
bonds with arginine-106 (Hamilton, 2004). Although it is known that rat IFABP and liver FABP (LFABP) bind a number of acidic and neutral drugs with varying affinities (Velkov et al., 2005 and 2007; Chuang et al., 2008), the binding of drugs to human IFABP has not been characterized.

In addition to its endogenous ‘binding’ roles, albumin has been included as a constituent of microsomal incubations in experiments to generate kinetic parameters for drug and xenobiotic metabolism. In vitro approaches for the prediction of drug pharmacokinetic parameters in vivo (in vitro – in vivo extrapolation; IV-IVE), particularly intrinsic clearance (CL_{int}) and hepatic clearance (CL_{H}), have attracted widespread interest in recent years (Houston, 1994; Iwatsubo et al., 1997; Obach et al., 1997; Miners et al., 2006). However, there is a bias towards under-prediction of CL_{int} and CL_{H} for drugs metabolized by UDP-glucuronosyltransferase (UGT) and cytochrome P450 (CYP), particularly when human liver microsomes (HLM) are employed as the enzyme source (Boase and Miners, 2002; Miners et al., 2004 and 2006; Ito and Houston, 2005; Brown et al., 2007).

Recent studies have demonstrated that this discrepancy arises to a large extent from the release of membrane long chain unsaturated fatty acids which act as potent competitive inhibitors of several UGT and CYP enzymes, resulting in over-estimation of the Michaelis constant (K_{m}) and hence under-prediction of microsomal CL_{int} (Tsoutsikos et al., 2004; Rowland et al., 2007, 2008a and 2008b). Addition of bovine serum albumin (BSA) or essentially fatty acid free HSA (HSA-FAF) to HLM sequesters the inhibitory long chain unsaturated fatty acids and improves prediction accuracy of the in vivo CL_{H} values for substrates of UGT1A9, UGT2B7 and CYP2C9.
(Rowland et al., 2007, 2008a and 2008b). For example, the $K_m$ value for the glucuronidation of the UGT2B7 substrate zidovudine (AZT) by HLM was decreased by an order of magnitude in the presence of BSA, with a corresponding increase in $CL_{int}$.

However, many drugs and non-drug xenobiotics that are metabolized by these enzymes bind extensively to albumin. Characterization of the kinetics of metabolite formation of such drugs by incubations of HLM supplemented with albumin presents significant difficulties given the requirement to measure the low unbound concentration of substrate in microsomal incubations. Thus, the availability of a protein which binds drugs to a lesser extent than fatty acids would be a valuable development in IV-IVE. Here, we report for the first time the binding of acidic, basic and neutral compounds (Figure 1) to purified human IFABP and, for comparison, to BSA, including measurement of the binding affinities of the ‘model’ acidic drugs phenytoin and torsemide to each protein. Furthermore, comparable enhancement of human liver microsomal AZT $CL_{int}$ by IFABP and BSA was demonstrated, indicating that IFABP may be substituted for BSA in microsomal kinetic studies.
Materials and Methods

Materials

Alamethicin (from *Trichoderma viride*), 1-anilino-8-naphthalene sulfonate (ANS), BSA (product no. A7906), caffeine, β-estradiol, frusemide (furosemide), lignocaine, naproxen (S-enantiomer), nortriptyline, propofol, propranolol, sulfinpyrazone, UDP-glucuronic acid (trisodium salt), zidovudine (AZT) and zidovudine β-D-glucuronide were purchased from Sigma-Aldrich (Sydney, Australia). Diazepam and torsemide were obtained from Hoffmann-La Roche (Basel, Switzerland) and Boehringer Mannheim International (Mannheim, Germany), respectively. Homo sapiens fatty acid binding protein 2 (intestine) TrueClone cDNA (reference sequence NM_000134.2) was purchased from Origene (Rockville, MD), and tetra His HPR-conjugate kit from Qiagen (Melbourne, Australia). Solvents and other reagents were of analytical reagent grade.

Human liver microsomes

Pooled HLM were prepared by mixing equal protein amounts of microsomes from five human livers (H7, 44yo female; H10, 67yo female; H12, 66yo male; H29, 45yo male; and H40, 54yo female), obtained from the human liver “bank” of the Department of Clinical Pharmacology (Flinders University, Adelaide, Australia). Approval for the use of human liver tissue in xenobiotic metabolism studies was obtained from both the Clinical Investigation Committee of Flinders Medical Centre and from the donors’ next of kin. HLM were prepared by differential centrifugation as described by Bowalgaha et al. (2005).

Expression of recombinant human IFABP
cDNA encoding IFABP was PCR amplified from the human IFABP cDNA. To facilitate the purification of the IFABP, six histidine residues were added to the C-terminus of wild-type IFABP cDNA using the primers:

**IFABP-His 5**
5’-ATTAGGATCCAAATGAGTTTCTCCGGCAAGTAC-3’

**IFABP-His 3**
5’-ATTATCTAGAGATCAGTGATGGTGATGGTGATGATCCTTTTTAAAGATCCTTTTGGCTTC-3’

The IFABP insert was ligated into the pCWori(+) bacterial expression plasmid. pCW-IFABP was transformed into DH5α *E.coli* cells and colonies were screened for the correct plasmid by restriction enzyme analysis. Plasmid DNA was purified and the nucleotide sequence was confirmed on both strands by sequencing (ABI Prism 3100).

Overnight sub-cultures (4mL) grown in Luria–Bertani broth with ampicillin (100mg/L) at 37°C were used to inoculate 400mL cultures of modified Terrific broth containing 100mg/L ampicillin. Cultures were grown at 37°C with shaking (180rpm) for 3hr, or until an optical density of approximately 0.7 at 600nm was attained, at which time the temperature was reduced to 30°C and isopropyl-b-D-thiogalactopyranoside (1mM) was added. Cultures were grown at 30°C with shaking (150rpm) for an additional 40hr. Cells were then harvested by three passages through a French press cell at a cell pressure of 8 to 11kPa. IFABP was recovered in the soluble fraction after centrifugation at 45000g for 90 min and then purified by chromatography on a pre-equilibrated Ni²⁺ affinity (Ni-NTA agarose) column according to the general procedure of Johnson et al. (2005). The concentration of purified IFABP protein was determined according to the Lowry procedure.
**Immunoblotting of IFABP**

Denatured purified protein (1μg) was separated by SDS-PAGE and then transferred onto nitrocellulose membranes. Membranes were subsequently washed and blocked in 1% (w/v) blocking reagent (Qiagen, Melbourne, Australia) for 2hr at room temperature, then washed (5min) with Tris buffered saline and probed (120min at room temperature) with a commercial anti-His antibody (Tetra-HIS HPR-conjugate; 1:1000 dilution). Prior to development, the membrane was washed in Tween 20. The BM chemiluminescence blotting substrate was used for immunodetection. The membrane was exposed to Kodak X-Omat films for 10sec and films were processed manually using AGFA G153 developer and G354 fixer.

**AZT glucuronidation assay**

Incubations, in a total volume of 0.2mL, contained phosphate buffer (0.1M, pH 7.4), MgCl₂ (4mM), HLM (0.05mg), BSA or IFABP (0 to 2.5%), and AZT (10 to 3000μM). HLM were fully activated by the addition of the pore-forming polypeptide alamethicin (50μg/mg protein) with incubation on ice for 30min (Boase and Miners, 2002). Following a 5min pre-incubation at 37°C, reactions were initiated by the addition of 5mM UDPGA. Incubations were performed at 37°C in a shaking water bath for 60min. Reactions were terminated by the addition of 6μl of perchloric acid (70%, v/v). Samples were subsequently centrifuged at 4000g for 10min, and a 30μL aliquot of the supernatant fraction was injected directly into the HPLC column. Zidovudine β-D-glucuronide formation was quantified by HPLC as described by Rowland et al. (2007).

**Measurement of ANS binding and competitive displacement by ligands**
The binding of ANS to IFABP and BSA was quantified by fluorescence spectroscopy using a modification of the method of Norris and Spector (2002). Spectrofluorometer (Perkin Elmer 300) excitation and emission wavelengths were set at 370 and 475nm, respectively, with respective excitation and emission slit widths of 2.5 and 5nm. Incubations to measure ANS binding to IFABP and BSA (3mL total volume) were performed at 20°C in a quartz cuvette (10mm path length) and contained phosphate buffer (0.1M, pH7.4) and IFABP or BSA (500nM). The incubation sample was titrated with fourteen 10μL aliquots of ANS (100μM), resulting in an ANS concentration range of 500nM to 10μM. ANS fluorescence was not detected in the absence of protein. Incubations to measure the displacement of ANS by arachidonic acid contained phosphate buffer (0.1M, pH7.4), IFABP (500nM) and ANS (1μM). The incubation samples were titrated with eight 10μL aliquots of arachidonic acid, resulting in an ANS concentration range of 25 to 1000nM. In drug displacement studies, incubation samples containing phosphate buffer (0.1M, pH7.4), IFABP or BSA (500μg/mL) and ANS (50μM) were titrated with competitor ligand (phenytoin and torsemide; 100nM to 50μM).

**Measurement of drug binding to IFABP and BSA**

The binding of drugs to IFABP and BSA was measured using Microcon centrifuge filter devices (Millipore Corporation, Bedford, MA), which comprise reservoir and filtrate compartments separated by a 3kDa cellulose membrane. The cellulose membranes were conditioned with 500μL of phosphate buffer (0.1M, pH 7.4). Incubation samples (total volume 110μL) contained the drug of interest, phosphate buffer (0.1M pH 7.4) and protein (IFABP or BSA; 0 to 0.5%). Samples were incubated in a shaking water bath at 37°C for 120min, after which time a 100μL
aliquot was added to the reservoir compartment and the device was centrifuged at 14,000g for 2min. Under these conditions, less than 15% of the sample passed from the reservoir compartment to the filtrate compartment. A 10μL aliquot was collected from each compartment and protein was precipitated with ice-cold methanol containing 4% glacial acetic acid (10-20μL), or ice-cold methanol (20μL) alone in the case of lignocaine and propofol. Samples were cooled on ice, centrifuged at 4000g for 10min at 4ºC, and aliquots of the supernatant fraction (5μL) were analyzed by HPLC. Drug and protein concentrations investigated are shown in Supplemental Table 1. Fraction unbound (fu) was calculated as 1 – ([drug_{reservoir}] – [drug_{filtrate}])/ [drug_{reservoir}].

It should be noted that conditioning of the membrane prior to use overcame the non-specific binding and poor recovery reported in certain previous studies that have measured protein binding using centrifugation filter devices. Recovery, assessed by mass balance against an unfiltered sample, was ≥ 95% for all drugs. In addition, the fu values for the binding of AZT, lignocaine, phenytoin and propofol to BSA measured here (see Results) were in close agreement to those determined previously in this laboratory using equilibrium dialysis (Rowland et al., 2007, 2008a and 2008b; JO Miners and DJ Elliot, unpublished data).

**Quantification of drug binding to IFABP and BSA**

Drug present in the filtrate from binding experiments was separated on a Waters NovaPak C18 analytical column (3.9 x 150mm, 4μm, Waters, Sydney, Australia) using a mobile phase comprising 10mM triethylamine (pH adjusted to 2.5 with perchloric acid; mobile phase A) and acetonitrile (mobile phase B), at a flow rate of 1mL/min (see Supplemental Table 2 for proportions). Column eluant was monitored
at the optimal wavelength (determined by spectroscopic analysis) for each drug (Supplemental Table 2). The concentration of drug in ultra-filtration samples was determined by comparison of peak areas to those of authentic standards using calibration curves that spanned the concentration ranges employed in binding studies.

**Data analysis**

All data points represent the mean of duplicate estimates (<10% variance). Kinetic constants for AZT glucuronidation by HLM, in the absence and presence of BSA and IFABP, were obtained by fitting experimental data to the Michaelis-Menten equation. The stoichiometry of ANS, arachidonic acid, phenytoin and torsemide binding to IFABP and BSA was determined by Scatchard ([S]_bound/[P] versus [S]_bound/[S]_total) analysis. When Kᵩ values for binding of substrate molecules at all binding sites are equivalent, a plot of [S]_bound/[P] versus [S]_bound/[S]_total gives a straight line with an x-intercept of n, where n is the number of substrate molecules bound to each protein molecule (Moller and Denicola, 2002). Kᵩ values for ANS and arachidonic acid binding to IFABP and BSA, and torsemide binding to IFABP, were determined using a single binding mode equation, while Kᵩ values for phenytoin binding to IFABP and BSA, and torsemide binding to BSA, were determined using a two binding mode equation:

\[
\Delta F = \sum_{n=1}^{or} \frac{C_n \times [S]}{K_{dn} + [S]}
\]

where \(\Delta F\) is the percent change in fluorescence upon addition of ANS or competitive ligand, \(C_n\) is the capacity of the nth binding mode, \(K_{dn}\) is the dissociation constant for the nth binding mode, \([S]\) is the concentration of added substrate, and n is the number of binding modes.
Results

IFABP expression and ANS and arachidonic acid binding

Expression of IFABP was demonstrated by immunoblotting the purified protein. A single band corresponding to the molecular mass of His-tagged IFABP (approximately 16kD) was detected by chemiluminescence following immunoblotting with a tetra-His antibody (Figure 2). No other bands were observed. Coomassie staining of the SDS-PAGE gel prior to transfer onto nitrocellulose was also consistent with the presence of a single protein with an approximate molecular mass of 16kD.

For characterization purposes, the binding capacity of IFABP was confirmed with the fluorescent probe ANS and by measuring the displacement of ANS by arachidonic acid. Titration with ANS resulted in a saturable increase in fluorescence in the presence of IFABP, which was not observed in the absence of protein (Figure 3A). The binding of ANS was well described by the single binding site equation. The derived dissociation constant (2.9µM) is comparable to Kd values reported previously for the unmodified rat protein (6.9µM, Kirk et al., 1996; 3.6 to 7.4µM, Velkov et al., 2005). Scatchard analysis indicated that each molecule of IFABP bound one molecule of ANS. Addition of arachidonic acid to samples containing IFABP and ANS resulted in a decrease in ANS fluorescence (Figure 3B), which was best fit to the single binding site equation. The derived Kd value for arachidonic acid binding to IFABP was 122nM, which is within the range reported for the binding of saturated and unsaturated long chain fatty acids to rat IFABP (Richieri et al., 1994; Velkov et al., 2005).

Comparative binding of drugs to IFABP and BSA
Preliminary experiments were performed to assess the binding of phenytoin (an acid), propofol (a neutral compound) and propranolol (a base) to IFABP and, for comparison, to BSA. Binding was measured at three drug concentrations which spanned the known $K_m$ for each substrate at each of four protein concentrations, ranging from 0.05% (0.5mg/mL) to 0.5% (5mg/mL). Values of $f_u$ for phenytoin, propofol and propranolol in the presence of IFABP and BSA are shown in Table 1. As observed previously (Rowland et al., 2008a), phenytoin bound appreciably to BSA; the binding was independent of ligand concentration, but increased with increasing BSA concentration. Phenytoin also bound appreciably to IFABP. Again, binding was independent of ligand concentration but increased with increasing protein (IFABP) concentration. Whereas the binding of propofol to IFABP was negligible, <10% at the protein concentrations employed (0.05 to 0.5%), binding to BSA was appreciable. Binding to BSA did not vary with propofol concentration, but increased with increasing albumin concentration. Binding of propranolol to IFABP was minor ($\leq$13%) at all protein concentrations (0.05 to 0.5%). Similarly, the binding of propranolol to BSA was minor at the low protein concentrations (0.05 and 0.25%), although binding was >20% at the highest BSA concentration (0.5%).

On the basis of the data for phenytoin, propofol and propranolol, the binding of ten further compounds to IFABP and BSA (0.5%) was assessed. The compounds (Figure 1) were classified as acids (frusemide, naproxen, sulfinpyrazone and torsemide), bases (lignocaine and nortriptyline) or neutrals (caffeine, diazepam, $\beta$-estradiol and AZT) based on the charge state at pH 7.4. Binding measurements were performed at three or four ligand concentrations that spanned the known $K_m$ for the major human liver
microsomal metabolic pathway of each substrate (and which included 100µM) at a
protein concentration of 0.5% (5mg/mL).

The binding of all acidic compounds to both proteins exceeded 10% over the ligand
concentration ranges investigated (Table 2). Fraction unbound was independent of
drug concentration, but generally varied between compounds and proteins. Frusemide,
naproxen, sulfinpyrazone, and torsemide bound extensively to BSA; mean fu values
ranged from 0.05 to 0.24. Phenytoin binding to both proteins and sulfinpyrazone
binding to IFABP was moderate, with mean fu values of approximately 0.4 – 0.6.
Whereas frusemide, naproxen and torsemide bound strongly to BSA, mean fu values
for the binding of these compounds to IFABP were approximately 0.8. The binding of
the basic compounds lignocaine, nortriptyline and propranolol to IFABP was
negligible (<10%). The binding of lignocaine to BSA was also minor, but
nortriptyline and propranolol bound appreciably to BSA. As observed for the acidic
compounds, binding of nortriptyline and propranolol to BSA was concentration
independent, but varied for each drug (Table 2). Differing patterns of binding were
observed for the neutral compounds: diazepam and β-estradiol bound appreciably to
both proteins, although binding was much higher with BSA; binding of AZT and
caffeine was minor with both BSA and IFABP; and, as observed in the screening
experiment, propofol bound appreciably only to BSA.

**Binding affinities of phenytoin and torsemide to IFABP and BSA**

The binding affinities of phenytoin and torsemide to IFABP and BSA were
determined by ANS (10µM) displacement at a protein concentration of 500µg/mL
(0.5% w/v). Experimental data for the binding of phenytoin to IFABP and BSA and
torsemide to BSA were best described by a two binding mode equation (Table 3 and Figure 4). In contrast, data for the binding of torsemide to IFABP were best fit to the single binding mode equation (Figure 4).

Effect of IFABP on AZT glucuronidation by HLM

As indicated in Methods, binding of AZT to BSA, IFABP and HLM was accounted for in the calculation of kinetic constants. The conversion of AZT to zidovudine β-D-glucuronide by HLM, in the absence and presence of IFABP and BSA (0.5-2.5%), was well described by the Michaelis-Menten equation (Figure 5). Derived $K_m$ and $V_{max}$ values for AZT glucuronidation by HLM in the absence of added protein and in the presence of BSA (0.5, 1.5 and 2.5%) were similar to previous reports from this laboratory (Boase and Miners, 2002; Rowland et al., 2007). Addition of BSA and IFABP (0.5, 1.5 and 2.5%) to incubations increased the rate of AZT glucuronidation by decreasing the $K_m$ for this pathway without an appreciable effect on $V_{max}$ (Table 4 and Figure 5). $K_m$ values for AZT glucuronidation in the presence of 0.5 and 1.5% IFABP were approximately 2 to 3-fold higher than those observed in the presence of the same concentration of BSA. However, $K_m$ values determined in the presence of 2.5% of each protein were similar (Table 4 and Figure 5).
Discussion

This study reports for the first time the binding, both extent and concentration dependence, of ‘model’ acidic, basic and neutral drugs to purified recombinant human IFABP and, for comparison, to BSA. Acidic drugs and two neutral compounds bound to IFABP to a minor or moderate extent (based on fu values), but binding was consistently lower than to BSA. The utility of IFABP as an alternative fatty acid sequestrant to BSA in incubations of HLM, and hence its potential application in IV-IVE, was confirmed with the glucuronidated drug AZT, although the content of IFABP required to produce a 10-fold reduction in the $K_m$ for AZT glucuronidation in incubations of HLM was higher than for BSA.

The comparative binding of drugs to IFABP and BSA was assessed as fraction unbound at three or four concentrations (always including 100μM) that spanned the known $K_m$ for the principal route of metabolism of each compound. Following screening studies with phenytoin, propofol and propranolol, which indicated that acidic drugs were likely to bind most extensively to IFABP, studies (at 0.5% w/v protein) were conducted with an additional 4 acids, 2 bases and 3 neutrals. Like propranolol, the two other bases (lignocaine and nortriptyline) bound negligibly to IFABP, despite moderate binding of nortriptyline to BSA. Of the four neutral compounds, only diazepam (mean fu, 0.80) and β-estradiol (mean fu, 0.56) bound appreciably to IFABP. Binding of the neutrals to IFABP was lower than to BSA.

As with the neutral compounds, the binding of acidic drugs to BSA (fu range 0.04 – 0.49) was higher than to IFABP (fu range 0.41 – 0.85). Indeed, mean fu values of frusemide, naproxen, sulfinpyrazone and torsemide were 4- to 10- fold higher with
IFABP. Binding affinities and capacities measured for phenytoin and torsemide at 0.5% w/v protein were in broad agreement with these observations. Based on fu values, phenytoin binds ‘moderately’ to both BSA and IFABP whereas torsemide binds much more avidly to BSA (Table 2). Binding of torsemide to BSA occurs at two sites, with high total capacity compared to the single IFABP site (Table 3). In contrast, $K_d$ values and capacities for the binding of phenytoin to BSA and IFABP were of a similar order (Table 3). The observation of two modes for the binding of phenytoin to IFABP is consistent with the docking of ibuprofen and bezafibrate into the binding cavity of rat IFABP x-ray crystal structure which suggests that, unlike fatty acids, some acidic drugs may bind to IFABP in two orientations (Velkov et al., 2005). It should be noted that the molecular masses of IFABP ($\approx 15\text{kDa}$) and albumin ($\approx 66\text{kDa}$) differ. Thus, the drug binding capacity of BSA substantially exceeds that of IFABP on a molar basis.

The human IFABP drug binding data reported here are in agreement with two recent studies with rat IFABP. Velkov et al. (2005) found that ibuprofen and bezafibrate bound strongly to rat IFABP, whereas binding of the neutral drugs nitrazepam and diltiazem was weak or absent. More recently, the same group characterized the binding of a larger group of compounds, mostly organic acids (Velkov et al., 2007). Highest binding to rat IFABP was generally observed with compounds possessing a carboxylic acid group, although binding affinities varied by an order of magnitude. In addition to drugs, rat IFABP is also known to bind butylated hydroxytoluene and phthalate esters (Kanda et al., 1990).
Although the primary ligands for IFABP are fatty acids, our work and that of Velkov et al. (2007) demonstrates that the presence of a carboxylic acid function is not a requirement for binding. Only two of our study drugs, naproxen and frusemide, contain the carboxylate group. Indeed, sulfinpyrazone and β-estradiol (a phenol essentially uncharged at pH 7.4) exhibited the lowest fu values. The acidity of sulfinpyrazone arises from the presence of a C-H bond α to two carbonyl groups, while phenytin and torsemide contain hydantoin and sulfonylurea features, respectively. The binding of bulky multi-ring structures (e.g. β-estradiol and sulfinpyrazone; Figure 1) is consistent with the known volume of the ligand binding domain of rat IFABP (≈ 850 Å³; Hamilton, 2004) and with a flexible ligand entry portal (Hodson and Cistola, 1997). Binding data for a broader range of substrates will be necessary to generate quantitative structure-activity relationships, particularly the comparative importance of polar and hydrophobic features for drug binding to IFABP.

The pharmacological significance of the binding of acidic and neutral drugs to IFABP, which together with LFABP comprises up to 6% of the cytosolic protein of enterocytes, is unknown. However, it has been proposed that binding to FABPs enhances the transcellular delivery of drugs to the enterocyte basolateral membrane, with subsequent uptake into the intracellular space (Velkov et al., 2007). Thus, IFABP may assist the absorption of acidic drugs and xenobiotics.

IV-IVE is a major research interest of this laboratory. Recent work has demonstrated that the addition of BSA or HSA-FAF to incubations of HLM markedly increases the prediction accuracy of in vivo CLint and CLH for substrates of UGT1A9, UGT2B7 and
CYP2C9 (Rowland et al., 2007, 2008a and 2008b), and the prediction of drug interactions arising from inhibition of UGT2B7 (Uchaipichat et al., 2006; Rowland et al., 2006). The ‘albumin’ effect arises from sequestration of long chain unsaturated fatty acids released from the microsomal membrane during the course of an incubation. In the absence of BSA or HSA-FAF these compounds act as potent competitive inhibitors of several CYP and UGT enzymes. Thus, experiments conducted in the presence of BSA or HSA-FAF provide a true estimate of $K_m$ and $K_i$. However, the limitations of the use of albumin were demonstrated in a recent study involving the UGT1A9 substrate frusemide. While the $K_m$ and $CL_{int}$ values generated in the absence of BSA were almost certainly overestimates, the extensive albumin binding of frusemide (> 97%) precluded in vitro kinetic studies in the presence of BSA (Kerdpin et al., 2008). In fact, most acidic and many neutral drugs bind extensively to albumin at concentrations added to incubations of HLM, which is apparent from data presented in Table 2.

The lower binding observed here with IFABP suggested that this protein may indeed represent an alternative to BSA for enhancing the prediction accuracy of IV-IVE based on human liver microsomal kinetic data. To confirm this, the kinetics of AZT glucuronidation by HLM were characterized in the absence and presence of BSA and IFABP (0.5 – 2.5% w/v). Both proteins decreased the $K_m$ for zidovudine glucuronidation without affecting $V_{max}$, as reported previously with BSA and HSA-FAF (Rowland et al., 2007). However, a higher content of IFABP (2.5% vs. 1%; Table 4 and Rowland et al., 2007) was necessary to reduce the $K_m$ for AZT glucuronidation by an order of magnitude. The increased requirement for IFABP is consistent with the known fatty acid binding capacity of albumin; whereas each
molecule of IFABP binds only a single fatty acid molecule, albumin contains at least seven binding sites for long chain unsaturated fatty acids (Hamilton, 2004). This difference is, however, partially compensated by the 4.4-fold lower molecular mass of IFABP.

The $K_m$s generated for microsomal AZT glucuronidation in the presence of 2.5% IFABP (74 µM) and BSA (69 µM) are in accord with that reported with human hepatocytes (87 µM; Engtrakul et al., 2005) as the enzyme source and thus appear to represent true hepatocellular $K_m$. Based on IV-IVE scaling factors for AZT given in Uchaipichat et al. (2006), predicted in vivo hepatic clearances for AZT glucuronidation were approximately 27 L/hr. While this still represents an underestimation of known in vivo CL$_{H}$ (82 L/hr), the prediction bias is substantially less than that normally associated with the scaling of intrinsic clearances derived using HLM and indeed human hepatocytes as the enzyme sources (Boase and Miners 2002; Miners et al., 2006; Brown et al., 2007).

In summary, it has been demonstrated that human IFABP has the capacity to bind acidic and neutral drugs to a minor or moderate extent. Binding is not dependent on the presence of the carboxylate group. At equivalent protein concentrations, binding to BSA exceeds that of IFABP. Based on AZT glucuronidation kinetic data, IFABP is able to substitute for BSA as a fatty acid sequestran in incubations of HLM, although there is a requirement for a higher content of IFABP in incubations. The utility of IFABP in IV-IVE is being investigated further with drugs that bind extensively to BSA.
Acknowledgements

Technical assistance from Mr DJ Elliot and Mr BC Lewis is gratefully acknowledged.
References


DMD 27656

Footnotes

This work was funded by a grant from the National Health and Medical Research Council of Australia. AR is the recipient of a Flinders University postgraduate research scholarship.
List of figures

Figure 1. Chemical structures of compounds used in IFABP and BSA binding studies.

Figure 2. Western blot of purified His tagged human intestinal fatty acid binding protein. The sample contained 1µg of purified protein.

Figure 3. Panel A. Plots for the binding of ANS to IFABP; Panel B. Percent ANS displacement versus ligand concentration ([S]) and Scatchard plots for the binding of arachidonic acid to IFABP. Points show experimentally determined values, while curves are from model fitting.

Figure 4. Percent ANS displacement versus ligand concentration ([S]) and Scatchard plots for the binding of phenytoin and torsemide to IFABP and BSA. Points show experimentally determined values, while curves are from model fitting.

Figure 5. Eadie-Hofstee (V versus V/[S]) plots for zidovudine (AZT) glucuronidation by HLM, in the absence and presence of IFABP and BSA (0.5, 1.5 and 2.5%). Points show experimentally determined values, while curves are from model fitting.
Table 1. Unbound fractions (fu) for the binding of phenytoin, propofol and propranolol to BSA and IFABP.

<table>
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<td></td>
<td></td>
<td></td>
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Table 2. Unbound fractions (fu) for the binding of drugs to BSA and IFABP (0.5%).

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### Table 3. Phenytoin and torsemide binding affinities and capacities.

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<th>Parameter</th>
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<th>BSA</th>
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<td>$C_1^a$</td>
<td>19.9</td>
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<td>$C_2^a$</td>
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<td>$C_2^a$</td>
<td>N/A$^b$</td>
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</table>

Units: $K_d$, μM; C, % ANS displacement. N/A – not applicable.

$^a$ C (capacity) was assessed as the maximal % capacity of the substrate to displace ANS from the protein.

$^b$ Experimental data for torsemide binding to IFABP were best described by a single binding mode equation.
Table 4. Kinetic parameters for zidovudine (AZT) glucuronidation by human liver microsomes in the absence and presence of IFABP and BSA (0.5, 1.5 and 2.5%)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
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<th>IFABP 1.5%</th>
<th>IFABP 2.5%</th>
<th>BSA 0.5%</th>
<th>BSA 1.5%</th>
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<td>604</td>
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</table>

Units: $K_m$, µM; $V_{max}$, pmol/min. mg.

Control refers to incubations of HLM conducted in the absence of BSA and IFABP.
Figure 1

caffeine

diazepam

β-estradiol

frusemide

lignocaine

naproxen

nortriptyline

phenytoin

propofol

propranolol

sulfinpyrazone

torsemide

zidovudine
Figure 2

[Image of a gel electrophoresis with molecular weight markers: 60kD, 45kD, 30kD, and 15kD. A band is visible at approximately 15kD labeled IFABP-His.]
Figure 3

A

Fluorescence Units

[ANS] (nM)

B

ANS Displacement (%)

[Arachidonic Acid] (nM)
Figure 4

**BSA**

- Phenytoin
- Torsemide

**IFABP**

- Phenytoin
- Torsemide

**[Substrate] (µM)**

**[ANS Displacement] (%)**

**[Substrate]_bound / [BSA]**

**[Substrate]_bound / [IFABP]**
Figure 5

**BSA**

- 0%
- 0.5%
- 1.5%
- 2.5%

**IFABP-His**

- 0%
- 0.5%
- 1.5%
- 2.5%