

***Impact of Intracellular Lipid Binding Proteins on Endogenous and Xenobiotic Ligand  
Metabolism and Disposition***

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## Running Title Page

**Running title:** *Impact of Lipid Binding Proteins on Ligand Disposition*

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**Abbreviations:** ANS: 8-anilinonaphthalene-1-sulfonic acid, *atRA*: *all-trans*-retinoic acid, CSP: chemical shift perturbation, CPTI: carnitine palmitoyltransferase I, CRABP: cellular retinoic acid binding protein, CRBP: cellular retinol binding protein, CYP: cytochrome P450, DAUDA: 11-(Dansylamino)undecanoic Acid, FABP: fatty acid binding protein, HSL: hormone sensitive lipase, iLBP: intracellular lipid binding protein, LCFA: long chain fatty acid, LRAT: lecithin retinol acyltransferase, NBD: nitrobenzoxadiazole, PPAR: peroxisome proliferator-activated receptor, RAR: retinoic acid receptor, THC:  $\Delta^9$ -tetrahydrocannabinol, WT: wildtype

## **Abstract**

The family of intracellular lipid binding proteins (iLBPs) is comprised of sixteen members of structurally related binding proteins that have ubiquitous tissue expression in humans. iLBPs collectively bind diverse essential endogenous lipids and xenobiotics. iLBPs solubilize and traffic lipophilic ligands through the aqueous milieu of the cell. Their expression is correlated with increased rates of ligand uptake into tissues and altered ligand metabolism. The importance of iLBPs in maintaining lipid homeostasis is well established. Fatty acid binding proteins (FABPs) make up the majority of iLBPs and are expressed in major organs relevant to xenobiotic absorption, distribution and metabolism. FABPs also bind a variety of xenobiotics including non-steroidal anti-inflammatory drugs, psychoactive cannabinoids, benzodiazepines, antinociceptives and peroxisome proliferators. FABP function is also associated with metabolic disease making FABPs currently a target for drug development. Yet, the potential contribution of FABP binding to distribution of xenobiotics into tissues and the mechanistic impact iLBPs may have on xenobiotic metabolism is largely undefined. This review examines the tissue specific expression and functions of iLBPs, the ligand binding characteristics of iLBPs, their known endogenous and xenobiotic ligands, methods for measuring ligand binding and mechanisms of ligand delivery from iLBPs to membranes and enzymes. Current knowledge of the importance of iLBPs in affecting disposition of xenobiotics is collectively described.

## **Significance Statement**

The data reviewed here show that FABPs bind many drugs and suggest that binding of drugs to FABPs in various tissues will affect drug distribution into tissues. The extensive work and findings with endogenous ligands suggest that FABPs may also alter the metabolism and transport of drugs. This review illustrates the potential significance of this understudied area.

## Introduction:

Intracellular lipid binding proteins (iLBPs) are a family of ubiquitous proteins in animals that solubilize essential cellular lipids (Schaap *et al.*, 2002; Storch and Corsico, 2008; Smathers and Petersen, 2011; Napoli, 2017). Together with avidins and lipocalins, iLBPs belong to the calycin superfamily of structurally related binding proteins. Despite low amino acid sequence homology (< 10%), avidins, lipocalins and iLBPs share a common  $\beta$ -barrel structural fold which makes up their ligand binding cavity (Flower *et al.*, 2000; Schaap *et al.*, 2002; Smathers and Petersen, 2011). Avidins and lipocalins are found in both prokaryotic and eukaryotic organisms but iLBPs are only present in vertebrate and invertebrate animals (Schaap *et al.*, 2002). The ancestral iLBP gene evolved after animals diverged from plants and fungi, and individual isoforms arose through gene duplication and diversification (Schaap *et al.*, 2002; Haunerland and Spener, 2004; Smathers and Petersen, 2011). The primary amino acid sequence identity for the sixteen known human iLBPs range from 21-77% (Figure 1A). Generally, the amino acid sequence identity for specific iLBPs across different species is greater than the sequence identity of all FABPs within the same species. For example, fatty acid binding protein 1 (FABP1) has > 60% amino acid sequence identity across 18 different species (Zhang *et al.*, 2020), but the sequence identity of all FABPs in humans is as low as 21%.

The human iLBPs are divided into four subfamilies (Figure 1B) based on phylogenetic analysis and amino acid sequences (Schaap *et al.*, 2002; Liu *et al.*, 2008; Smathers and Petersen, 2011; Ragona *et al.*, 2014). Subfamily I is comprised of the cellular retinol binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs). Subfamily II contains liver FABP (FABP1) and ileal FABP (FABP6, also called I-BABP). Intestinal FABP (FABP2) is the lone iLBP to make up Subfamily III and heart (FABP3), adipocyte (FABP4), epidermal

(FABP5), brain (FABP7), myelin (FABP8) and testis (FABP9) FABPs, and FABP12 make up Subfamily IV (Schaap *et al.*, 2002; Smathers and Petersen, 2011). The FABPs were originally named after the organs from which they were cloned but have been later found to have broader expression. The human iLBP genes are located in several different chromosomes (Table 1) and, like most iLBP genes in animals, have four exons with three introns (Schaap *et al.*, 2002; Babin, 2009; Smathers and Petersen, 2011; Zhang *et al.*, 2020). The second and third exons are conserved in nearly all FABP genes (Zhang *et al.*, 2020). Phylogenetic studies suggest that FABP genes evolved from a common ancestor likely through tandem duplication (Babin, 2009; Zhang *et al.*, 2020). FABP4, 5, 8, 9 and 12 form a gene cluster on the same chromosome in humans and several other mammals. Some of these FABP genes also form clusters in aves, amphibians and reptiles. This supports the hypothesis that vertebrate FABP genes may have arisen through continuous tandem duplication from a common ancestor (Zhang *et al.*, 2020).

The complete physiological functions of iLBPs have yet to be defined but iLBPs appear to facilitate the efficient uptake of endogenous lipids into tissues, acting as carriers to shuttle ligands through the cytosol and modulating rates of ligand metabolism (Kushlan *et al.*, 1981; Luxon and Weisiger, 1993; Martin *et al.*, 2003; Kaczocha *et al.*, 2009; Yu *et al.*, 2014; Gajda and Storch, 2015). Altered iLBP function and expression have been associated with dyslipidemia, metabolic syndrome, obesity, diabetes, atherosclerosis and inflammation (Furuhashi and Hotamisligil, 2008; Atshaves *et al.*, 2010; Peng *et al.*, 2012; Wang *et al.*, 2016; Furuhashi, 2019; Valizadeh *et al.*, 2021). Several iLBP isoforms also bind xenobiotics (Chuang *et al.*, 2008; Trevaskis *et al.*, 2011; Velkov, 2013; Lee *et al.*, 2015; Huang *et al.*, 2018; Elmes *et al.*, 2019). Based on their high and ubiquitous expression in tissues, iLBPs may be determinants of xenobiotic distribution and uptake into tissues. This review focuses on ligand binding to iLBPs,

tissue expression of iLBPs, methods to determine ligand binding and the biochemical roles of iLBPs as they relate to the potential of iLBPs to be determinants of drug disposition.

### **Intracellular Lipid Binding Protein Structures and Endogenous Ligand Binding**

The tertiary structures of iLBPs are virtually superimposable and have two characteristic structural features, a  $\beta$ -barrel domain and helix-turn-helix motif (Figure 2). Ten anti-parallel  $\beta$ -strands fold into two nearly orthogonal  $\beta$ -sheets to form the  $\beta$ -“clam-like” cavity of the iLBPs (Figure 2) (Furuhashi and Hotamisligil, 2008; Storch and McDermott, 2009; Ferrolino *et al.*, 2013; Napoli, 2016). The two alpha-helices along with nearby loops form a portal region for ligand entry and egress into the interior binding cavity (Figure 2). The iLBPs have a characteristic fingerprint composed of three separate motifs termed FATTYACIDBP1-3 (Figure 1A). The G-x-W triplet in the first FATTYACIDBP1 motif is highly conserved between iLBP members (Figure 1A) and homologous with a similar motif in lipocalin family of binding proteins (Smathers and Petersen, 2011). FABP5 is unique in the iLBP family in that it is the only FABP known to form an intramolecular disulfide bond (C120-C127) (Hohoff *et al.*, 1999). The dynamics of iLBP structures and consequences on ligand binding have been extensively studied and several comprehensive reviews are available on this topic (Storch and McDermott, 2009; Atshaves *et al.*, 2010; Smathers and Petersen, 2011; Ragona *et al.*, 2014).

#### *iLBP Structures*

Crystal structures and NMR solution structures of iLBPs show that ligands are stabilized within the binding cavity by ionic interactions, hydrogen bonding networks with water molecules and interactions with hydrophobic regions (Kleywegt *et al.*, 1994; Cai *et al.*, 2012; Nossoni *et al.*, 2014; Silvaroli *et al.*, 2016). Hydrophobic interactions between the ligand and amino acid

sidechains that line the iLBP binding cavity are important for ligand binding (Thumser *et al.*, 1996). The residues identified as part of the hydrophobic interaction network are shown for representative iLBPs in Figure 3A. The importance of the hydrophobic interactions is also illustrated in the general observation that binding affinities with FABPs correlate with increasing hydrophobicity (Storch and Corsico, 2008; Smathers and Petersen, 2011). Ionic and hydrogen bonding interactions for ligand binding typically involve charged residues. The arginine residue in the FATTYACIDBP3 (Figure 1A) is highly conserved in the iLBPs that bind acidic ligands (R122 in FABP1, R126 in FABP3 and FABP4, R129 in FABP5 and R132 in CRABP2) and is located on the  $\beta$ J strand of these proteins (Figure 3A). For CRBP1 that binds nonacidic ligands *all-trans*-retinol and *all-trans*-retinal, the Q128 appears to be the corresponding residue important for ligand binding (Silvaroli *et al.*, 2016). For CRABP1 and CRABP2 the amino acids R132, and Y134 coordinate with the carboxylic acid of *all-trans*-retinoic acid (*atRA*) and R111 appears to coordinate with the carboxylic acid via an ordered water molecule (Figure 3C) (Kleywegt *et al.*, 1994). Similarly, for some FABPs analogous amino acids also coordinate with the carboxylate of fatty acids bound to FABPs (Hanhoff *et al.*, 2002; Smathers and Petersen, 2011). However, these residues are not essential for ligand binding in all iLBPs. Mutations of the conserved arginine in the  $\beta$ J strand confer different effects on ligand binding depending on the iLBP isoform and the ligand in question. A single R132A or R132Q mutation completely abolishes binding of *atRA* to CRABP2 (Chen *et al.*, 1995). Similarly, an R126Q mutation on the analogous residue in FABP4 reduces the binding affinity for *cis*-parinaric acid by > 10-fold (Sha *et al.*, 1993). In contrast an R122Q mutation in FABP1 only moderately decreases fatty acid binding, and increases binding of bulkier ligands (Thumser *et al.*, 1996). Charged or polar residues in the  $\beta$ H strand also interact with hydroxy and carbonyl head groups (Figure 3A)

except for Subfamily II FABPs (FABP1 and FABP6), and likely contribute to ligand binding. The hydroxy group of *all-trans*-retinol interacts with Q108 in CRBP1 (Figure 3A) and Q109 in CRBP2. Ligands appear to interact with the conserved residue R111 in CRABPs (Figure 1A and Figure 3A). In addition, ligands interact with R106 in FABP2 and this residue is also conserved in subfamily IV FABPs (Figure 1A and 3A).

The helix-turn-helix motif, in conjunction with nearby  $\beta$ C- $\beta$ D and  $\beta$ E- $\beta$ F loops, form the portal region of the iLBP that permits ligand entry and egress from the interior binding cavity (Figure 2) (Vaezslami *et al.*, 2006; Storch and Corsico, 2008; Silvaroli *et al.*, 2016). Different hypotheses describe the extent of the dynamics and flexibility of the portal region. Early observations from NMR solution structures of FABP2 showed disorder and flexibility in the portal region leading to the “dynamic portal hypothesis” (Hodsdon and Cistola, 1997a; b). This hypothesis suggests that the disordered portal region in the apo-protein could undergo large structural fluctuations to permit ligand entry, but shifts to an ordered closed state upon ligand binding. Processes that destabilize the helical cap, such as interactions with cationic membranes, would then shift the protein toward the disordered state, and hence, facilitate ligand release. Later studies with FABP1 supported this hypothesis and showed the apo- and holo-protein structures to have an open and closed “helix cap”, respectively (He *et al.*, 2007). However, the dynamic portal hypothesis is not sufficient to reconcile observations that some FABPs have similar structures between ligand bound and unbound forms (Vaezslami *et al.*, 2006; Gillilan *et al.*, 2007; Cai *et al.*, 2012).

Internal proteins dynamics may also have a major role in influencing ligand accessibility since major fluctuations in the portal region are not observed in structural studies with both retinoid binding proteins and FABPs (Vaezslami *et al.*, 2006; Cai *et al.*, 2012; Ragona *et al.*,



2014). In CRABP2, the portal appears large enough to allow entry of *all-trans*-retinoic acid in both the apo- and holo-structures with little change in the overall protein backbone (Vaezeslami *et al.*, 2006). However, the sidechain of R59 (Figure 3) which is located at the entry of the portal region ( $\beta$ C- $\beta$ D loop) in the apo-protein appears to rotate its position in the holo-protein to form stabilizing interactions with *all-trans*-retinoic acid (Figure 3). An analogous residue (F57) in FABP4 appears to have a similar function and supports the importance of sidechain dynamics in the internal binding cavity. Structural studies with FABP4 suggest that locking the internalized ligand in the holo-protein is controlled by the F57 sidechain on the  $\beta$ C- $\beta$ D loop that rotates into the binding cavity in the holo-conformation (Gillilan *et al.*, 2007) despite little conformational change between apo- and holo-proteins. Indeed, ligand binding kinetics with the fluorescent ligand 8-anilinoanthracene-1-sulfonic acid (ANS) are faster in an FABP4 portal mutant (V32G, F57G, K58G), which has an enlarged portal region, than with wildtype (WT) FABP4 (Jenkins *et al.*, 2002).

Solution NMR studies show that the backbone dynamics in the portal region in apo-proteins vary between iLBP isoforms. FABP6 has a relatively rigid portal region while FABP1, 3, and 4 portal regions are more flexible, and the FABP2 portal is virtually disordered (Ragona *et al.*, 2014). However, in general the changes in the backbone dynamics of FABPs upon ligand binding are consistent with disordered to ordered stabilizing interactions.

### *Endogenous Ligand Binding in iLBPs*

The divergence of ligand specificity in iLBPs arise from nuanced differences in the  $\beta$ -barrel cavity and portal regions. Figure 4 shows representative structures of CRBP1, FABP1, FABP2 and FABP4 bound with their endogenous ligands illustrating general features of ligand binding with iLBPs. Historically protein fractionation, gel filtration, ion-exchange

chromatography and electrophoresis techniques were used to isolate and identify iLBPs in tissues and tissue homogenates, and bound ligands were identified from this isolated protein (Bashor *et al.*, 1973; Ockner and Manning, 1974; Maatman *et al.*, 1991; Veerkamp and Maatman, 1995). Subsequent characterization of ligand binding has been largely done with fluorescent probes or radiolabeled ligands in tissue homogenates or with purified recombinant iLBPs (MacDonald and Ong, 1987; Giguère *et al.*, 1990; Nemezc *et al.*, 1991; Sanquer and Gilchrest, 1994; Folli *et al.*, 2001) and with x-ray crystallography and NMR (Kleywegt *et al.*, 1994; LaLonde *et al.*, 1994; Thompson *et al.*, 1997; Hohoff *et al.*, 1999).

The known endogenous ligands of iLBPs are summarized in Table 1. The retinoid binding proteins appear to be selective toward vitamin A and its metabolites while all FABPs bind long chain fatty acids (LCFA) (Table 1). Some FABPs also bind a variety of other endogenous ligands (Table 1). It is important to note that the list of known ligands is limited to those ligands that have been explicitly tested for their binding and may not be comprehensive for all endogenous ligands. Several studies have explored synthetic derivatives of the endogenous ligands of FABPs (Wang *et al.*, 2016; Floresta *et al.*, 2017) but these synthetic derivatives are not discussed in this review. Additionally, the summary below includes binding data from species other than human proteins, as many ligand binding studies with iLBPs were done with rat, mouse and bovine recombinant protein.

#### *Endogenous Ligands of Subfamily I*

Vitamin A (retinol) or its biologically important metabolites retinaldehyde and the pharmacologically active *at*RA bind the proteins in subfamily I with high affinity. Notably, all proteins in this subfamily are intracellular, in contrast to the lipocalin retinol binding protein 4 (RBP4) which is the circulating carrier for retinol. *all-trans*-retinol, *all-trans*-retinaldehyde and

their 13-*cis* and 9-*cis* isomers bind to CRBP1 (Figure 4A) and CRBP2 with nanomolar affinity. Yet *atRA* nor its 13-*cis* and 9-*cis*-isomers bind to CRBPs (Kane *et al.*, 2011; Napoli, 2016, 2017; Menozzi *et al.*, 2017). *all-trans*-retinol binds to CRBP3 (Folli *et al.*, 2001) and *all-trans*-retinol along with 13-*cis* and 9-*cis* retinol bind to CRBP4 (Vogel *et al.*, 2001; Folli *et al.*, 2002).

Although CRBPs appear to be specific for retinol and retinal ligands, monoacylglycerols were also recently shown to bind to CRBPs (Lee *et al.*, 2020). This suggests CRBPs and CRABPs may have broader ligand specificity than previously assumed. *atRA* and its isomers and metabolites bind specifically to CRABP1 and CRABP2 with *atRA* having higher binding affinity toward CRABP1 and CRABP2 ( $K_d = 0.4\text{-}39$  nM) (Fiorella and Napoli, 1991; Fogh *et al.*, 1993; Norris *et al.*, 1994; Wang *et al.*, 1997) than 9-*cis*-RA ( $K_d = 51\text{-}69$  nM) (Norris *et al.*, 1994) or 13-*cis*-RA ( $K_d = 156\text{-}238$  nM) (Fiorella *et al.*, 1993). Generally, *atRA* appears to bind slightly tighter to CRABP1 than CRABP2 (Fiorella *et al.*, 1993; Dong *et al.*, 1999; Yabut and Isoherranen, 2022). Retinol or retinal isomers do not bind to CRABP1 or CRABP2 (Fiorella and Napoli, 1991; Fiorella *et al.*, 1993; Napoli, 2017).

### *Endogenous Ligands of Subfamily II*

FABP1 and FABP6 make up subfamily II. Generally, bulky ligands in addition to LCFA bind to FABP1 and FABP6 (Smathers and Petersen, 2011). The binding pockets of FABP1 and FABP6 are larger ( $\geq 639$  and  $460 \text{ \AA}^3$ , respectively) (Lücke *et al.*, 2000) than other FABPs that have solvent accessible binding pockets of  $\sim 230\text{-}330 \text{ \AA}^3$  (Smathers and Petersen, 2011). FABP1 and FABP6 can accommodate larger ligands found in the liver such as bile acids, cholesterol, bilirubin and heme (Bernlohr *et al.*, 1997; Smathers and Petersen, 2011). Other ligands of FABP1 include branched fatty acids, endocannabinoids, acyl-CoA, prostaglandins and vitamin K (Khan and Sorof, 1990; Thumser and Wilton, 1996; Martin *et al.*, 2003; Storch and Corsico, 2008;

Atshaves *et al.*, 2010; Smathers and Petersen, 2011). While fatty acid ligands appear to bind to all other FABPs in a 1:1 ratio, two fatty acids can bind to FABP1 simultaneously (Figure 4B) (Bernlohr *et al.*, 1997; Thompson *et al.*, 1997; Cai *et al.*, 2012). FABP1 has a high affinity fatty acid binding site ( $K_d = 4\text{-}60$  nM) located deep within its interior cavity, and a low affinity site (0.3-12  $\mu\text{M}$ ) closer in proximity to the alpha-helical domain and opening of the portal region (Figure 4B) (Atshaves *et al.*, 2010; Smathers and Petersen, 2011; Cai *et al.*, 2012). With larger ligands such as bile acids, this stoichiometry appears to be reduced (1:1) along with reduced binding affinities ( $K_d$  4-50  $\mu\text{M}$ ) (Richieri *et al.*, 1995). FABP6 is structurally similar to FABP1 but due to differences in interior amino acid side chains between the two proteins, preferential ligands of FABP6 include bile acids over long chain fatty acids (Lücke *et al.*, 2000). Due to the size of bile acids only a single ligand is typically observed in the FABP6 binding cavity.

#### *Endogenous Ligands of Subfamily III*

FABP2 is the sole member of subfamily III. In contrast to the iLBPs in subfamily II, FABP2 has a small solvent accessible binding pocket (234  $\text{\AA}^3$ ) (Smathers and Petersen, 2011) and its preferential ligands include saturated LCFAs (Figure 4C) (Lowe *et al.*, 1987; Richieri *et al.*, 1994; Velkov *et al.*, 2005; Smathers and Petersen, 2011). Measured fatty acid binding affinities with FABP2 range between 0.02-1.5  $\mu\text{M}$  based on fluorescence displacement assays (Nemecz *et al.*, 1991; Velkov *et al.*, 2005, 2007).

#### *Endogenous Ligands of Subfamily IV*

The seven members of subfamily IV collectively bind diverse lipids. The size of the solvent accessible binding pockets of subfamily IV FABPs appear to be larger than subfamily III (FABP2) but smaller than subfamily II (FABP1 and FABP6). FABP3, 4 and 8 have 323, 310 and

330 Å<sup>3</sup> binding pockets, respectively (Smathers and Petersen, 2011). Saturated and unsaturated fatty acids bind to FABP3 with nanomolar affinity, and oxygenated fatty acids (epoxyeicosatrienoic acid, hydroxyeicosatetraenoic acid, dihydroxyeicosatrienoic acid) bind to FABP3 with  $K_d$ 's from 0.4 to 14 μM (Widstrom *et al.*, 2001; Smathers and Petersen, 2011). FABP4 appears to be more ligand selective, and only LCFAs bind to FABP4 with nanomolar affinity ( $K_d = 22-196$  nM) (Figure 4D) (Richieri *et al.*, 1994; Gillilan *et al.*, 2007; Storch and Corsico, 2008; Smathers and Petersen, 2011). However, other ligands such as *atRA* also bind to FABP4 but with a considerably lower binding affinity ( $K_d = 50$  μM) (Matarese and Bernlohr, 1988; Veerkamp *et al.*, 1999).

With FABP5, stearic acid and docosahexaenoic acid have nanomolar affinity to FABP5 ( $K_d = 0.17-0.29$  and  $0.16$  μM, respectively) and oleic acid, lauric acid and arachidonic acid binding affinity range from nanomolar to micromolar ( $K_d = 0.15-1.6$ ,  $2.5$  and  $0.12-1.7$  μM, respectively) (Hohoff *et al.*, 1999; Smathers and Petersen, 2011; Kaczocha *et al.*, 2012; Pan *et al.*, 2015; Lee *et al.*, 2018). *atRA* has also been reported to bind to FABP5 in fluorescence displacement assays with ANS ( $K_d = 35$  nM) (Schug *et al.*, 2007). However, FABP5 did not to sequester *atRA* from metabolism by cytochrome P450 enzymes suggesting binding may not be as tight as suggested by the displacement assay (Yabut and Isoherranen, 2022). FABP7 prefers polyunsaturated fatty acids with longer chains (docosahexaenoic acid, eicosapentaenoic acid, arachidonic acid) and these fatty acids bind to FABP7 with affinities ranging from 27-250 nM (Smathers and Petersen, 2011).

In addition to the fatty acid ligands of FABPs, FABP3, FABP5 and FABP7 have also been shown to bind the endocannabinoids 2-archidonylglycerol (2-AG) and anandamide (AEA), and FABPs have been proposed to have a role in modulating endocannabinoid metabolism and

signaling. 2-AG and AEA bind to FABP7 with higher affinity ( $K_d = 0.2$  and  $0.8 \mu\text{M}$ , respectively) than to FABP3 ( $K_d = 1.63$  and  $3.07 \mu\text{M}$ , respectively) and to FABP5 ( $K_d = 1.45$  and  $1.26 \mu\text{M}$ , respectively) (Kaczocha *et al.*, 2012; Elmes *et al.*, 2015). FABP8, 9 and 12 have not been extensively studied and the binding of their endogenous ligands is not well characterized (Storch and Corsico, 2008; Smathers and Petersen, 2011).

### **Tissue distribution and expression of iLBPs**

The tissue distribution of iLBPs is broad and expression patterns have been studied in several mammalian species including rat, mice, pig and human (Paulussen *et al.*, 1988, 1990; Gong *et al.*, 1994; Sanquer and Gilchrest, 1994). However, species differences in tissue expression have not been comprehensively compared for all iLBPs. The following is a summary of the tissue expression of iLBPs in adult mammals determined using a combination of techniques including western and northern blot analysis, immunohistochemistry, enzyme-linked immunosorbent assay, reverse transcriptase-quantitative polymerase chain reaction and binding assays with radiolabeled ligands. Some iLBPs are expressed in multiple tissues while others are expressed in specific tissues and cell types which may be indicative of specialized biological functions (Storch and Corsico, 2008). The expression pattern of the FABPs is sometimes evident from the original name of the FABP as FABPs were named after the tissues from where they were first identified. However, multiple FABPs are often expressed in the same tissues and the expression patterns are typically broader than what is implied from the original names of the FABPs. Hence, early studies identifying FABPs in tissues often required confirming the specificity of antisera against multiple FABPs (Paulussen *et al.*, 1990; Maatman *et al.*, 1991; Gong *et al.*, 1994).

Although the iLBPs are generally considered to be intracellular, FABP1, 2, 3, 4 and 5 have also been measured in plasma in humans (0.3-13 ng/mL) (Pelsers *et al.*, 2003; Ishimura *et al.*, 2013). Yet their concentrations are much lower than other circulating proteins such as albumin that bind fatty acids in plasma, and the importance of the circulating FABPs is unknown. FABP4 is the only isoform shown to be secreted from tissues (adipose) into circulation (Hotamisligil and Bernlohr, 2015; Shrestha *et al.*, 2018; Villeneuve *et al.*, 2018). For this review only CRABPs and those FABPs that xenobiotics have been shown to bind to are discussed, but the tissue expression for all iLBPs is summarized in Table 1.

CRABP1 protein is found in various tissues including liver, kidney, stomach, lymph, eye, and brain, but it is most abundant in skin and reproductive tissues (seminal vesicles, vas deferens and testis) (Kato *et al.*, 1985). CRABP2 protein expression appears to be limited to skin (Giguère *et al.*, 1990).

FABP1, or liver FABP, is the major FABP in the liver and the intestine, but is also found in the kidney, lung, pancreas, and stomach (Besnard *et al.*, 2002; Pelsers *et al.*, 2003; Gajda and Storch, 2015; Wang *et al.*, 2015). FABP1 is most abundant in the liver and comprises 2-11% of all cytosolic protein in the liver (Wang *et al.*, 2015; Schroeder *et al.*, 2016). Expression of FABP1 in the liver is zonal, possibly indicating a unique role in specific areas of the liver (Bass *et al.*, 1989). Peroxisome proliferators, female sex steroids, retinoids and a diet high in fat increase the expression of FABP1 mRNA and protein in the liver (Poirier *et al.*, 1997; Hung *et al.*, 2003; Trevaskis *et al.*, 2011; Velkov, 2013). Interestingly, FABP1 mRNA and protein expression is decreased after dexamethasone treatment likely due to altered lipid metabolism and concentrations (Foucaud *et al.*, 1998). In the gut, FABP1 mRNA is expressed throughout the length of the small intestines but is highest in the duodenum and jejunum (Agellon *et al.*, 2002;

Gajda and Storch, 2015). The expression pattern of FABP1 in the liver and intestines suggests FABP1 may also impact drug metabolism in the liver and drug absorption in the intestines. Additionally, FABP1 expression and function may have a role in metabolic disease progression as FABP1 polymorphisms in humans are associated with dyslipidemia, non-alcoholic fatty liver disease and hepatocellular carcinoma (Peng *et al.*, 2012; Schroeder *et al.*, 2016; McKillop *et al.*, 2019; Valizadeh *et al.*, 2021). For example, the T94A mutation (allele frequency 26-38%) in FABP1 alters FABP1 expression, ligand binding characteristics, protein structure and stability and protein function (Schroeder *et al.*, 2016). The T94A SNP is associated with elevated triglycerides, LDL cholesterol and altered response to fenofibrate (Schroeder *et al.*, 2016).

FABP2, also called intestinal FABP, is solely expressed in the gut and its expression appears to be similar to FABP1 in rodent intestine but lower than FABP1 in human intestine. FABP2 mRNA is expressed throughout the length of the small intestine and its expression is highest in the jejunum (Sacchettini *et al.*, 1990; Gajda and Storch, 2015). Along with FABP1, FABP2 expression is highest in the villi of enterocytes, and it is not expressed in the crypt. FABP2 expression in enterocytes may be regulated by the gut peptide tyrosine tyrosine (Halldén and Aponte, 1997). FABP2 expression appears to be diffused throughout enterocytes but localized to the apical side in a fasted state (Alpers *et al.*, 2000). Similar to FABP1, an A54T polymorphism in FABP2 appears to be associated with dyslipidemia, insulin resistance, obesity, cardiovascular disease and may increase the risk of colorectal cancer (McKillop *et al.*, 2019; Huang *et al.*, 2022). FABP2 has been proposed as a potential biomarker for disruption of intestinal epithelial integrity as FABP2 is released to circulation when intestinal epithelium is compromised (Huang *et al.*, 2022).



FABP3, or heart FABP, protein has been found in the heart, skeletal muscle, brain, kidney, liver, lung, spleen and placenta (Paulussen *et al.*, 1990). FABP3 is most abundant in the heart where its expression is nearly 2-fold greater than in skeletal muscle. Protein abundance in the kidney and brain is about half of that in the muscle and even less in the liver and placenta. FABP3 is also found to circulate at elevated levels in plasma in response to myocardial injury, presumably due to release from the heart. As such it may be a potential biomarker for cardiovascular disease (Pelsers *et al.*, 2005). In the kidney, FABP3 is found to be expressed in the distal and proximal convoluted tubules (Maatman *et al.*, 1991) suggesting FABP3 could play a role in renal handling of drugs and xenobiotics.

FABP4, known as adipocyte FABP, is abundantly expressed in adipose tissue and is also the major FABP found in macrophages (Pelton *et al.*, 1999; Furuhashi and Hotamisligil, 2008). FABP4 is the most abundant FABP in circulation (Ishimura *et al.*, 2013), and is secreted from adipocytes via a membrane-bound pathway independent of the canonical endoplasmic reticulum-Golgi-plasma membrane secretion pathway (Villeneuve *et al.*, 2018). Secreted FABP4 may serve as an adipokine, and lipolysis increases secretion of FABP4 from adipocytes (Furuhashi *et al.*, 2015). Exogenous FABP4 influences hepatocyte glucose production, insulin secretion by pancreatic  $\beta$  cells, and cellular functions of cardiomyocytes and smooth muscle cells (Furuhashi *et al.*, 2015). Indeed, circulating FABP4 levels are associated with the development of insulin resistance, diabetes, atherosclerosis, cardiac dysfunction and inflammation (Furuhashi and Hotamisligil, 2008; Ishimura *et al.*, 2013; Furuhashi *et al.*, 2015; Saito *et al.*, 2021). Reduced FABP4 function appears to reduce the risk of metabolic and cardiovascular disease (Hotamisligil *et al.*, 1996; Furuhashi and Hotamisligil, 2008; Furuhashi *et al.*, 2015), and hence, FABP4 has been explored as a potential therapeutic target (Floresta *et al.*, 2017). Due to its small size,

FABP4 found in circulation is subject to glomerular filtration, but it accumulates in the kidney via megalin-mediated reabsorption from the tubular lumen (Shrestha *et al.*, 2018). Notably, circulating FABP4 levels also showed a sex difference with females having higher concentrations than males (Ishimura *et al.*, 2013).

FABP5, epidermal FABP, is the major FABP found in the epidermis but FABP5 tissue expression is broad and not restricted to the skin (Table 1). FABP5 mRNA along with FABP3 and FABP4 mRNAs are found in human brain endothelial cells (hCMEC/D3) and FABP5 protein appears to be more abundant than FABP3 and FABP4 in these cells (Lee *et al.*, 2015).

FABP7, brain FABP, is largely expressed in the brain and central nervous system but is also found in the skeletal muscle (Shimizu *et al.*, 1997; Veerkamp and Zimmerman, 2001; Owada *et al.*, 2006) with diurnal variation in its expression (Gerstner *et al.*, 2008). FABP7 mRNA in the brain increases during light periods and declines in the dark period. This leads to an accumulation of FABP7 protein in dark periods, and a decrease in protein in the light period. Yet, the biological role of this diurnal variation has not been defined.

## **Xenobiotic Ligands of iLBPs and Methods to Characterize Ligand Binding**

### *Known Xenobiotic Ligands of iLBPs and Their Binding Characteristics*

The literature is rich with binding and structural studies of endogenous ligands of iLBPs, but binding of xenobiotics to iLBPs has not been as extensively studied. This is despite clear evidence of xenobiotics binding to iLBPs. For example, synthetic retinoid drugs (agonists of retinoic acid receptors and retinoid X receptors) bind to retinoid binding proteins (Ferreira *et al.*, 2020), but the clinical relevance of the binding is not known. Whether retinoid binding proteins

bind other classes of therapeutic drugs has not been explored. The majority of xenobiotic binding studies with iLBPs have been done with FABPs, likely due to their broad ligand specificity and high expression in tissues relevant for drug disposition and pharmacological activity. Of the ten FABPs, xenobiotics have been shown to bind to FABP1-5 and FABP7 *in vitro* (Table 2).

Xenobiotic binding to FABP6, 8, 9 and 12 has not been reported to our knowledge.

Initial binding studies of lipophilic drugs to FABP1 and FABP2 were done to explore the potential of FABP1 and 2 to facilitate drug absorption into enterocytes (Velkov *et al.*, 2005, 2007; Chuang *et al.*, 2008). A broad range of therapeutic drugs such as non-steroidal anti-inflammatory drugs, peroxisome proliferator-activated receptor (PPAR) agonists (fibrates and glitazones) and benzodiazapines were shown to bind to FABP1 and FABP2 (Table 2). Additionally, due to the high expression of FABP1 in the liver, the role of FABP1 binding as the rate limiting step in hepatocyte uptake has been explored (Rowland *et al.*, 2015). FABP3, 4 and 5 were found to be expressed in brain endothelial cell lines, and hence, the potential of drugs to bind to these FABPs at the blood-brain-barrier was evaluated (Lee *et al.*, 2015). Similar drugs were shown to bind to FABP3, 4 and 5 as to FABP1 and FABP2. Xenobiotic cannabinoids  $\Delta^9$ -tetrahydrocannabinol and cannabidiol also bind to FABP1 and the brain FABPs, FABP3, 5, and 7 (Elmes *et al.*, 2015, 2019; Huang *et al.*, 2018). Due to its role in metabolic disease, FABP4 has become a potential therapeutic target and a variety of inhibitor ligands have been developed and their binding to FABP4 characterized (Floresta *et al.*, 2017).

FABP1 has been a focus of binding and structural studies with PPAR agonists (fibrates, glitazones and synthetic agonists) to probe PPAR binding specificities as they relate to interactions with residues within the binding cavity of FABP1. Ester and carboxylic acid fibrates showed distinct differences in chemical shift perturbations (CSPs) within the FABP1 binding

cavity in NMR studies (Chuang *et al.*, 2009). Carboxylic acid fibrates showed significant CSPs with residues S39, R122, S124 in FABP1 that directly interact with the carboxylate of fatty acids while ester fibrates showed far less CSPs at these residues. Additionally, thermodynamic analysis showed that binding of carboxylic acid fibrates to the high affinity site of FABP1 was mainly driven by enthalpic interactions while ester fibrate binding had a much larger entropic component (Chuang *et al.*, 2009). These data suggest that while ionic interactions play a role in the recognition and binding specificity of non-fatty acid ligands, they are not essential for ligand binding. Hydrophobic interactions are a large component of xenobiotic binding to FABP1.

The importance of hydrophobic interactions in ligand binding is evident in structural studies with FABP4 and (S)-ibuprofen (Figure 5A). (S)-ibuprofen binding to FABP4 is stabilized by both ionic and edge-to-face aromatic interactions with FABP4 sidechains (Figure 5B and 5C) (González and Fisher, 2015). Similar to binding of endogenous ligands, internal protein dynamics also appear to play an important role in xenobiotic binding to FABPs. NMR solution structures of FABP1 with the synthetic PPAR agonist GW7647 bound (Figure 5D) demonstrate that significant sidechain conformational changes occur within the binding cavity of holo-FABP1 upon ligand binding. This is despite there being little change in the overall backbone structure with ligand binding (Patil *et al.*, 2019). Knowledge of the structures and ligand binding characteristics of individual FABPs can aid in designing FABP isoform specific ligands. For example, the synthetically designed FABP4 ligand BMS309403 binds to FABP4 (Figure 5E) with a binding affinity of less than 2 nM but binds to FABP3 and FABP5 with > 100-fold weaker affinity (Sulsky *et al.*, 2007).

### *Methods to Measure Ligand Binding with iLBPs*

Historically, measuring free ligand concentrations using separation techniques such as Lipidex-1000 (Glatz and Veerkamp, 1983; Vork *et al.*, 1990) were used to determine ligand binding affinities to iLBPs that were isolated from tissue homogenates or recombinantly expressed and purified. However, as the concentration of free ligand is decreased via partitioning to Lipidex, these techniques generally disturb the equilibrium between ligand and iLBP and the binding affinities are generally underestimated (apparent  $K_d > \text{true } K_d$ ) using this technique (Kane and Bernlohr, 1996; Veerkamp *et al.*, 1999). Most of the recent work to characterize xenobiotic binding to iLBPs has been done using *in vitro* spectrophotometric assays. The following is a brief description of the direct and indirect spectrophotometric approaches to determine xenobiotic equilibrium binding affinities along with potential caveats associated with these methods.

#### *Direct Binding Assays*

Binding affinities ( $K_d$ ) of retinoids with retinoid binding proteins are typically determined via direct fluorescence titration assays. These monitor either the increase in retinoid fluorescence upon binding to the binding protein or the quenching of intrinsic protein fluorescence (from tryptophan or tyrosine) as a result of retinoid binding (Fiorella *et al.*, 1993; Norris *et al.*, 1994; Wang *et al.*, 1997; Dong *et al.*, 1999; Vogel *et al.*, 2001; Folli *et al.*, 2002; Yabut and Isoherranen, 2022). These methods work well for retinoid binding proteins such as CRBP1, CRABP1 and CRABP2 which have five or more fluorescent (tryptophan and tyrosine) amino acids in their primary sequence. The intrinsic fluorescence spectra of tryptophan and tyrosine (excitation peak 280-290 nm, emission peak 330-355 nm) and the fluorescence spectra of retinoids (excitation peak 348-360 nm and emission peak 450-480 nm) (MacDonald and Ong,

1987; Fiorella and Napoli, 1991; Dong *et al.*, 1999; Herr *et al.*, 1999; Folli *et al.*, 2002) are amenable for monitoring binding via fluorescence resonance energy transfer from protein to retinoid ligands (Peterson and Rask, 1971).

Because retinoids bind to retinoid binding proteins tightly (nanomolar affinities), performing the fluorescence titrations under steady state assumptions can be challenging. Relatively low concentrations of protein (ideally subnanomolar) are necessary to obtain accurate  $K_d$  value estimates, and hence, protein fluorescence signal and instrument (fluorimeter) sensitivity can become a limitation. Therefore, retinoid binding assays are often done with protein concentrations that are much greater than the estimated  $K_d$ -values. This approach may lead to inaccuracies in  $K_d$  estimates. These inaccuracies may be compounded by the use of kinetic binding models that assume steady state and that ligand binding to the binding protein does not alter free ligand concentrations in solution ( $[L]_{total} \approx [L]_{free}$ ). These experimental challenges likely partially explain the wide range of binding affinities reported in the literature (Norris *et al.*, 1994; Napoli, 2016). The impact of protein concentrations and model fitting on the error in determination of the  $K_d$  values is illustrated in Figure 6. For an iLBP-ligand interaction with a true  $K_d$  of 10 nM, using 100 nM iLBP protein (10-fold  $> K_d$ ) in the experiment can result in an error as high as 5-fold when a simple binding model ( $\% iLBP\ bound =$

$\frac{\%iLBP\ Bound_{max} \times [L]}{K_d + [L]}$ ) is fit to the data (Figure 6B). The error becomes negligible when the quadratic binding equation

( $\% iLBP\ bound = \%iLBP\ bound_{max} \frac{[P] + [L] + K_d - \sqrt{([P] + [L] + K_d)^2 - 4 \times [P] \times [L]}}{2 \times [P]}$ ) is fit to the data as it

accounts for ligand depletion when the iLBP concentration ranges from 0.01-10 times the true  $K_d$  value (Figure 6B). However, caution should be used when using the quadratic binding equation

for tight binding ligands as the dependence of  $K_d$  for the model fit becomes negligible when  $[P] \gg K_d$ , and hence, a  $K_d$  estimate may not be meaningful (Jarmoskaite *et al.*, 2020). As such, even when the quadratic equation is used, the  $K_d$  values estimated should be assumed to be the upper limit of the true  $K_d$  value if the iLBP concentration in the assay exceeds the determined  $K_d$  value. Nevertheless, direct fluorescence titration assays have provided extensive information of the ligand binding characteristics and binding specificities of iLBPs.

### *Fluorescence Displacement Assays*

Measuring direct protein fluorescence is not always feasible due to a lack of fluorescent amino acids or lack of fluorescence of the ligand. For example, FABP1 has no tryptophan residues and only one tyrosine residue preventing the use of direct fluorescence measurements in evaluating ligand binding to FABP1. Hence, one approach for measuring direct ligand binding to FABP1 is to introduce tryptophan mutations to increase intrinsic protein fluorescence (Thumser and Wilton, 1994). However, such mutations may also affect ligand binding and hence indirect fluorescence displacement assays are more commonly used.

Indirect measurements of ligand binding for FABPs have been a common approach for estimating binding affinities for FABPs (Schug *et al.*, 2007; Smathers and Petersen, 2011; Kaczocha *et al.*, 2012; Elmes *et al.*, 2015, 2019; Huang *et al.*, 2016, 2018; Schroeder *et al.*, 2016). Fluorescence displacement assays using fluorescent probes such as ANS or fluorophore conjugated fatty acids such as 11-(dansylamino)undecanoic acid (DAUDA) and nitrobenzoxadiazole (NBD)-stearate are commonly used due to the low intrinsic fluorescence of FABPs, and the lack of measurable fluorescence from fatty acid ligands upon FABP binding. In these assays, a fluorescent probe is first bound to the FABP at a pre-determined concentration, and the shift in the fluorescence of the ligand upon protein binding is measured. The drug of

interest is then titrated into the sample, and the decrease in the probe fluorescence due to probe displacement by the drug is measured. Because displacement of the probe is assumed to be a purely competitive interaction, inhibitory constants ( $K_i$ ) are either determined from a direct fit of a competitive binding model to the fluorescence data, or are calculated from  $IC_{50}$  values assuming competitive inhibition (Velkov *et al.*, 2005; Chuang *et al.*, 2008; Lee *et al.*, 2015; Elmes *et al.*, 2019). Interfering fluorescence at similar wavelengths as the probe from the ligand of interest should be considered in these assays as this may confound the binding data. For example, *atRA* has a similar fluorescence emission peak (475 nm) as the fluorescent probe ANS (480 nm) with excitation wavelengths at 350 and 380 nm, respectively (Fiorella *et al.*, 1993; Huang *et al.*, 2014; Vogler, 2015). This fluorescence overlap may affect the interpretation of ANS displacement data for *atRA* binding to FABPs.

For most FABPs the assumption of competitive binding is likely appropriate as only one ligand appears to bind at a time to FABPs (Figure 4 and 5). However, with FABP1 which can have multiple ligands bound to it simultaneously (Figure 4B), a simple competitive binding model may not be appropriate and  $EC_{50}$  or  $IC_{50}$  values determined with ligand displacement assays with FABP1 should not be directly translated to  $K_d$  or  $K_i$  values. It is possible that a ligand can bind simultaneously with fluorescent probe to the FABP1, or that the binding of one fluorescent probe molecule affects the binding affinity of the ligand tested that may bind to a second binding site.

Similar concerns may be relevant for FABP2, although endogenous ligands appear to bind to FABP2 with 1:1 stoichiometry, possibly due to the size of the ligands. In FABP2, ANS and ketorolac have been shown to bind to different binding sites based on fluorescence and isothermal titration calorimetry data (Patil *et al.*, 2014). This suggests that the two ligands could



also bind simultaneously. However, there is currently no structural evidence that two ligands bind to FABP2 simultaneously. In the case of FABP1 and FABP2, it is unclear whether it is possible for a ligand to bind to the second binding site without affecting the binding of the fluorescent ligand in the first, high affinity binding site. The probe fluorescence intensity or the wavelength maxima of the probe fluorescence may be allosterically affected by binding of a ligand at an additional binding site, or complete displacement of the fluorescent probe may only occur when all binding sites are occupied by the xenobiotic ligand. Hence, alternative kinetic binding models may be more appropriate than a purely competitive one.

Like the direct binding assays described above, limitations with fluorescence signal, instrument sensitivity, and protein and probe concentrations should be carefully considered with displacement assays. Figure 6C shows simulated fluorescence displacement assay where the true affinity for the probe ( $K_d$ ) and test ligand ( $K_i$ ) are the same (10 nM). Using an iLBP concentration of 100 nM (10-fold greater than the  $K_d$ ) can result in > 10-fold error in the estimated  $K_i$  value illustrating the potential confounding effects of experimental design on the data collected (Figure 6C and D).

### **Impact of iLBPs on Ligand Distribution and Metabolism**

One of the biological functions of iLBPs is to serve as lipid carriers that bind, solubilize, and shuttle their ligands to relevant cellular compartments (Storch and Corsico, 2008; Storch and Thumser, 2010). iLBPs may simply bind their often unstable or toxic ligands to stabilize the ligand or prevent ligand interactions with nonspecific proteins in a cell. However, iLBPs have been shown to interact with phospholipid membranes, associate with cellular compartments such

as mitochondria and lysosomes and interact with different metabolic enzymes and nuclear receptors suggesting more broad functions in a cell. Three different mechanisms for the impact of iLBPs on ligand disposition have been proposed (Figure 7) (Smith and Storch, 1999; Storch and Corsico, 2008). In the first model, the iLBPs release their ligands to solution (diffusional model). Alternatively, iLBPs may interact directly with the phospholipid membranes via direct protein-membrane interactions to accept their ligands from or release their ligands directly to the membrane (collisional model). Finally, iLBPs may participate in direct protein-protein interactions, and channel their ligands directly to catalytic enzymes or transporters. Since FABPs are highly expressed in tissues relevant to xenobiotic disposition, it is likely that xenobiotics bind to FABPs in these tissues, and the three models of iLBP functions may also be relevant for xenobiotic disposition. The following sections summarize various studies on the impact of iLBPs on ligand distribution and metabolism and the possible mechanisms of ligand delivery.

### *Impact of FABPs on Ligand Uptake into Tissues*

The role FABPs have in regulating the uptake and tissue distribution of their endogenous ligands has been studied for many FABPs to which xenobiotic ligands also bind to. It is well established that FABP1 facilitates lipid uptake into the liver, and FABP1 expression in the liver correlates with uptake of fatty acids (Kushlan *et al.*, 1981; Hung *et al.*, 2003; Newberry *et al.*, 2003). Induction of FABP1 expression by PPAR agonists in HepG2 cells resulted in increased rates of oleate uptake, while knocking down FABP1 expression significantly reduced rates of uptake (Wolfrum *et al.*, 1999). Changes in uptake appear to also alter lipid metabolic products. FABP1-knockout mice have decreased rates of  $[H]^3$ oleate uptake to the liver, which corresponds to decreased fatty acid  $\beta$ -oxidation and incorporation of  $[H]^3$ oleate into triglycerides (Newberry *et al.*, 2003). In rat perfused livers, higher expression of FABP1 in the liver correlated with

greater palmitate clearance and higher retention of palmitate and its metabolites in the liver (Hung *et al.*, 2003). Similarly, FABP5 and FABP7 that are expressed in the brain appear to enhance endocannabinoid uptake into cells and endocannabinoid metabolism. The cellular uptake of AEA and subsequent metabolism by fatty acid amide hydrolase was greater in N18TG2 (mouse neuroblastoma) and COS-7 cells transfected with FABP5 and FABP7 when compared to mock transfected cells while FABP3 had no effect (Kaczocha *et al.*, 2009).

FABP2 appears to play a role in the cellular uptake and distribution of xenobiotic ligands in the gut, and many orally administered drugs bind to FABP2 (Table 2). The potential role of FABP2 in modulating apical and basolateral transport of drugs in the intestine was studied in the parallel artificial membrane permeability assays (PAMPA) where an artificial phospholipid membrane separates donor and acceptor reservoirs (Velkov *et al.*, 2007). PAMPA studies were designed to test the effect of FABP2 on the rates of diffusion across an artificial phospholipid membrane mimicking the apical membrane of enterocytes. For apical membrane permeability, drugs were added to the donor side and physiological concentrations of FABP2 in the enterocytes (0.33 mM) were present in the acceptor side. The rates of drug uptake from the apical membrane were increased for drugs that bound to FABP2, with tighter binding drugs showing higher rates of uptake. This suggests FABP2 may facilitate drug absorption in the small intestine. In support of these findings, FABPs appear to also increase rates of drug uptake in perfused rat intestines. In rats, FABP1 and FABP2 mRNA expression in the gut increased ~1.5-2 fold by feeding a high fat diet. Compared to control fed rats, this higher expression of FABP1 and FABP2 correlated with nearly two-fold higher rates of disappearance of ibuprofen (disappearance  $P_{app}$  158 vs 97 x 10<sup>6</sup> cm/sec) and midazolam (disappearance  $P_{app}$  239 vs 143 x 10<sup>6</sup> cm/sec) from intestinal perfusate, and increased accumulation of the drugs in the intestinal tissue (Trevaskis *et al.*, 2011). This

suggests that FABPs may facilitate the uptake of these drugs into the enterocytes (Trevaskis *et al.*, 2011). Interestingly, significantly less 4-hydroxy-midazolam was quantified in mesenteric blood in animals with elevated FABP and the extraction ratio of midazolam by the intestine was decreased from 11% to 7% in rats with higher FABP1 expression compared to control fed rats. This suggests that midazolam likely bound to FABP1 in the enterocytes altering midazolam metabolism in the enterocytes (Trevaskis *et al.*, 2011). Despite these findings, the contribution of FABPs to rate and extent of drug absorption has received relatively little attention.

The potential impact of FABP binding on drug distribution is clear from the high expression of FABPs in different tissues and their capacity of FABPs for drug binding in variety of tissues throughout the body. Binding of numerous drugs that target the central nervous system to FABP3, FABP4 and FABP5, that are expressed in the brain, was proposed to impact the distribution of drugs across the blood-brain barrier (Lee *et al.*, 2015). This process is similar to the regulation of endogenous docosahexaenoic acid concentrations in the brain by FABP5 (Pan *et al.*, 2015, 2016). Current models (Utsey *et al.*, 2020) for predicting tissue distribution of drugs and tissue partition coefficients ( $K_p$  values) do not account for specific protein binding sinks in tissues and hence extensive FABP binding in any tissue is not considered when distribution kinetics are modeled. As physiologically based pharmacokinetic models of drug distribution become more mainstream, incorporation of FABP binding into tissue distribution models and considering FABP binding when rates of distribution are considered will become increasingly important.

### *Ligand Delivery to Membranes*

It remains unclear whether the observed changes in lipid metabolism that correlate with FABP expression are simply because FABPs provide an intracellular reservoir to increase uptake of their ligands to cells, and hence, the availability of ligands to sites of metabolism (diffusional model, Figure 7A), or if FABPs deliver ligands via specific interactions to enzymes or to cellular membranes. The mechanism of ligand transfer from FABPs to model phospholipid membranes has been studied for FABPs 1-5 using fluorescent fatty acids as ligands (Storch and Thumser, 2000). The rates of ligand transfer from FABPs to phospholipid membranes could be measured via quenching of fluorescence upon fatty acid ligand incorporation into the phospholipid membrane. The rate of ligand transfer from FABP1 to acceptor membranes was not affected by the concentrations or composition of phospholipid. However, FABP1 transfer rates were substantially impacted by the ionic strength of the surrounding aqueous medium. These data suggest that FABP1 does not interact directly with phospholipid membranes and that ligand delivery to model membranes requires release of ligand into solution (Hsu and Storch, 1996).

In contrast, the transfer rates of FABPs 2-5 were proportional to phospholipid concentration in acceptor membranes and affected by membrane phospholipid composition (Kim and Storch, 1992; Wootan *et al.*, 1993; Hsu and Storch, 1996; Storch and Thumser, 2000). FABP2 was shown to also accept fatty acids from donor membranes. Faster fatty acid transfer rates from donor membranes to FABP2 were observed with negatively charged membranes compared to zwitterionic membranes (Thumser and Storch, 2000). Taken together, these data suggest that FABPs 2-5 directly deliver their ligands to phospholipid membranes via ionic interactions. The structural basis for this interaction has been elucidated with mutagenesis studies with FABP2. These studies suggest that the alpha-helical domain in FABP2 is important for these interactions (Corsico *et al.*, 1998; Falomir-Lockhart *et al.*, 2006). The rate of fatty acid transfer

from a helix-less FABP2 variant to phospholipid model membranes was unaffected by increasing phospholipid concentration compared to WT, suggesting the loss of the helices also eliminates the membrane interactions (Corsico *et al.*, 1998). Additionally, WT FABP2 could outcompete cytochrome c interactions with anionic membranes, but this function was severely disrupted with the helix-less variant. These findings were corroborated with later mutational studies showing that charged lysine residues in the alpha-helical region are critical for efficient fatty acid transfer (Falomir-Lockhart *et al.*, 2006). The significance of this protein-membrane interaction *in vivo* is unknown but may play a role in the uptake and targeting of ligands to specific cellular organelles (Hsu and Storch, 1996).

#### *Ligand Delivery by iLBPs to Enzymes and Receptors*

The role of iLBPs in delivering their ligands to metabolic enzymes or receptors via protein-protein interactions and substrate channeling has been most extensively studied with the retinoid binding proteins (Napoli, 2017). Possibly due to the reactivity and potential toxicity of retinoids, the retinoid binding proteins appear to modulate and direct retinoid metabolism and signaling via a network of protein-protein interactions. Extensive kinetic and metabolic studies have been conducted (Napoli, 2016, 2017) in rat and human intestinal and liver microsomes with holo-CRBPs. In these studies, despite the tight binding of the ligands with the CRBPs, the apparent  $K_m$  values for the total ligand are often significantly decreased or unaltered when the ligand is entirely bound to the CRBP in comparison to free ligand (Ong *et al.*, 1987; Herr *et al.*, 1999; Napoli, 2016, 2017). This kinetic data cannot be explained by the diffusional model (free drug hypothesis) and have been interpreted through protein-protein interactions between the CRBPs and retinoid metabolizing enzymes. Consistent with the protein-protein interaction model, apo-CRBP1 appears to also inhibit retinol esterification by lecithin retinol acyltransferase

enzyme suggesting a function of the apo-CRBP1 in regulating metabolism even in the absence of its ligand. These data suggest that the ratio of apo- to holo-CRBP1 or the ratio of CRBP1 to its ligand may have an important role in regulating vitamin A homeostasis in the cell. This concept is illustrated via kinetic simulations in Figure 8. Yet, these observations are limited to endogenous retinoids and their specified metabolic enzymes and the importance to drug metabolism by major drug metabolizing enzymes is unknown.

Protein-protein interactions between CRABPs and nuclear retinoic acid receptors (RAR) have also been extensively studied (Dong *et al.*, 1999; Budhu *et al.*, 2001; Schug *et al.*, 2007; Majumdar *et al.*, 2011). Expression of CRABP2 but not CRABP1 in Cos7 cells enhances RAR transactivation (Dong *et al.*, 1999), and the transfer rate of *atRA* from CRABP2 to RAR appears to be dependent on RAR receptor concentration while transfer rates from CRABP1 are unaffected by changes in RAR concentration. Holo-CRABP2 also appears to translocate to the nucleus via a SUMOylation dependent mechanism to channel *atRA* directly to RAR (Majumdar *et al.*, 2011). These findings demonstrate the potential role that iLBPs may have in cellular targeting of their ligands and delivery of their ligands to target receptors, and suggest that iLBP interaction may be protein specific.

The impact of the CRABPs on *atRA* hydroxylation has also been studied in rodent microsomes (Napoli *et al.*, 1991; Fiorella and Napoli, 1994), with recombinant drug metabolizing CYPs, CYP3A4 and CYP2C8, and with the *atRA* hydroxylases CYP26A1, CYP26B1 and CYP26C1 (Nelson *et al.*, 2016; Zhong *et al.*, 2018; Yabut and Isoherranen, 2022). Recently holo-CRABP2 was also shown to be a substrate of CYP27C1, a retinoid desaturase in the skin (Glass and Guengerich, 2021). As expected from the tight binding of *atRA* to CRABP1 and CRABP2, CYP3A4 and CYP2C8 mediated metabolism of *atRA* was nearly completely

abolished when *atRA* was bound to the CRABPs consistent with the free drug hypothesis (Nelson *et al.*, 2016; Yabut and Isoherranen, 2022). However, with the CYP26 enzymes efficient *atRA* formation was observed also when *atRA* was completely bound to CRABPs. The apparent  $K_m$  values for holo-CRABPs were either unchanged or decreased when compared to free ligand in solution. Surprisingly the  $k_{cat}$  values for *atRA* hydroxylation were also significantly decreased in the presence of CRABPs for all three CYP26 enzymes. This suggests that apo-CRABPs inhibit CYP26 enzymes via a noncompetitive mechanism similar to the inhibition observed between CRBPs and lecithin retinol acyltransferase. The observed kinetics could be explained using a substrate channeling model incorporating direct protein-protein interactions between CYP26 and apo- and holo-CRABPs (Nelson *et al.*, 2016; Yabut and Isoherranen, 2022).

The binding protein (CRBP, CRABP)-enzyme interactions may be critical modulators of ligand metabolism and vitamin A homeostasis in cells in a ligand concentration dependent manner, and the phenomenon may be important for other iLBPs as well. This hypothesis was explored via kinetic simulations of the effect of the binding protein-ligand ratio on the metabolic rates and ligand clearance in a cell (Figure 8). The simulations show how altered expression of the binding proteins will change ligand metabolism and concentrations through direct protein-protein interactions between the apo- and holo-binding protein and the metabolic enzyme. When substrate is in excess to the binding protein, the substrate is relatively freely metabolized (Figure 8, blue and green lines) allowing for homeostasis to be maintained. However, under circumstances of substrate deficiency when the binding protein is in excess to substrate, nearly all of the enzyme is bound by the apo-binding protein severely inhibiting metabolism (Figure 8, red and purple lines).



In addition to the retinoid binding proteins, the FABPs have also been shown to directly interact with nuclear receptors and metabolic enzymes. Similar to holo-CRABP2 channeling *atRA* to RARs, FABP1, FABP4 and FABP5 have been shown to translocate to the nucleus upon ligand binding to enhance PPAR transactivation (Wolfrum *et al.*, 2001; Tan *et al.*, 2002; Schug *et al.*, 2007; Hostetler *et al.*, 2009; Velkov, 2013). Physical interactions between FABPs and PPARs have been demonstrated using biochemical and biophysical assays (co-immunoprecipitation, circular dichroism, fluorescence resonance energy transfer and NMR). These studies suggest that FABP-PPAR interactions are protein specific. FABP1, FABP4 and FABP5 specifically activate and interact with PPAR $\alpha$ , PPAR $\gamma$  and PPAR $\beta$ , respectively, and the extent of transactivation appears to be ligand dependent.

FABP4 and FABP5 have been shown to directly interact with hormone sensitive lipase (HSL) (Jenkins-Kruchten *et al.*, 2003; Storch and Corsico, 2008; Storch and Thumser, 2010) to promote the liberation of free fatty acids from triglycerides in times of fatty acid scarcity. FABP4 and FABP5 showed ligand dependent interactions with HSL in isothermal titration calorimetry experiments and increased HSL catalytic activity by ~ 2-fold (Jenkins-Kruchten *et al.*, 2003). Similarly, FABP1 has been shown to interact with carnitine palmitoyl transferase I (CPTI), a key mitochondrial enzyme for fatty acid  $\beta$ -oxidation (Hostetler *et al.*, 2011). Significant deviation from the theoretical circular dichroism (CD) spectra of the C-terminal and active domain of CPTI was observed in the presence of FABP1. The affinity ( $K_d$ ) between FABP1 and CPTI was 2.5 nM as determined by fluorescence resonance energy transfer binding assays. Notably, FABP1 enhanced CPTI activity to metabolize long chain fatty acid (LCFA)-CoA to LCFA-carnitine demonstrating facilitation of the rate-limiting step in fatty acid  $\beta$ -oxidation. Given the broad binding specificity of FABP1 and FABP2 for various xenobiotics and their high abundance in the

liver and intestine, it is likely that FABPs also impact drug metabolism via similar mechanisms *in vivo*. This hypothesis is supported by the finding that FABP1 binds  $\Delta^9$ -tetrahydrocannabinol (THC) and the rate of THC metabolism is altered in FABP1-knockout mice (Elmes *et al.*, 2015).

While FABP interactions with transporters have not been extensively studied, several groups have reported that FABP4 directly interacts with the fatty acid uptake transporter CD36 to mediate fatty acid metabolism (Spitsberg *et al.*, 1995; Glatz and Luiken, 2018; Gyamfi *et al.*, 2021). CD36 appears to act as an intracellular docking site for FABP4 to facilitate fatty acid transfer to the cytoplasm where FABP4 may then shuttle fatty acids to the peroxisomes or mitochondria for fatty acid metabolism.

## Conclusions

iLBPs are ubiquitously expressed small binding proteins in tissues, that bind a variety of lipophilic compounds and facilitate the cellular uptake, diffusion and subsequent metabolism of their endogenous ligands. Yet, despite the plethora of work that exists to define biochemical functions of iLBPs, their impact on xenobiotic disposition is poorly defined and very few studies have explored the binding characteristics of various drugs with FABPs. Many xenobiotics also bind to FABPs that are highly expressed in major organs that govern drug absorption and clearance with micromolar to submicromolar affinity *in vitro*. Based on the high expression of FABPs (up to 11% of all cytosolic protein) it is likely that FABPs also bind xenobiotics *in vivo*.

The importance of FABP binding in drug disposition is not understood, however, limited studies have shown that absorption and clearance of drugs that bind to FABPs is linked to FABP expression in animal models. These findings suggest that FABPs have the potential to be determinants of xenobiotic disposition. Variability in FABP binding/expression may explain

some intra- and inter-individual variability in drug disposition as FABP expression changes with diet, disease states and administration of other therapeutics. Whether FABPs directly affect xenobiotic access to drug metabolizing enzymes remains a knowledge gap. It is unclear if FABPs may simply provide an intracellular “sink” to increase the partitioning and availability of free drug accessible for metabolism within cells, or if FABPs directly interact with metabolic enzymes to alter rates of drug metabolism. Further studies are needed to elucidate these mechanisms which would provide insight on how FABPs may regulate xenobiotic disposition.

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### **Data Availability Statement:**

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

### **Authorship Contributions:**

Participated in research design: Yabut, Isoherranen

Participated in data collection: Yabut

Performed data analysis: Yabut, Isoherranen

Wrote or contributed to the writing of the manuscript: Yabut, Isoherranen

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## Footnotes

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## Legends to Figures

**Figure 1:** Sequence alignment (A) and phylogenetic tree (B) of human intracellular lipid binding proteins (iLBPs). The primary amino acid sequences for all human iLBP family members were collected from the National Center for Biotechnology Information protein database (<https://www.ncbi.nlm.nih.gov/protein/>). The accession numbers for the amino acid sequences used were P09455.2 (CRBP1), P50120.3 (CRBP2), NP\_113679.1 (CRBP3), Q96R05.1 (CRBP4), P29762.2 (CRABP1), P29373.2 (CRABP2), P07148.1 9 (FABP1), P12104.2 (FABP2), P05413.4 (FABP3), P15090.3 (FABP4), Q01469.3 (FABP5), P51161.2 (FABP6), O15540.3 (FABP7), P02689.3 (FABP8), Q0Z7S8.1 (FABP9), A6NFH5.2 (FAPB12). The sequences were aligned using Clustal Omega Multiple Sequence Alignment (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) (Sievers *et al.*, 2011) and visualized using JalView (Waterhouse *et al.*, 2009). The black bars above the sequence alignment show the three motifs (FATTYACIDBP1-3) that make up the highly conserved fingerprint common to all iLBPs. The colored residues indicate conserved residues based on thresholds set by the Clustal X Colour Scheme (<https://www.jalview.org/help/html/colourSchemes/clustal.html>). Red indicates positively charged residues, blue residues are hydrophobic, magenta are negatively charged, green are polar, orange are glycines, yellow are prolines and cyan are aromatic. Boxed residues indicate locations of a highly conserved G-x-W triplet common to iLBPs and lipocalins and highly conserved residues involved in ionic interactions with hydroxy and carbonyl groups of ligands. The phylogenetic tree shown in (B) was calculated using the UPGMA clustering method in Simple Phylogeny ([https://www.ebi.ac.uk/Tools/phylogeny/simple\\_phylogeny/](https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/)) using the multiple sequence alignment data for human iLBPs. Evolutionary distances and phylogenetic relationships should not be inferred from this tree (figure created with BioRender.com).

**Figure 2:** The crystal structure of human holo-CRABP2 (PDB 1CBS) showing the overall structural features of intracellular lipid binding proteins (iLBPs). The figure shows the  $\beta$ -barrel cavity composed of ten  $\beta$ -strands ( $\beta$ A- $\beta$ J), the helix-turn-helix cap consisting of the alpha helices ( $\alpha$ 1 and  $\alpha$ 2) and the portal to the ligand binding domain with the neighboring loops (loop  $\beta$ C- $\beta$ D and  $\beta$ E- $\beta$ F). Two  $\beta$ -sheets, each made up of five  $\beta$ -strands, fold to form the  $\beta$ -clam of the iLBP structure (Structures generated from PDB using ChimeraX, figure created with BioRender.com).

**Figure 3:** Binding characteristics of endogenous ligands to iLBPs. **(A)** The distribution of residues shown to interact with endogenous ligands for iLBPs are depicted along their structural features. Residues labeled in red font are involved in coordinating with the hydroxy or carbonyl groups of endogenous ligands via ionic interactions. **(B)** A top-down perspective into the *all-trans*-retinoic acid (*atRA*) binding site of hCRABP2 (PDB 1CBS) with side chains that interact with *atRA*. The position of R111, R132 and Y134 residues that coordinate with carboxylate of *atRA* are shown along with the position of R59 which interacts with the  $\beta$ -ionone ring. **(C)** Side view and positions of residues R111, R132 and Y134 are shown relative to the carboxylate of *atRA* along with hydrogen bonding interactions. **(D)** The amino acid sidechains that interact with *atRA* and form the *atRA* binding pocket in CRABP2 are shown (Structures generated from PDB using ChimeraX, figure created with BioRender.com).

**Figure 4.** Binding orientations of endogenous ligands in the binding cavity of iLBPs for **(A)** hCRBP1 with *all-trans*-retinol (PDB 5H8T), **(B)** hFABP1 with two oleate molecules (PDB 2LKK), **(C)** rFABP2 with myristate (PDB 1ICM) and **(D)** mFABP4 with arachidonic acid (PDB 1ADL) (Structures generated from PDB using ChimeraX, figure created with BioRender.com).

**Figure 5:** Binding characteristics of xenobiotic ligands of FABPs. **(A)** Crystal structure of hFABP4 complexed with (S)-ibuprofen (PDB 3P6H). **(B)** Amino acid side chains that line the

binding pocket of (S)-ibuprofen in FABP4. (C) (S)-ibuprofen is stabilized in the binding pocket via ionic interactions between its carboxylate group and R126 and Y128, and edge-to-face aromatic interactions with residue F16 in FABP4. Structures for (D) hFABP1 in complex with PPAR $\alpha$  agonist GW7647 (PDB 6DRG), (E) hFABP4 complexed with the inhibitor BMS309403 (PDB 2NNQ), (F) hFABP5 complexed with the antinociceptive SBFI-26 (PDB 5URA) and (G) hFABP3 complexed with ANS (PDB 3WBG) (Structures generated from PDB using ChimeraX, figure created with BioRender.com)

**Figure 6:** Impact of experimental conditions and model fitting on determination of ligand binding affinities. (A) Simulated binding curves for a hypothetical probe with varying iLBP concentrations for direct titrations. (B) Simulation of the fold error in  $K_d$  determination for a hypothetical ligand-iLBP interaction with increasing [iLBP] concentrations used in the binding experiments in relation to different  $K_d$  values (symbols) for the ligand. The solid lines show simulated  $K_d$  values obtained using a one-site simplified hyperbolic binding equation ( $\% \text{ iLBP bound} = \frac{\% \text{ iLBP bound}_{max} \times [L]}{K_d + [L]}$ ) while the dotted line shows the  $K_d$  values obtained with a one-site ligand depletion quadratic binding equation

( $\% \text{ iLBP bound} = \% \text{ iLBP bound}_{max} \frac{[P] + [L] + K_d - \sqrt{([P] + [L] + K_d)^2 - 4 \times [P] \times [L]}}{2 \times [P]}$ ) fit to the data. (C)

Simulated fluorescence displacement data shown with varying [iLBP] concentrations in relation to the  $K_d$  of the fluorescent probe. (D) Simulations of the fold error in  $K_i$  determination with varying [iLBP] concentrations relative to the  $K_d$  of the fluorescent probe. The  $K_d$  used in the simulations for direct binding titrations in (A) was 10 nM. The  $K_d$  of the probe and  $K_i$  of the drug used in simulations for (C) were 10 and 10 nM, respectively (figure created with BioRender.com). MATLAB code used for simulations is provided in Supplementary Data.

**Figure 7:** Three proposed models of ligand delivery by iLBPs. **(A)** The diffusional model requiring ligand release into solution, **(B)** the collisional model where iLBPs directly interact with phospholipids to directly transfer substrates to organelle membranes, and **(C)** direct transfer of substrates to metabolic enzyme via direct protein-protein interactions (Figure created with BioRender.com).

**Figure 8:** Simulation of the impact of varying ligand to iLBP -ratios on metabolic enzyme activity under the circumstances that the iLBP interacts directly with the metabolic enzyme. **(A)** An enzyme kinetic scheme showing the overall model used for the simulations where apo-iLBP directly inhibits the enzyme and holo-iLBP can deliver substrate to the enzyme via protein-protein interactions. **(B)** Simulated concentrations of the metabolite (product) formation, enzyme-substrate complex concentrations, concentrations of the ternary iLBP-substrate-enzyme complex, iLBP-enzyme complex, free substrate in solution and iLBP-substrate complex as a function of time when the ratio of the substrate concentration to binding protein concentration is varied, and all the processes are simulated according to the scheme in A. The substrate concentrations were 1 nM (red line), 10 nM, (blue, orange and purple lines) and 20 nM (green line). The iLBP concentration was either 1 nM (blue), 10 nM (green, orange and red lines) or 20 nM (purple line). The kinetic and catalytic rate constants used in the simulations are listed in a table in (A). The enzyme concentration in all simulations was 0.5 nM. (Figure created with BioRender.com). MATLAB code used for simulations is provided in Supplementary Data.



**Table 1: Tissue Expression patterns, genomic localization and Endogenous Ligands of iLBPs.**

\*LCFAs – long chain fatty acids; EETs - epoxyeicosatrienoic acids; HETEs - hydroxyeicosatetraenoic acid; DHETs - dihydroxyeicosatrienoic acids; NSAIDs – nonsteroidal anti-inflammatory drugs

<i>Gene</i>	<i>Protein Name</i>	<i>Molecular Weight (kDa)<sup>b</sup></i>	<i>Human Gene Locus<sup>a</sup></i>	<i>Tissue Expression</i>	<i>Known Endogenous Ligands</i>
<b>Subfamily I</b>					
<i>RBP1</i>	CRBP1	15.9	3q23	Adipose, brain, heart, kidney, liver, lung, mammary gland, muscle, ovary, pituitary, spinal cord, skin, spleen, stomach, testis	retinol, retinaldehyde
<i>RBP2</i>	CRBP2	15.7	3q23	Heart, muscle, small intestine, placenta	retinol, retinaldehyde, monoacylglycerols
<i>RBP5</i>	CRBP3	15.9	12p13.31	Adipose, heart, muscle	retinol, retinaldehyde
<i>RBP7</i>	CRBP4	15.5	1p36.22	Large intestine, heart, kidney	retinol, retinaldehyde
<i>CRABP1</i>	CRABP1	15.6	15q25.1	Adipose, adrenal, brain, eye, kidney, liver, lung, lymph nodes, muscle, pancreas, skin, small intestine, spleen, stomach, testis, thymus	retinoic acid, retinoic acid metabolites
<i>CRABP2</i>	CRABP2	15.7	1q23.1	Skin, testis	retinoic acid, retinoic acid metabolites
<b>Subfamily II</b>					
<i>FABP1</i>	Liver FABP	14.2	2p11.2	Kidney, liver, lung, pancreas, small intestine, stomach	LCFAs, fatty acyl CoA, fatty acyl-carnitines, monoacylglycerols, lysophospholipids, bile acids, cholesterol, heme, bilirubin, retinoic acid, endocannabinoids, prostaglandins, vitamin K
<i>FABP6</i>	Ileal FABP	14.4	5q33.3	Adrenal, ovary, small intestine, stomach	Bile acids, LCFAs
<b>Subfamily III</b>					
<i>FABP2</i>	Intestinal FABP	15.2	4q26	Small intestine, liver	LCFAs
<b>Subfamily IV</b>					
<i>FABP3</i>	Heart FABP	14.9	1p35.2	Adipose, adrenal, brain, heart, kidney, liver, lung, mammary gland, muscle, ovary, placenta, spleen, stomach, testis	LCFAs, EETs, HETEs, DHETs
<i>FABP4</i>	Adipose FABP	14.7	8q21.13	Adipose, dendritic cells, macrophages, muscle	LCFAs, retinoic acid
<i>FABP5</i>	Epidermal FABP	15.2	8q21.13	Adipose, brain,	LCFAs,

				dendritic cells, eye, heart, kidney, liver, lung, macrophages, mammary gland, muscle, placenta, skin, small intestine, spleen, stomach, testis, tongue	endocannabinoids, retinoic acid
<i>FABP7</i>	Brain FABP	14.9	6q22.31	Brain, central nervous, mammary gland, muscle, system glial cells, eye	LCFAs
<i>FABP8</i>	Myelin FABP	14.9	8q21.13	Peripheral nervous system myelin	Cholesterol, LCFAs
<i>FABP9</i>	Testis FABP	15.1	8q21.13	Mammary gland, salivary gland, testis	LCFAs
<i>FABP12</i>	FABP12	15.6	8q21.13	Retina, testis	LCFAs

<sup>a</sup>Gene location obtained from the National Center of Biotechnology and Information (NCBI) gene database

<sup>b</sup>Molecular weight determined by ExPASy ProtParam (Gasteiger *et al.*, 2005) based on reference amino acid sequence

References (Veerkamp and Zimmerman, 2001; E *et al.*, 2002; Pelsers *et al.*, 2005; Schug *et al.*, 2007; Liu *et al.*, 2008; Storch and Corsico, 2008; Noiri *et al.*, 2009; Rowland *et al.*, 2009; Smathers and Petersen, 2011; Wang *et al.*, 2014; Lee *et al.*, 2015; Rezar *et al.*, 2020)

**Table 2: Binding Affinities of Xenobiotic Ligands with different FABPs**

Ligand	Range of Reported $K_i$ and $K_d$ Values ( $\mu$ M)					
	FABP1	FABP2	FABP3	FABP4	FABP5	FABP7
Acicfluorfen		4.2-8.9				
ANS	1.1-6.0 1.1 <sup>a</sup>	12 <sup>b</sup>	3.0-31 <sup>c</sup>	0.6	0.03-32	0.07-1.3
Aspirin	348 <sup>a</sup>	3780 <sup>b</sup>	300-460 NB <sup>c</sup>			
Atenolol	717					
Benzafibrate	NB <sup>a</sup>	44.4 <sup>b</sup>	26-100 <sup>c</sup>	NB	12	NB
Benzilic acid			110.8-200 <sup>c</sup>			
Benzyl salicylate			NB			
Bifenox			NB			
BMS309403					0.9	
CBD	4.0			1.7	1.9	1.5
Ciprofibrate			24-72 <sup>c</sup>			
Clofibrate	6.9					
Clofibric acid			17.7-110 <sup>c</sup>	NB	17	NB
Cortexolone			1600-1900			
DAUDA	0.4-1.4		0.3-0.7 <sup>c</sup>			
Dexamethasone	22.1 <sup>a</sup>	41.3 <sup>b</sup>	1100-1200			
Diazepam	0.5 <sup>a</sup>	115 <sup>b</sup>	1980-2200 NB <sup>c</sup>	NB	NB	325
Diclofenac	3.2 <sup>a</sup>	35 <sup>b</sup>	86.3-520 <sup>c</sup>			
Dilitiazem			NB <sup>c</sup>			
Fenbofibric acid	1-1.6, 0.3 <sup>a</sup>	27.5 <sup>b</sup>	1-6.1 <sup>c</sup>	33	24	3.3
Fenofibrate	2.9 0.02 <sup>a</sup>	0.4 <sup>b</sup>	0.8 NB <sup>c</sup>			
Fenoprofen			14-64 <sup>c</sup>			
Flufenamic acid			3.7-15.5			
(R/S) Flurbiprofen	1.2 <sup>a</sup>	222 <sup>b</sup>	20-70			
Gemfibrozil	1.9 <sup>a</sup>	179 <sup>b</sup>	110.5-121.3 <sup>c</sup>	NB	3.8	6.1
GW7647	0.3-0.6		1.3 <sup>c</sup>	25	7.6	0.7-8.9
(R/S) Ibuprofen	47.6 <sup>a</sup>	448 <sup>b</sup>	32.2-263 <sup>c</sup>	325	2.6	138
3-indolacetic acid			93-200			
Indole-3-butyric acid			72-170			
Indoprofen	1.27 <sup>a</sup>	161 <sup>b</sup>	129-520.1			
Jasmonic acid			140-350			
Ketoprofen			24-82.4 <sup>c</sup>			
Ketorolac	11.6 <sup>a</sup>	119 <sup>b</sup>	9.4-2300 <sup>c</sup>			
Lorazepam	12.9 <sup>a</sup>	140 <sup>b</sup>	2100-2500			
Meclofenamic acid	0.4 <sup>a</sup>	0.3 <sup>b</sup>	8.9-21 <sup>c</sup>			
Mefenamic acid			63-110	5.8	1.1	4.3
Meprofil			NB			
Midazolam	7.9		12			
Nabumetone			NB <sup>c</sup>			
Nadolol	2310					
Nalidixic acid			NB			
2-naphthoxyacetic acid			7.2-14			
(S)-(-)-Naproxen	0.06 <sup>a,c</sup>	2.8 <sup>b,c</sup>	56-180			
Nitrazepam			1200-2300 <sup>c</sup>	28	36	20
Perfluorononanoic acid	1.3-3.1 <sup>c</sup>					
Perfluorooctanoic acid	2.4-6.5 <sup>c</sup>					

Phenytoin			0.2 <sup>a</sup>	4.7 <sup>b</sup>		
			NB			
Pioglitazone					33	NB 11
Prednisolone	2.7 <sup>a</sup>	101 <sup>b</sup>	95-113			
Progesterone	0.03		20-32			
Propranolol	NB		NB			
Pyrilamine			NB			
Rosiglitazone	2.8				NB	NB 28.8
Sulfinpyrazone	0.1 <sup>c</sup>	8.2 <sup>c</sup>				
THC	0.1-2.9				2.0	3.1 1
11-COOH-THC	11.2					
	NB					
11-COOH-THC-gluc	NB					
11-OH-THC	5-7.2					
	NB					
Tolfenamic acid			2.8-8.2 <sup>c</sup>		1.9	0.1 2.9
Tolmetin			1300-2200			
Torsemide	0.2 <sup>c</sup>	12.3 <sup>c</sup>	0.8			
Troglitazone	1.7				11	0.02-16 1
Valproate			240-470			
Verapamil			NB			

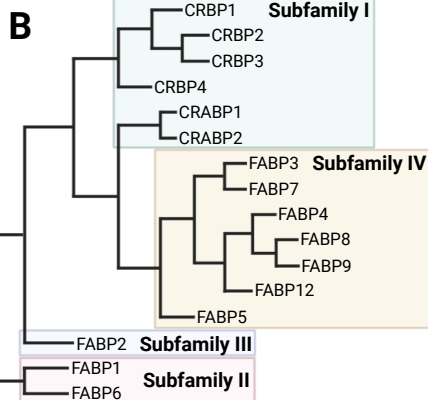
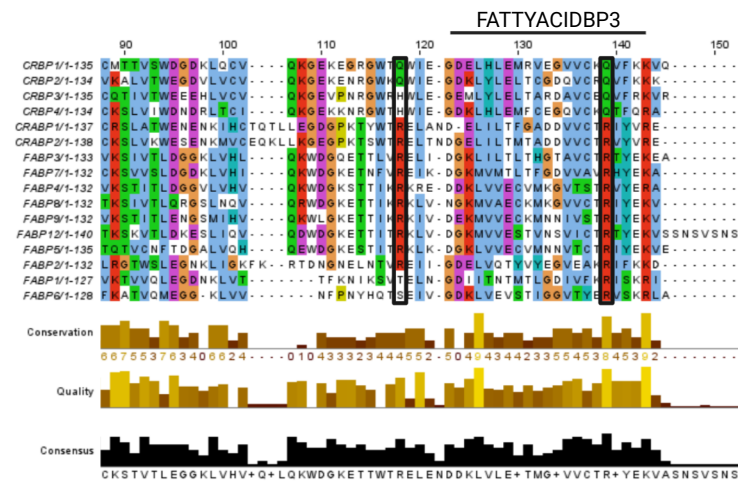
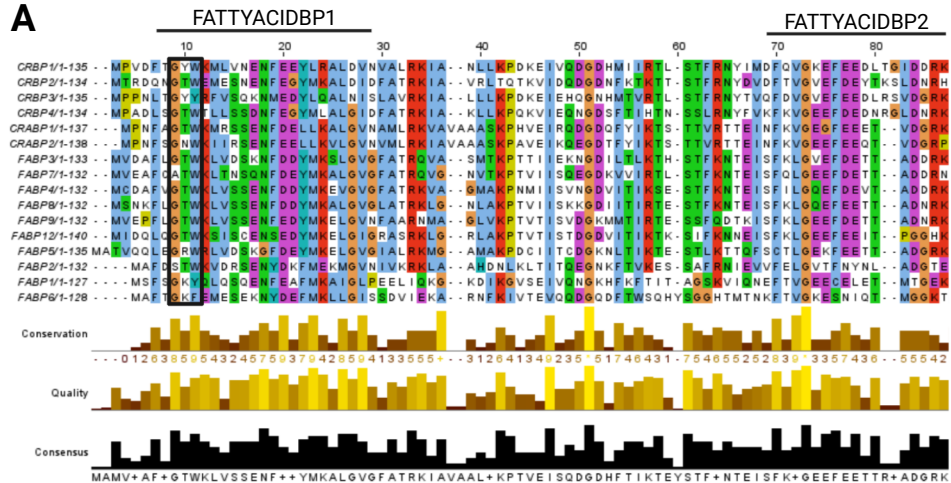
<sup>a</sup>Affinity for first, high affinity binding site <sup>b</sup>affinity for second, low affinity binding site respectively, determined in the same study

<sup>c</sup>Includes studies where binding affinities were determined by SPR, ITC, or thermal shift with SYPRO Orange NB indicates that binding was tested but no binding was observed in at least one study

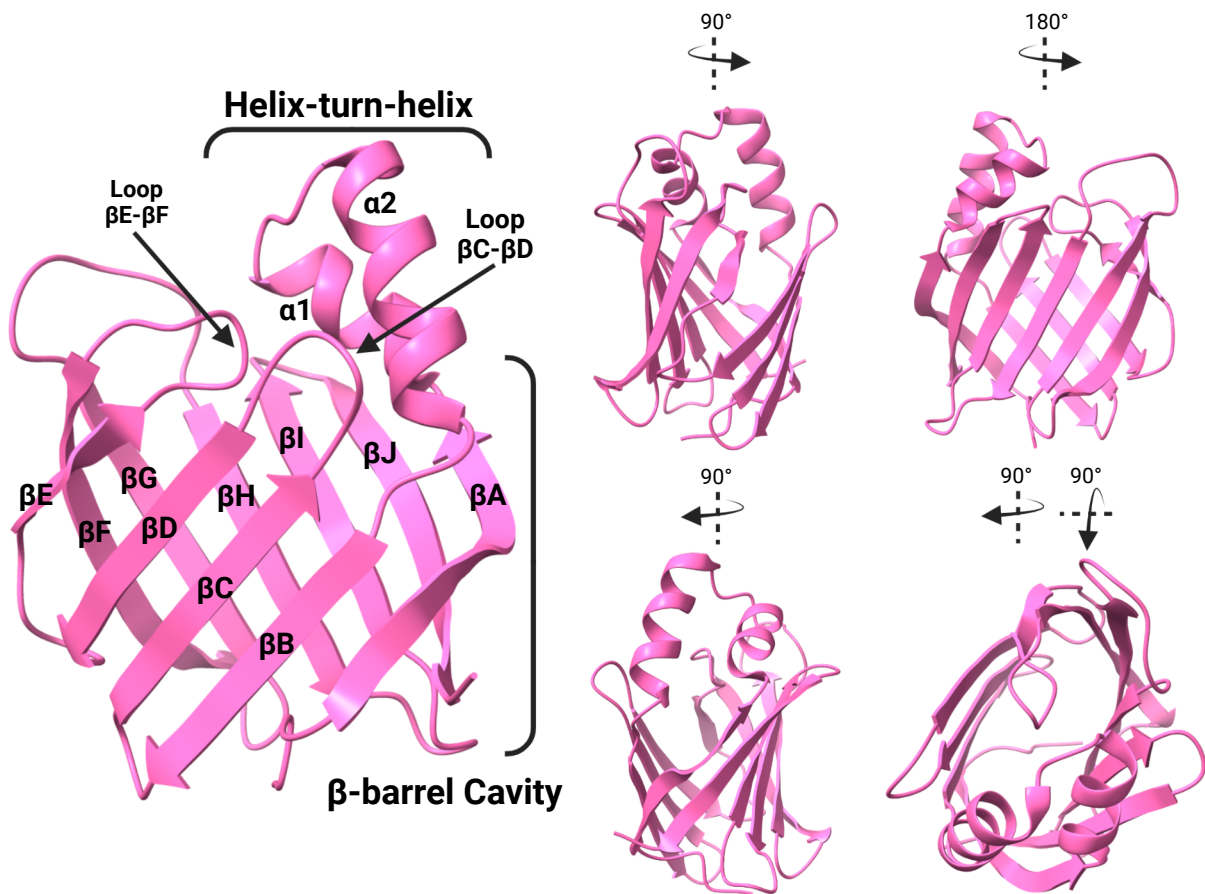
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### **Supplemental Data:**

MATLAB code for the simulations shown in Figure 6 and Figure 8 are provided as supplementary data.



**Figure 1**



**Figure 2**

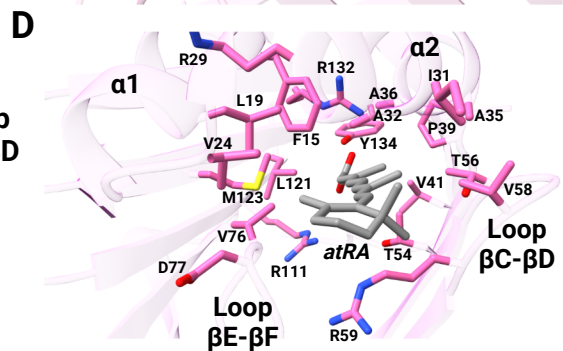
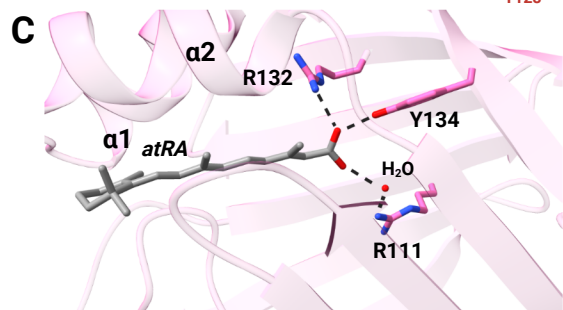
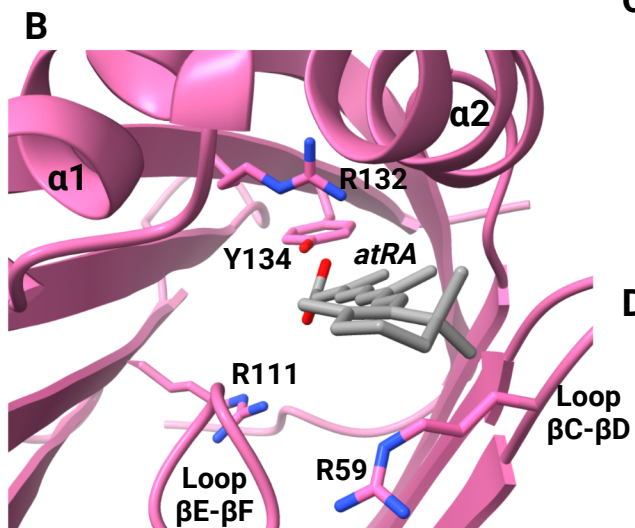
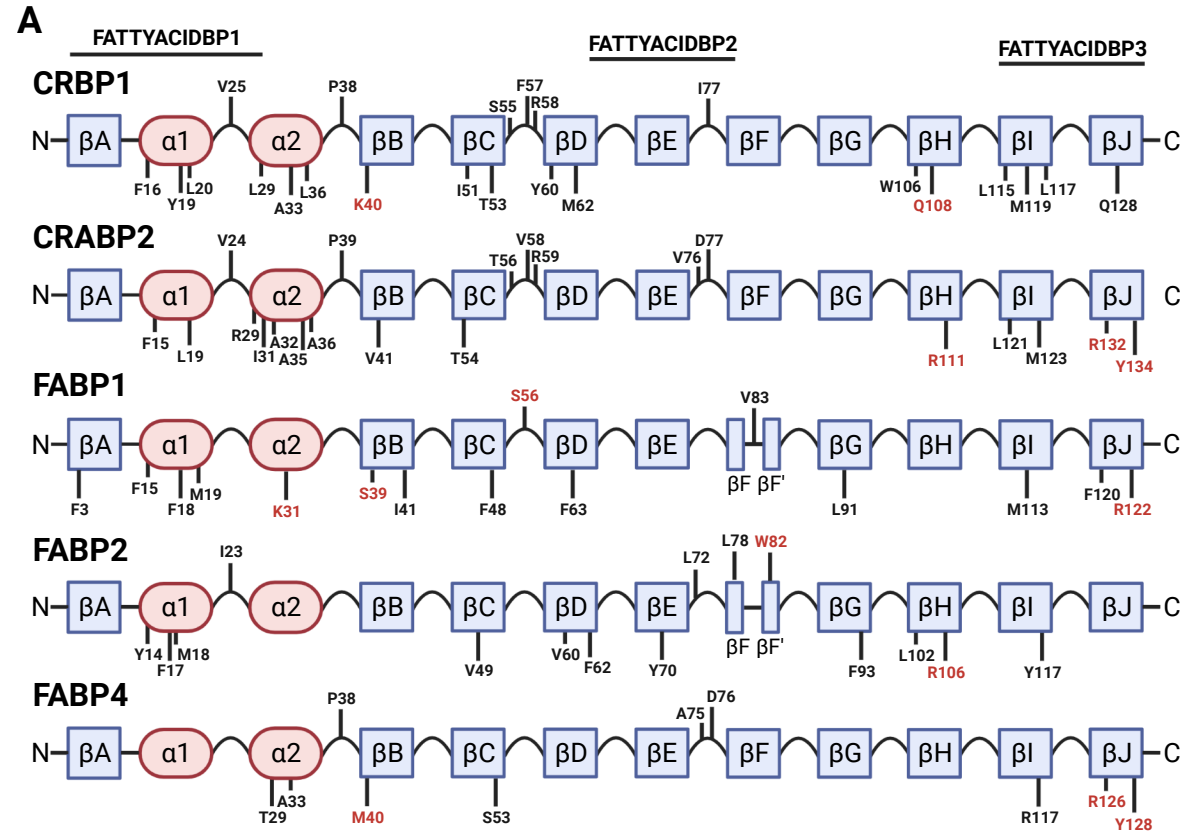
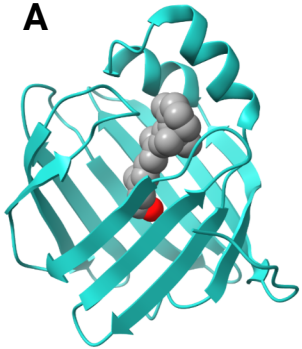
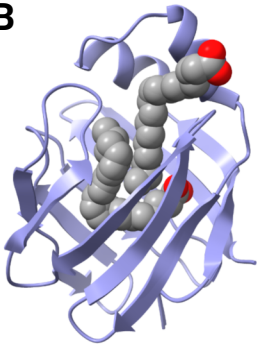
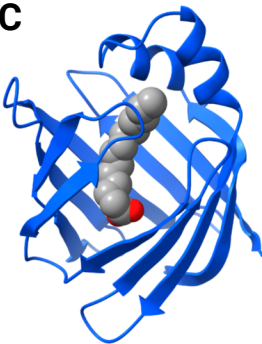
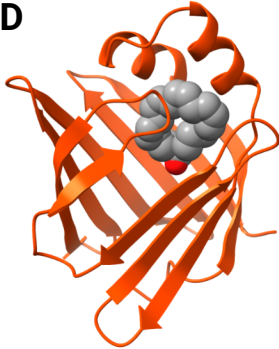
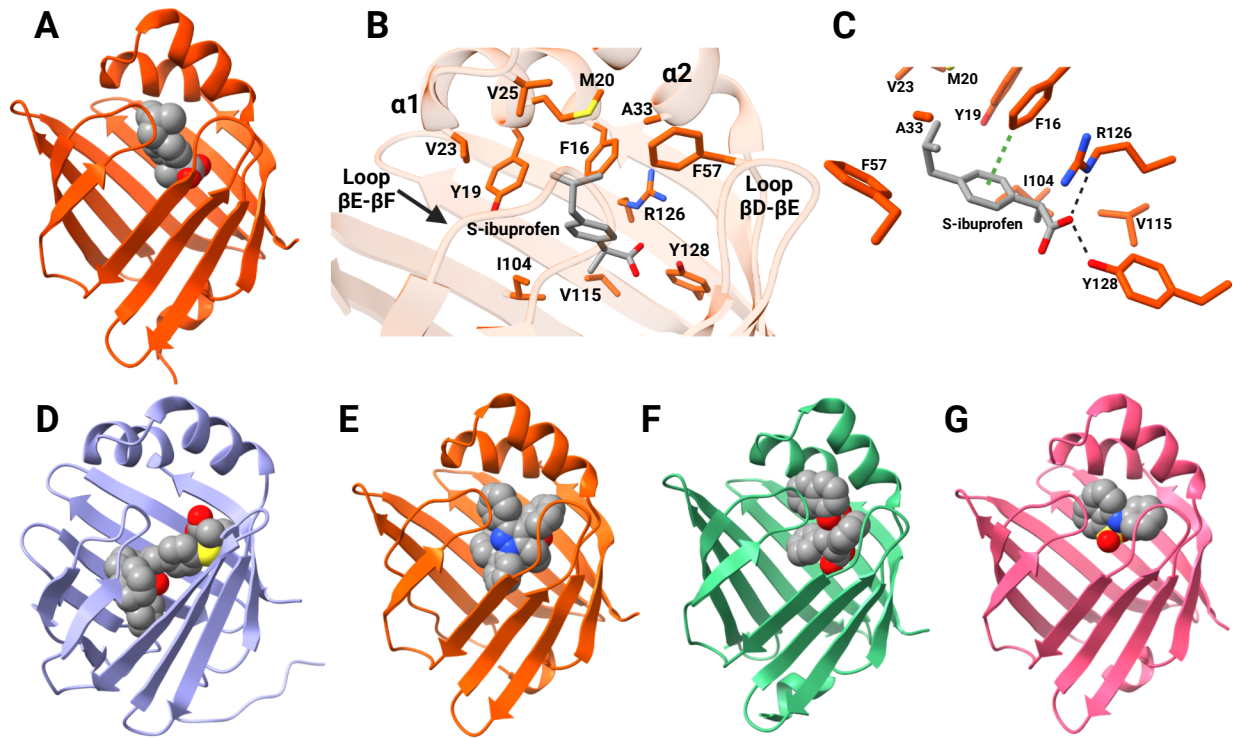


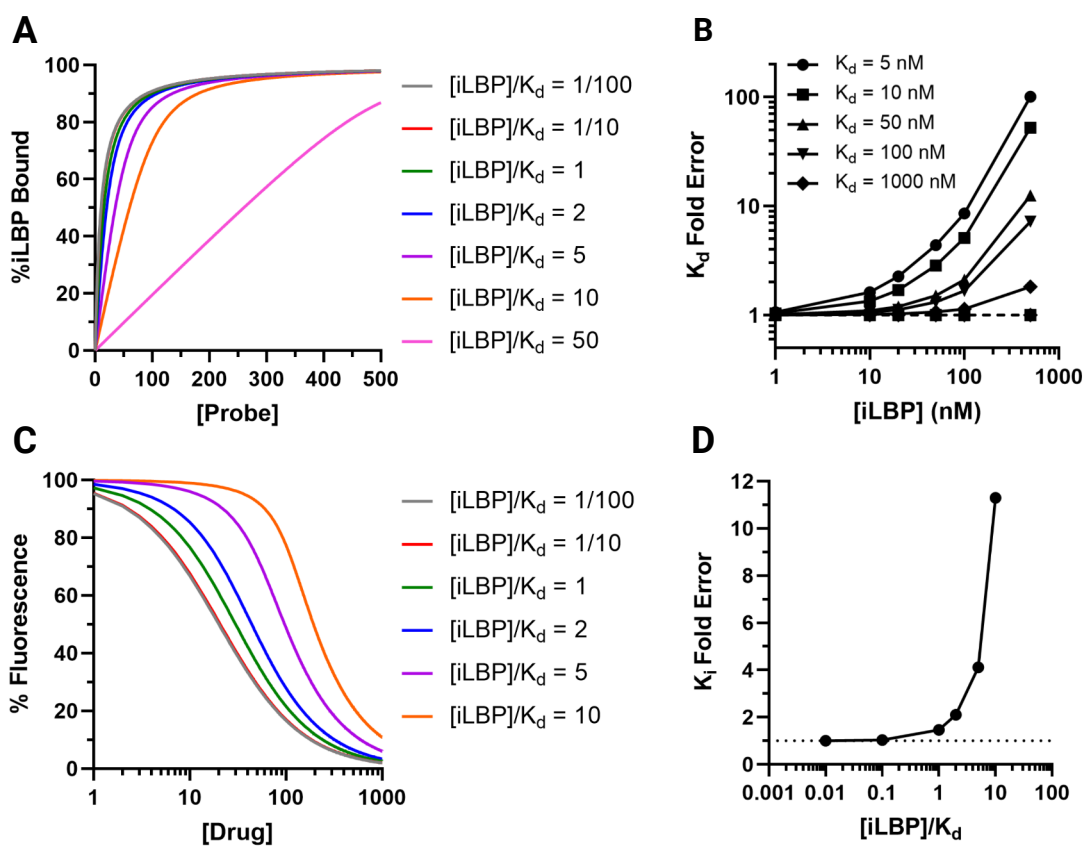
Figure 3

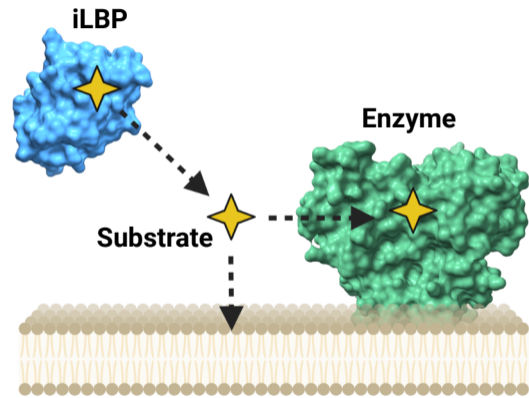
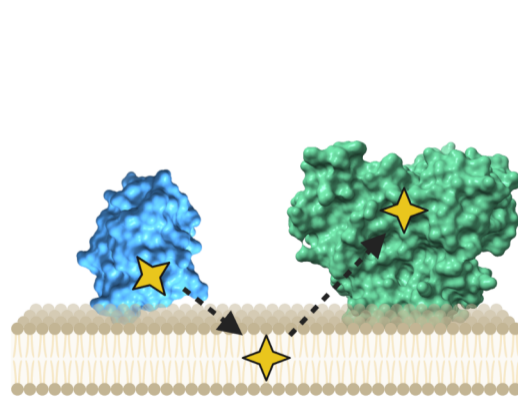
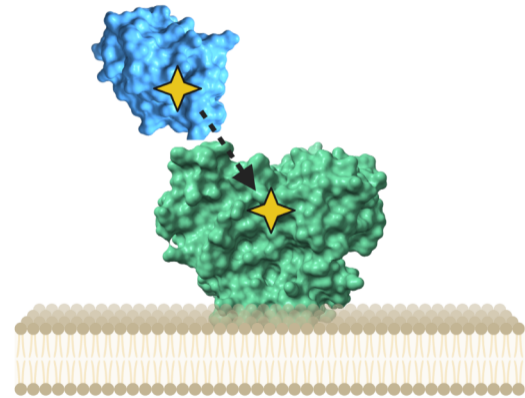


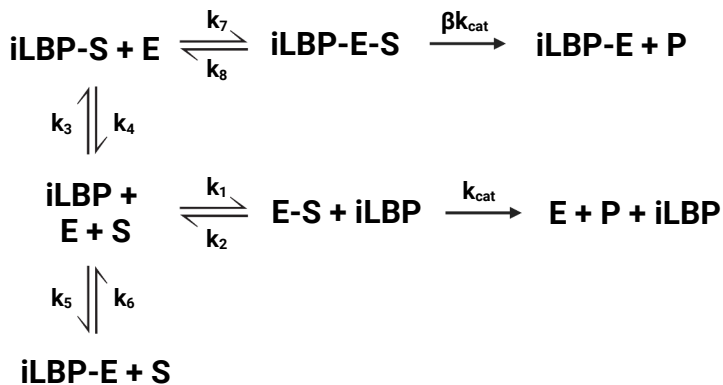
**A****B****C****D****Figure 4**



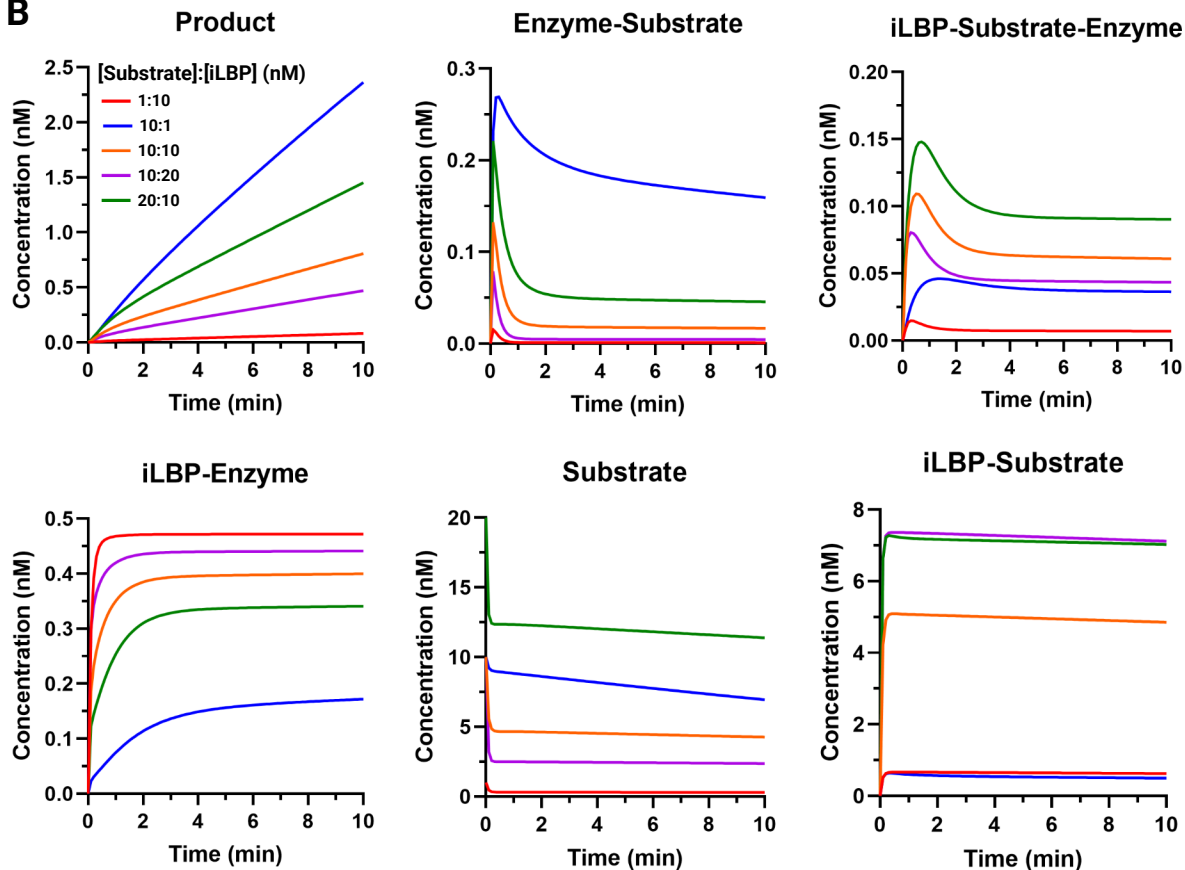
**Figure 5**



**A****Diffusional****B****Collisional****C****Protein-protein****Figure 7**

**A**

Rate	
$k_1$	$1.00 \text{ nM}^{-1} \text{ min}^{-1}$
$k_2$	$4.70 \text{ min}^{-1}$
$k_3$	$1.07 \text{ nM}^{-1} \text{ min}^{-1}$
$k_4$	$4.40 \text{ min}^{-1}$
$k_5$	$1.00 \text{ nM}^{-1} \text{ min}^{-1}$
$k_6$	$0.39 \text{ min}^{-1}$
$k_7$	$1.00 \text{ nM}^{-1} \text{ min}^{-1}$
$k_8$	$0.99 \text{ min}^{-1}$
$k_{\text{cat}}$	$1.10 \text{ min}^{-1}$
$\beta k_{\text{cat}}$	$0.83 \text{ min}^{-1}$

**B****Figure 8**