Structural analysis of ibuprofen binding to human adipocyte fatty-acid binding protein (FABP4)

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3-(4-methoxy-3-methylphenyl)propionic acid,
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Inhibition of human adipocyte fatty-acid binding protein (FABP4) has been proposed as a treatment for type 2 diabetes, fatty liver disease and atherosclerosis. However, FABP4 displays a naturally low selectivity towards hydrophobic ligands, leading to the possibility of side effects arising from cross-inhibition of other FABP isoforms. In a search for structural determinants of ligand-binding selectivity, the binding of FABP4 towards a group of small molecules structurally related to the nonsteroidal anti-inflammatory drug ibuprofen was analyzed through X-ray crystallography. Several specific hydrophobic interactions are shown to enhance the binding affinities of these compounds, whereas an aromatic edge-to-face interaction is proposed to determine the conformation of bound ligands, highlighting the importance of aromatic interactions in hydrophobic environments.

1. Introduction

Cytosolic fatty-acid binding proteins (FABPs) are lipid chaperones that are able to reversibly bind a wide variety of hydrophobic ligands (Furuhashi & Hotamisligil, 2008). As such, FABPs are central to key cellular processes, including intracellular lipid trafficking, lipid metabolism, membrane synthesis, response to lipid signals and ultimately regulation of gene expression, through targeting lipids to transcription factors. Although FABPs have been shown to interact with many carboxylic acids, steroids, retinoids and drugs, their precise biological functions are poorly understood. There are at least nine human FABP isoforms, the expression of which is more or less tissue-specific (Smathers & Petersen, 2011). Adipocyte FABP (A-FABP or FABP4) is expressed mainly in adipose tissue, transcriptionally controlled by fatty acids, peroxisome proliferator-activated receptor γ and insulin (Krusinova & Pelikanova, 2008), as well as in macrophages, where it mediates inflammatory responses (Makowski *et al.*, 2001). In pioneering work by Hotamisligil *et al.* (1996), FABP4-deficient mice with diet-induced obesity were shown not to develop insulin resistance or diabetes compared with wild-type mice, turning the spotlight onto FABP4 inhibition as a treatment for type 2 diabetes, fatty liver disease and atherosclerosis (Furuhashi *et al.*, 2007).

The crystallographic structure of human FABP4 has been solved as a complex with palmitic acid (Marr *et al.*, 2006). The protein exhibits a typical lipocalin fold (Lehmann *et al.*, 2004): a ten-strand antiparallel β -sheet folded into a flattened barrel sometimes termed a ' β -clam' fold (Supplementary Fig. S1). The resulting cavity is partially lined with hydrophobic amino-acid side chains, giving rise to an amphipathic ligand-binding pocket in which the ligand is flanked between a layer of hydrophobic amino-acid side chains on one side and a layer of structured water molecules on the other (Reese-Wagoner *et*

al., 1999). A helix–turn–helix motif acts as the barrel lid at one end, whereas the opposite end is closed by a short α -helical segment and holds the conserved Arg126 and Tyr128 residues that interact electrostatically with the fatty-acid carboxyl group. This structure also showed the aliphatic chain of palmitate in close proximity to Phe57, which exhibited significantly higher *B* factors. This led to the so-called ‘portal hypothesis’ by which residues in this region, namely loops β 3– β 4 (54–59) and β 5– β 6 (74–79), together with the helix–turn–helix lid motif (14–36) comprise the gate for ligand entrance and exit (Reese-Wagoner *et al.*, 1999).

Of the nine human FABP isoforms identified to date, FABP4 is the best characterized, and a number of inhibitors have been reported (Barf *et al.*, 2009; Cai *et al.*, 2010; Furuhashi *et al.*, 2007; Lehmann *et al.*, 2004; Liu *et al.*, 2011; Ringom *et al.*, 2004; Sulsky *et al.*, 2007), including the potent rationally designed BMS-309403 developed at Bristol-Myers Squibb (Sulsky *et al.*, 2007), which displays a K_i of <2 nM for FABP4 and comparable K_i values for FABP3 (250 nM) and FABP5 (350 nM). Nonetheless, given the structural similarity among FABP isoforms, highly selective inhibitors are needed in order to avoid cross-inhibition side effects. This feature is critically important given the intricate and poorly understood network of intracellular lipid-binding proteins. For instance, mouse cardiac myocytes have been shown to depend exclusively on FABP3 for long-chain fatty-acid utilization, which is compensated by glucose oxidation (Schaap *et al.*, 1999), suggesting that FABP3 inhibition might pose serious side effects in some individuals. These unwanted reactions would need to be addressed before the approval of FABP inhibitors for use in human subjects. In this report, we explore the structural determinants of FABP4 ligand-binding affinity and specificity through analyzing the binding mode of a series of compounds structurally related to ibuprofen. A key role of residue Phe16 is proposed to significantly influence the conformation of bound ligands, illustrating how aromatic interactions are able to grant binding specificity in hydrophobic environments.

2. Materials and methods

2.1. Chemicals

All chemicals were of the best quality available. Ligands L2 [3-(4-methoxy-3-methylphenyl)propionic acid], L3 [3-(4-methoxyphenyl)propionic acid], L4 (*S*-3-phenylbutyric acid), L5 (*R*-ibuprofen) and L6 (*S*-ibuprofen) were purchased from Sigma–Aldrich and dissolved to 100 mM in dimethylsulfoxide (DMSO). 8-Anilino-1-naphthalenesulfonic acid (ANS) was purchased from Fluka.

2.2. Protein expression and purification

Recombinant FABP4 was obtained as follows. Chemically competent *Escherichia coli* Rosetta2 (DE3) pLysS cells (Novagen) were transformed with vector pET-FABP4 (K_m^R) encoding the human FABP4 amino-acid sequence (UniProt FABP4_HUMAN) cloned between the EcoRI and XhoI

restriction sites of plasmid pET-28a (EMD Millipore) with an N-terminal His tag cleavable by bovine thrombin protease (Calbiochem). The plasmid construct was custom-synthesized by DNA 2.0 (Menlo Park, California, USA). A stop codon was introduced after the XhoI site in order to eliminate the C-terminal His tag encoded in the pET-28a vector. Starting from an overnight saturated inoculum, cells were grown at 310 K in selective medium (1 l Luria–Bertani medium supplemented with 35 $\mu\text{g ml}^{-1}$ chloramphenicol and 50 $\mu\text{g ml}^{-1}$ kanamycin) with shaking (250 rev min $^{-1}$) for ~2 h until an optical density at 600 nm of 0.8 was reached. Protein overexpression was then induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside and the culture was incubated for an additional 4 h under the same conditions. The cells were then harvested, resuspended in 50 mM NaH_2PO_4 pH 7.2 supplemented with 1 mM MgCl_2 , 100 units of Benzonase (Novagen) and one tablet of cComplete EDTA-free protease-inhibitor cocktail (Roche) and frozen at 193 K overnight. Next, the cells were thawed on ice and subjected to sonication (Misonix Sonicator 3000) for five cycles comprising 20 s sonication at power 5 plus 20 s on hold.

All remaining purification steps were performed at 277 K. Cell debris was separated after centrifugation at 20 000g for 30 min; NaCl was then added to 500 mM to the soluble cell extract fraction, which was filtered through a 0.45 μm pore-size filter and loaded onto an Ni–NTA His-Bind resin column (Qiagen) pre-equilibrated with buffer WB (50 mM NaH_2PO_4 pH 7.2, 500 mM NaCl, 50 mM imidazole). The column was washed with 20 column volumes of buffer WB and eluted with buffer WB with an additional 500 mM imidazole. Eluates were pooled and buffer-exchanged into TBS buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl) by dialysis using a 3 kDa cutoff regenerated cellulose membrane (Millipore). The sample was then concentrated to ~4 ml with 3 kDa cutoff centrifugal filters (Millipore), digested with 100 NIH units of bovine thrombin (Calbiochem) by incubation at 298 K for 2 h and applied onto a size-exclusion Sephacryl S-200 HR 26/60 column (GE Healthcare) mounted in a BioLogic chromatography system (Bio-Rad) and pre-equilibrated with TBS buffer. Finally, the protein was eluted with TBS buffer, concentrated to 5 mg ml $^{-1}$ and stored at 277 K. Protein purity was checked by SDS–PAGE. Protein concentration was determined spectrophotometrically using a calculated molar extinction coefficient of 14 100 $\text{M}^{-1} \text{cm}^{-1}$ at 280 nm (Gasteiger *et al.*, 2005).

2.3. Determination of ligand-binding affinities

FABP4 dissociation constants for ligands L2, L3, L4, L5 and L6 were determined spectrofluorometrically by competition assays with 8-anilino-naphthalene-1-sulfonic acid (ANS). A 1 μM solution of FABP4 with a twofold excess of ANS in CIT buffer (0.1 M sodium citrate pH 6.5, 2% DMSO) was loaded into a rectangular 10 mm path-length Spectrosil Far-UV cuvette with a magnetic stirrer (Starna Cells), thoroughly washed and treated with a 0.3 mg ml $^{-1}$ solution of porcine gelatin (Sigma) in CIT buffer in order to prevent nonspecific

Table 1

Summary of FABP4 ligand-binding parameters.

Dissociation constants were determined as described in the text. V is the ligand volume. The parameters d (distance between aromatic centers), s (shift with respect to the perpendicular direction of interaction) and θ (angular shift with respect to the perpendicular orientation) are descriptors for the corresponding ligand edge-to-face aromatic interaction with the Phe16 side chain (see Supplementary Fig. S4). For the parameters d , s and θ corresponding to L2, L3 and L4 the values for each alternate conformation are indicated separately.

	K_L (μM)	V ($\text{\AA}^3$)		d ($\text{\AA}$)	s ($\text{\AA}$)	θ ($^\circ$)
L4	148 ± 7.5	161	a	5.35	1.90	48
			b	5.12	1.37	9.5
L3	73.0 ± 7.3	170	a	5.04	1.09	7.7
			b	5.15	1.17	2.7
L2	$(737 \pm 46) \times 10^{-3}$	187	a	5.10	1.28	16
			b	5.00	0.35	4.4
L5	$(133 \pm 6.6) \times 10^{-3}$	211		5.79	2.01	54.8
L6	$(94.9 \pm 5.9) \times 10^{-3}$	211		5.22	1.44	24.9

binding of ANS and protein to the cuvette. Fluorescence emission at 475 nm was measured after excitation at 380 nm at 298 K throughout titrations with stock ligand solutions in CIT buffer using a PC1 Photon Counter spectrofluorometer (ISS) configured in the L format. Ligand dissociation constants, K_L , were determined by nonlinear least-squares regression using the *DynaFit* software (Kuzmič, 1996). A competition model of the form $[\text{FABP4-ANS} \rightleftharpoons \text{FABP4} + \text{ANS}, K_{\text{ANS}}; \text{FABP4-L} \rightleftharpoons \text{FABP4} + \text{L}, K_{L_n} (n = 2, 3, 4, 5, 6)]$ was assumed, in which the fluorescence at 475 nm is directly proportional to the concentration of the FABP4-ANS complex and the emission of free ANS is negligible. The dissociation constant for ANS, K_{ANS} , was determined separately in analogous conditions with the model $(\text{FABP4-ANS} \rightleftharpoons \text{FABP4} + \text{ANS}, K_{\text{ANS}})$ through titration of FABP4 with ANS and was considered constant in the data processing of the competition assays. Graphic data representation and additional processing was performed with *SigmaPlot* 11 (Systat Software).

2.4. Crystallization and X-ray diffraction data processing

Crystals of FABP4 were grown using the sitting-drop vapor-diffusion method in 96-3 well Intelli-Plate trays (Art Robbins Instruments). Sitting drops consisted of 1 μl mixtures of protein sample (5 mg ml^{-1} FABP4 in TBS buffer) and reservoir solution in a 1:1 ratio prepared using an Oryx8 liquid pipettor robot (Douglas Instruments). The reservoir contained 100 μl of a 1.6 M solution of sodium citrate pH 6.5. Crystals appeared after 3–4 d of incubation at 293 K and exhibited an elongated rod shape. The crystals were mounted in nylon loops (Hampton Research) and flash-cooled in liquid nitrogen cryoprotected by the mother liquor. Ligand complexes were obtained through soaking apoprotein crystals with 1 mM ligand solutions in mother liquor with an additional 2% DMSO. Crystals of apo FABP4 were obtained by soaking for 10 min in a cryoprotectant solution consisting of 3.4 M sodium malonate pH 7 in order to avoid the binding of citrate to FABP4, followed by flash-cooling in liquid nitrogen. Diffraction data were collected remotely on beamline BL7-1 of the Stanford Synchrotron Radiation Light Source (Cohen

et al., 2002; Soltis *et al.*, 2008). Diffraction anisotropy was assessed with the Diffraction Anisotropy Server hosted at the UCLA Molecular Biology Institute (<http://services.mbi.ucla.edu/anisotropy/>). Reflections were indexed, integrated and scaled with *XDS* (Kabsch, 2010) and *HKL-2000* (Otwinowski & Minor, 1997). The data were phased by molecular replacement with *Phaser* (McCoy *et al.*, 2007). The structure with PDB code 2hnx (Marr *et al.*, 2006) was used as a phasing model, in which all noncovalently bound ligands, alternate conformers and solvent molecules were removed and a random shift of 0.3 $\text{\AA}$ was added to the remaining protein atom coordinates in order to minimize model bias. Structure refinement was performed with *REFMAC5* (Murshudov *et al.*, 2011) and the *CCP4* suite of programs (Winn *et al.*, 2011). Manual building was conducted with *Coot* (Emsley *et al.*, 2010) using σ_A -weighted $2F_o - F_c$ and $F_o - F_c$ Fourier difference maps. Throughout the final cycles of restrained refinement, the geometry weight matrix was fixed and anisotropic atomic B factors were refined for all atomic coordinates. For ligands with two conformations (L3 and L4) occupancies were set manually to 0.5, except for ligand L2, for which conformation L2a was more populated (set to 70% occupancy). Ligand structures and geometric restraints were calculated with the *PRODRG* server (Schüttelkopf & van Aalten, 2004). Structure validation was performed with *SFCHECK* (Vaguine *et al.*, 1999) and the built-in functions implemented in *Coot*. Figures were prepared with *PyMOL* v.1.2 (Schrödinger).

2.5. Structure and sequence analyses

Root-mean-square deviation values for fitting of each FABP4 ligand-bound forms compared with the apo form were calculated and analyzed with the program *ProteinRanger* (<http://shakerr.sourceforge.net>). Molecular-surface areas and curvature were determined with *Surface Racer* (Tsodikov *et al.*, 2002). Secondary-structure elements were calculated with the *PROMOTIF* program (Hutchinson & Thornton, 1996) available in the PDBSUM database (Laskowski, 2009). Binding data processing and graphical representation was performed with *SigmaPlot* 11 (Systat Software). Ligand volumes were calculated with *MIPC* (Molinspiration Cheminformatics). FABP sequences were aligned with *Expresso* (Armougom *et al.*, 2006) and the alignment representation and coloring was prepared with *Jalview* (Waterhouse *et al.*, 2009).

3. Results and discussion

FABP4 ligand-binding affinities were determined through competition assays in the presence of 8-anilino-naphthalene-1-sulfonic acid (ANS; Fig. 1). These compounds were found to display unexpectedly high affinities, with dissociation constants in the nanomolar to micromolar range (Table 1), comparable to those of native fatty-acid ligands (Kane & Bernlohr, 1996). *R*- and *S*-ibuprofen (*i.e.* compounds L5 and L6) showed the highest binding affinities (with dissociation constants of 133 and 95 nM, respectively), whereas ligands L3

and L4 displayed lower affinities (dissociation constants in the micromolar range: 73 and 148 μM , respectively) and ligand L2 showed an intermediate binding affinity (dissociation constant of 0.74 μM). Although the general trend may be partially explained in terms of hydrophobic interactions directly correlated with ligand size (Supplementary Fig. S2), additional structural information is needed in order to explain affinity differences between ligands of similar size. For instance, compounds L2 and L3 differ only by the presence of one methyl group, which suffices to cause a 100-fold increase in the binding affinity of L2 compared with L3 (about 2.7 kcal mol⁻¹ change in binding free energy). Analogously, given that L5 and L6 differ only in the stereochemical configuration of their 2-methyl group, the small observed difference in dissociation constants (about 40 nM, or 0.2 kcal mol⁻¹ difference in binding free energy) is suggestive of nearly identical binding modes for both enantiomers, which is far from being the case (see below).

Crystallographic structures were obtained for all FABP4–ligand complexes, which comprise the highest resolution

atomic coordinates reported for this protein thus far (Table 2). FABP4 crystals grow as elongated needle-like rods, leading to anisotropic diffraction patterns. Depending on the degree of anisotropy, spherical truncation of ellipsoidally distributed anisotropic data may produce truncation artefacts. In order to quantitatively assess the degree of anisotropy, the reflection data were tested with the Diffraction Anisotropy Server hosted at the UCLA Molecular Biology Institute (<http://services.mbi.ucla.edu/anisotropy/>). The anisotropy statistic ΔB is defined as the difference in directional B factor between the crystal directions with the most extreme values (Strong *et al.*, 2006). ΔB values are classified as low ($\Delta B < 10 \text{ \AA}^2$), mild ($\Delta B = 10\text{--}25 \text{ \AA}^2$), strong ($\Delta B = 25\text{--}50 \text{ \AA}^2$) or severe ($\Delta B > 50 \text{ \AA}^2$), but only strong and severe anisotropies can be significantly improved through ellipsoidal truncation and anisotropic scaling (Strong *et al.*, 2006). The ΔB values obtained for our data indicated low to mild anisotropy (Table 2, Supplementary Fig. S5). Therefore, we decided to proceed with the refinements based on spherically truncated data, choosing resolution cutoffs that assure artefact-free electron-density maps, particularly for the cases of mild anisotropy (the structures of FABP4 with L1 to L5). Considering the excellent quality of the electron-density and difference Fourier maps obtained for all structures, and that the adequate way to deal with anisotropic data is still a matter of debate (Evans & Murshudov, 2013), we believe that this is a suitable approach.

Typically, ligand-bound FABP4 showed minimal conformational changes with respect to the apoprotein, a common feature among all FABPs. Parameters such as per-residue root-mean-square deviation (r.m.s.d.) for backbone and side-chain atoms, molecular surface area, surface curvature and accessible surface area showed no significant changes upon ligand binding compared with the apoprotein structure (Fig. 2). Nevertheless, all compounds are clearly defined in their corresponding electron-density maps (Supplementary Fig. S3). In general, ligands L2–L6 display electrostatic interactions between the terminal carboxyl group and residues Arg126 and Tyr128, and an aromatic edge-to-face interaction between the Phe16 side chain (electron acceptor) and the ligand phenyl group (electron donor), together with diverse van der Waals hydrophobic interactions.

Ligands L2 and L3 differ by the presence of a methyl group at position 3 in the phenyl moiety of L2 (Figs. 1a and 3). The (3)-methyl and (4)-methoxyl groups of L2 (numbers in parentheses correspond to positions in the phenyl group) display alternate hydrophobic interactions with Tyr19, Val23 and Val25 (conformer L2a) or with Ala33, Thr29 and Phe57 (conformer L2b), leading to a rotation of $\sim 180^\circ$ between the conformers. The Phe57 side chain appears to adapt to these changes, as it displays two conformations shifted by 0.94 $\text{\AA}$. In contrast, conformers L3a and L3b display minimal conformational differences, with the (4)-methoxyl group interacting with a hydrophobic patch centered on Ala33 (L3a) or Val23 and Val25 (L3b) and shifted by about 1.6 $\text{\AA}$. Although the Phe57 side chain displays two alternate conformations, they are shifted by only 0.33 $\text{\AA}$, indicating that L3 does not interact significantly with this residue. Notably, compound L3 displays

Figure 1
(a) Molecular structures of ligands co-crystallized with FABP4. Numbers in parentheses correspond to positions in the phenyl group. (b) Dissociation constants (K_L) were determined fluorometrically for ligands L2–L6 by competition assays with ANS. A value of $K_{\text{ANS}} = 15.3 \text{ nM}$ was determined for ANS and was assumed to be constant throughout the competition assays. Relative fluorescence at 475 nm ($F_{475 \text{ nm}}$) is expressed as a percentage of the initial value (for ligands; circles) or final value (for ANS; squares). The medium is 0.1 M sodium citrate buffer pH 6.5, 2% DMSO, 1 μM protein at 298 K. Nonlinear regression was performed with DynaFit (Kuzmič, 1996). Dissociation constants are summarized in Table 1.

Table 2
FABP4 X-ray diffraction data-collection and refinement statistics.

Values in parentheses are for the outer resolution bin.

	Apo	L1 (CIT†)	L2 (ZGB)	L3 (ZGC)	L4 (FBZ)	L5 (IZP)	L6 (IBP)
Data collection							
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters							
a (Å)	32.52	32.24	32.24	32.07	32.36	32.29	32.33
b (Å)	53.42	53.32	53.39	53.21	53.39	53.55	53.38
c (Å)	74.95	75.00	75.01	74.98	74.94	74.93	75.03
$\alpha = \beta = \gamma$ (°)	90	90	90	90	90	90	90
Resolution range (Å)	43.5–1.08 (1.10–1.08)	43.5–1.25 (1.28–1.25)	43.5–1.06 (1.09–1.06)	43.5–1.08 (1.11–1.08)	43.5–1.20 (1.23–1.20)	43.5–1.20 (1.23–1.20)	43.5–1.15 (1.18–1.15)
Unique reflections	53669 (4191)	36082 (3264)	59308 (5735)	53671 (4149)	40514 (3900)	40748 (3986)	38274 (2896)
Wilson B factor (Å ²)	12.6	14.1	9.55	13.1	10.1	9.82	15.4
R_{merge} (%)	11.9 (51.2)	9.2 (26.8)	10.3 (36.2)	12.2 (43.2)	7.5 (33.8)	11.5 (32.8)	12.4 (65.5)
Completeness (%)	94.2 (67.3)	98.6 (98.1)	99.4 (84.3)	96.0 (77.1)	95.9 (69.6)	96.3 (82.6)	98.4 (94.3)
Average $I/\sigma(I)$ ‡	11.7 (1.6)	9.9 (6.5)	14.4 (3.7)	11.8 (2.4)	10.4 (2.3)	10.1 (2.6)	6.1 (1.8)
Anisotropy§ (ΔB) (Å ²)	6.21	12.7	12.3	11.2	16.4	18.9	4.55
Refinement							
PDB code	3rzy	3p6c	3p6d	3p6e	3p6f	3p6g	3p6h
Resolution (Å)	1.08	1.25	1.06	1.08	1.20	1.20	1.15
R_{cryst} (overall) (%)	15.4 (26.0)	16.8 (19.0)	14.0 (20.1)	16.5 (25.9)	15.5 (13.3)	16.9 (22.1)	15.8 (24.1)
R_{free} ¶ (%)	18.9 (29.6)	21.2 (24.4)	16.1 (21.8)	19.9 (26.0)	19.0 (16.6)	19.4 (24.0)	19.1 (25.9)
No. of atoms							
Protein	1073	1072	1072	1063	1063	1072	1072
Solvent	147	162	230	196	202	200	172
Ligands	—	26 (a and b)	28 (a and b)	26 (a and b)	24 (a and b)	15	15
Mean B factor (Å ²)	14.2	21.4	16.9	18.7	18.0	18.0	17.0
Deviations from ideality							
R.m.s.d., bonds (Å)	0.025	0.022	0.021	0.022	0.020	0.022	0.021
R.m.s.d., angles (°)	2.172	2.259	2.016	2.020	1.995	2.021	2.010

† The ligand name as appearing in the corresponding PDB file is shown in parentheses. ‡ For spherically truncated data. § Diffraction anisotropy, ΔB , is defined as the difference in directional B factor between the two reciprocal axes with the most extreme values (Strong *et al.*, 2006). See the text and Supplementary Fig. S5 for additional details. ¶ For 5% of data.

Figure 2

Comparison of liganded FABP4 structures. All values were calculated per amino-acid residue using the apoprotein structure as a reference. Each row corresponds to FABP4 protein with ligands L1 to L6, as indicated by the numbers on the left (from 1 to 6, respectively). Parameters calculated are the root-mean-square deviations of backbone atoms ('RMSD bb') and of backbone plus side-chain atoms ('RMSD bb+sch'), molecular surface area ('MSA'), curvature ('CUR') and accessible surface area ('ASA') of each protein without ligand. All parameter values are indicated by shading from white to black corresponding to the minimum and maximum values observed for each protein. In addition, curvature values are depicted as upward or downward triangles indicating positive or negative values, respectively. The protein sequence (bottom) is scaled to the protein topology diagram (top). Most changes are associated with mobile loops and residues with solvent-exposed side chains, indicative of minimal conformational changes upon ligand binding.

an overall bent conformation, with a dihedral angle of 100° around the C2—C3 axis, compared with L2, which displays a dihedral angle of 166° . Consequently, the C2—C3 region of L3 makes hydrophobic contacts with the Ile104 side chain. Presumably, a (3)-methyl substituent on the phenyl ring of L2 enables it to make additional hydrophobic contacts which result in a more extended conformation compared with L3. In addition, this conformational change also improves the edge-to-face aromatic interaction between L3 and Phe16 compared with L2 and Phe16: the average angular shift for the aromatic interaction with L2 is 10.2° , whereas the corresponding value for L3 is 5.2° (see Table 1, Fig. 4a and Supplementary Fig. S4).

Compound L4 is the smallest ligand tested (Fig. 1a). Owing to the lack of substituents on the central phenyl group, its hydrophobic interactions are restricted to the 3-methyl group, which makes contacts with the Ile104 side chain, and those of the ligand phenyl group with Tyr19 and Phe57 (Fig. 3). Owing to intramolecular steric hindrance between the 3-methyl and phenyl groups, L4a does not display the edge-to-face aromatic interaction with the Phe16 side chain. However, this hindrance is overcome in L4b, which rotates by $\sim 38^\circ$ to properly interact with the Phe16 side chain, highlighting its role in restraining the ligand conformation (see Table 1 and Fig. 4b).

The enantiomers L5 and L6 have opposite stereochemical configurations at C2 (Fig. 1a). These compounds display the highest number of hydrophobic interactions with the protein,

both through their 2-methyl groups (with Ile104 and Val115) and (4)-isobutyl groups (with Tyr19, Met20, Val23, Val25, Ala33 and Phe57) (Fig. 3), in agreement with their binding affinities being significantly higher than those of ligands L2, L3 and L4. However, despite their structural resemblance, each enantiomer adopts a notably different conformation. L6 displays an aromatic edge-to-face interaction with Phe16, as witnessed for L2, L3 and L4b. In contrast, the L5 phenyl group rotates $\sim 30^\circ$ with respect to the L6 phenyl group (Table 1), leaving the 2-methyl group properly positioned to interact with Ile104 and Val115, the carboxyl group forming a bidentate salt bridge with the Arg126 guanidinium group and the (4)-isobutyl group rotated $\sim 180^\circ$ compared with L6 (Figs. 3 and 4c). As a result, the aromatic edge-to-face interaction between L5 and Phe16 is disrupted, resembling the situation witnessed for L4a. Thus, the hydrophobic interactions mediated by the (4)-isobutyl and 2-methyl groups appear to be the main binding-affinity determinants, given the unfavorable orientations for edge-to-face aromatic interactions (Table 1).

Overall, these scenarios illustrate how weak aromatic interactions can significantly affect the conformation of a ligand located in a hydrophobic environment. In the case of FABP4, the cation– π interaction between Arg126 and Phe16 is most likely to result in electron delocalization from the aromatic ring into the guanidinium group, enhancing the electrophilic character of H atoms in the Phe16 phenyl side

Figure 3

FABP4 binding mode of the studied compounds as revealed by X-ray crystallography. Compounds L2–L6 are highlighted in orange ball-and-stick representation. L2, L3 and L4 displayed two alternate conformations, labeled a and b. For each panel, the names of residues interacting significantly with the corresponding ligand are shown. Electrostatic interactions between ligands and residues Arg126 and Tyr128 are depicted as yellow dotted lines. Aromatic edge-to-face interactions between Phe16 and the ligand phenyl group are indicated as cyan dotted lines.

Figure 4

Aromatic interactions around Phe16. Cation– π aromatic interactions (green dotted lines) between the Arg126 guanidinium and the Phe16 phenyl groups and edge-to-face aromatic interactions (cyan dotted lines) between Phe16 and ligands (a) L2a and L3a, (b) L4b and (c) L6 are shown. Ligands L4a and L5 are colored gray to indicate their lack of aromatic interaction with Phe16.

chain. Consequently, aromatic interactions in which a ligand donates electron density to the Phe16 side chain will also be strengthened. Considering that FABP4 comprises about 5% of the total adipocyte protein content, inhibitor selectivity might be more important than inhibitor affinity in order to avoid cross-inhibition side effects. Optimization of aromatic interactions constitutes an attractive approach for selectivity enhancement of drugs targeting hydrophobic sites in proteins.

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References

- Armougom, F., Moretti, S., Poirot, O., Audic, S., Dumas, P., Schaeli, B., Keduas, V. & Notredame, C. (2006). *Nucleic Acids Res.* **34**, W604–W608.
- Barf, T., Lehmann, F., Hammer, K., Haile, S., Axen, E., Medina, C., Uppenberg, J., Svensson, S., Rondahl, L. & Lundbäck, T. (2009). *Bioorg. Med. Chem. Lett.* **19**, 1745–1748.
- Cai, H., Yan, G., Zhang, X., Gorbenko, O., Wang, H. & Zhu, W. (2010). *Bioorg. Med. Chem. Lett.* **20**, 3675–3679.
- Cohen, A. E., Ellis, P. J., Miller, M. D., Deacon, A. M. & Phizackerley, R. P. (2002). *J. Appl. Cryst.* **35**, 720–726.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Evans, P. R. & Murshudov, G. N. (2013). *Acta Cryst.* **D69**, 1204–1214.
- Furuhashi, M. & Hotamisligil, G. S. (2008). *Nature Rev. Drug Discov.* **7**, 489–503.
- Furuhashi, M., Tuncman, G., Görgün, C. Z., Makowski, L., Atsumi, G., Vaillancourt, E., Kono, K., Babaev, V. R., Fazio, S., Linton, M. F., Rae, F., Sulsky, R., Robl, J. A., Parker, R. A. & Hotamisligil, G. S. (2007). *Nature (London)*, **447**, 959–965.
- Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D. & Bairoch, A. (2005). *The Proteomics Protocols Handbook*, edited by J. M. Walker, pp. 571–607. Humana Press.
- Hotamisligil, G. S., Johnson, R. S., Distel, R. J., Ellis, R., Papaioannou, V. E. & Spiegelman, B. M. (1996). *Science*, **274**, 1377–1379.
- Hutchinson, E. G. & Thornton, J. M. (1996). *Protein Sci.* **5**, 212–220.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 133–144.
- Kane, C. D. & Bernlohr, D. A. (1996). *Anal. Biochem.* **233**, 197–204.
- Krusinova, E. & Pelikanova, T. (2008). *Diabetes Res. Clin. Pract.* **82**, S127–S134.
- Kuzmič, P. (1996). *Anal. Biochem.* **237**, 260–273.
- Laskowski, R. A. (2009). *Nucleic Acids Res.* **37**, D355–D359.
- Lehmann, F., Haile, S., Axen, E., Medina, C., Uppenberg, J., Svensson, S., Lundbäck, T., Rondahl, L. & Barf, T. (2004). *Bioorg. Med. Chem. Lett.* **14**, 4445–4448.
- Liu, X., Huang, X., Lin, W., Wang, D., Diao, Y., Li, H., Hui, X., Wang, Y., Xu, A., Wu, D. & Ke, D. (2011). *Bioorg. Med. Chem. Lett.* **21**, 2949–2952.
- Makowski, L., Boord, J. B., Maeda, K., Babaev, V. R., Uysal, K. T., Morgan, M. A., Parker, R. A., Suttles, J., Fazio, S., Hotamisligil, G. S., Linton, M. F. & Rae, F. (2001). *Nature Med.* **7**, 699–705.
- Marr, E., Tardie, M., Carty, M., Brown Phillips, T., Wang, I.-K., Soeller, W., Qiu, X. & Karam, G. (2006). *Acta Cryst.* **F62**, 1058–1060.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Reese-Wagoner, A., Thompson, J. & Banaszak, L. (1999). *Biochim. Biophys. Acta*, **1441**, 106–116.

- Ringom, R., Axen, E., Uppenberg, J., Lundbäck, T., Rondahl, L. & Barf, T. (2004). *Bioorg. Med. Chem. Lett.* **14**, 4449–4452.
- Schaap, F. G., Binas, B., Danneberg, H., van der Vusse, G. J. & Glatz, J. F. (1999). *Circ. Res.* **85**, 329–337.
- Schüttelkopf, A. W. & van Aalten, D. M. F. (2004). *Acta Cryst.* **D60**, 1355–1363.
- Smathers, R. L. & Petersen, D. R. (2011). *Hum. Genomics*, **5**, 170–191.
- Soltis, S. M. *et al.* (2008). *Acta Cryst.* **D64**, 1210–1221.
- Strong, M., Sawaya, M. R., Wang, S., Phillips, M., Cascio, D. & Eisenberg, D. (2006). *Proc. Natl Acad. Sci. USA*, **103**, 8060–8065.
- Sulsky, R. *et al.* (2007). *Bioorg. Med. Chem. Lett.* **17**, 3511–3515.
- Tsodikov, O. V., Record, M. T. Jr & Sergeev, Y. V. (2002). *J. Comput. Chem.* **23**, 600–609.
- Vaguine, A. A., Richelle, J. & Wodak, S. J. (1999). *Acta Cryst.* **D55**, 191–205.
- Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M. & Barton, G. J. (2009). *Bioinformatics*, **25**, 1189–1191.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.