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Discovery of highly selective inhibitors of human fatty acid binding protein 4 (FABP4) by virtual screening

Haiyan Cai [a,†], Guirui Yan [a,†], Xiaodong Zhang [a], Olena Gorbenko [b], Heyao Wang [a,*], Weiliang Zhu [a,*]

[a] Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China
[b] Department of Cell Signaling, Institute of Molecular Biology and Genetics NASU, 150 Zabolotnogo Str., 03143 Kyiv, Ukraine

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ABSTRACT

In this study, a series of small molecule inhibitors of human FABP4 were identified through virtual screening. Compound **1** is the most potent hit against FABP4 with a selectivity of more than 144-fold preferences over human FABP3. In addition, MD simulation and mutation studies revealed key residues for inhibitory potency and selectivity, which provides a guideline for further drug design against obesity, diabetes and atherosclerosis.

Fatty acid binding proteins (FABPs) are small (14–15 kDa) cytoplasmic proteins that bind reversibly with high affinity to hydrophobic ligands, such as long chain fatty acids. It has been suggested that FABPs act as fatty acids shuttles and play important roles in metabolic diseases.[1] Since the initial discovery of FABPs in 1972, at least nine members have been identified, of which fatty acid binding protein 4 (FABP4) is the predominant FABP in adipocytes and also expressed in activated macrophages.[1–3]

FABP4-deficient mice, including both dietary and genetic obese mice, exhibited reduced hyperinsulinemia and insulin resistance.[4,5] Macrophage-specific lack of FABP4 could protect mice deficient in apolipoprotein E from atherosclerosis.[6] These findings indicated an important role for FABP4 in the development of major components of metabolic syndrome through its distinct functions in adipocytes and macrophages. Thus, inhibition of FABP4 with small inhibitors may lead to a new class of powerful therapeutic agents to prevent and treat metabolic diseases such as type 2 diabetes and atherosclerosis. Recently, a small series of FABP4 inhibitors have been reported,[7–11] among which small molecule BMS309403, a rationally designed, potent and selective inhibitor of FABP4, seems to be effective to improve glucose metabolism, enhance insulin sensitivity in both dietary and genetic mouse models of obesity and diabetes, and ameliorate the symptom of atherosclerosis.[12]

It has been reported that FABP3-deficient mice reduced exercise tolerance and at old age they developed a regional cardiac hypertrophy, indicating an important role of FABP3 in metabolic homeostasis.[13] Therefore, the selectivity of the FABP4 inhibitors over FABP3 is fateful factor for their drugability. However, the sequence identities among FABPs are pretty high,[14] which raises a challenge to achieve good selectivity for FABP4 over other members especially FABP3. Up to now, several crystal structures of various ligand-bound human FABP4 have been determined[7–10,15] (PDB code: 1TOW, 1TOU, 2HNX, 2NNQ, 3FR2, 3FR4 and 3FR5), which provide insights into the structural foundation underlying the binding modes of endogenous ligands and small molecule inhibitors in the pocket of FABP4. FABP4 is composed of a well-known assembly of 10 antiparallel β-strands and a helix-loop-helix cap in the N-terminal, and ligands lie in the internal pocket of FABP4, usually make polar interactions with the side chains of Arg126 and Tyr128. Comparing the sequences and structures of FABP3 and FABP4 shows that the overall conformation is quite similar except that several residues in the binding pocket of FABP4 are substituted in FABP3, such as Met40 and Ser53, especially Ser53, which is substituted by Thr53 in FABP3. These residues may contribute to the selectivity for ligands of FABP4 over FABP3.

Here, we report a series of novel FABP4 inhibitors identified through virtual screening and similarity search. Two of them exhibited high selectivity over FABP3. The binding modes of these

* Corresponding authors. Fax: +86 21 50807088 (H.W.), +86 21 50805020 (W.Z.).
E-mail addresses: hywang@mail.shcnc.ac.cn (H. Wang), wlzhu@mail.shcnc.ac.cn (W. Zhu).
† These authors contributed equally to the work.

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novel inhibitors in the pocket of FABP4 were explored using molecular dynamics (MD) simulation, and were further verified by mutation technology.

Virtual screening (via software DOCK4.0,[16] GLIDE[17,18] and AUTODOCK3.05[19]) was employed to search for novel FABP4 inhibitors. The X-ray structure of FABP4 binding with BMS309403 (PDB code: 2NNQ[9]) was retrieved from the Protein Data Bank (http://www.rcsb.org/pdb) for docking calculation. All crystallographic water and SO_4^{2-} buffer molecules and ions were removed. The residues within a radius of 3.5 Å around the ligand were used to construct the grids for docking screening. First, DOCK4.0[18] was employed to search the Maybridge database (http://www.maybridge.com) for potential binders of FABP4. Subsequently, the top 10% ranked compounds with the highest score obtained by DOCK were selected for the second round docking by GLIDE[17,18] (www.schröinger.com), then the top 800 molecules were filtered by AUTODOCK3.05.[21] Finally, according to the diversity and solubility of molecules and their potential to form hydrogen bonds with the residues in the binding pocket of FABP4, we manually selected and purchased 52 molecules for inhibitory activity assay. All compounds were tested for their inhibitory activity using the 8-anilino-1-naphthalene-sulfonic acid (1,8-ANS) displacement assay developed by Kane and Bernlohr.[20] Five of them were proved to be inhibitors of FABP4 at 100 μM. Based on the five inhibitors, a structural similarity-based search in Maybridge database was performed, leading to the discovery of another five hits (Fig. 1). The IC_{50} value was then determined, which showed that four compounds have IC_{50} values ranging from 14 to 60 μM (Table 1). Impressively, these compounds have similar structures, indicating that this series of compounds might be potent FABP4 inhibitors. Remarkably, the compound **1** is as potent as the endogenous ligand of FABP4. The selectivity assay against FABP3 was performed on the most potent compounds **1**–**4**. It showed that **1** and **4** could hardly inhibit FABP3 while **2** and **3** presented nearly the same potency on both FABP3 and FABP4 (Table 2). Among these compounds, **1** was the most potent hit with IC_{50} of 13.5 μM against FABP4 and displayed more than 144-fold preferences over human FABP3, therefore, **1** is a potential lead structure and valuable for further drug development. Note that the four compounds have the same scaffold in structure and only vary with the group to which the amide linkage connect (aromatic ring in **1** and **4** while diphenyl ether and *N*-phenylpiperidine in **2** and **3**, respectively).

As selectivity is a very important issue for further structural optimization, the difference of their binding modes in FABP4 and FABP3 as well as the structure–activity relationships (SARs) of the four compounds should be useful information. Hence, using **1** and **2** as examples, four molecular dynamics (MD) simulation studies were performed using the AMBER 9.0 simulation package[21,22] to figure out the specific residues important for ligands binding in FABP4 and FABP3, which are FABP4-**1** (complex of FABP4 and **1**),

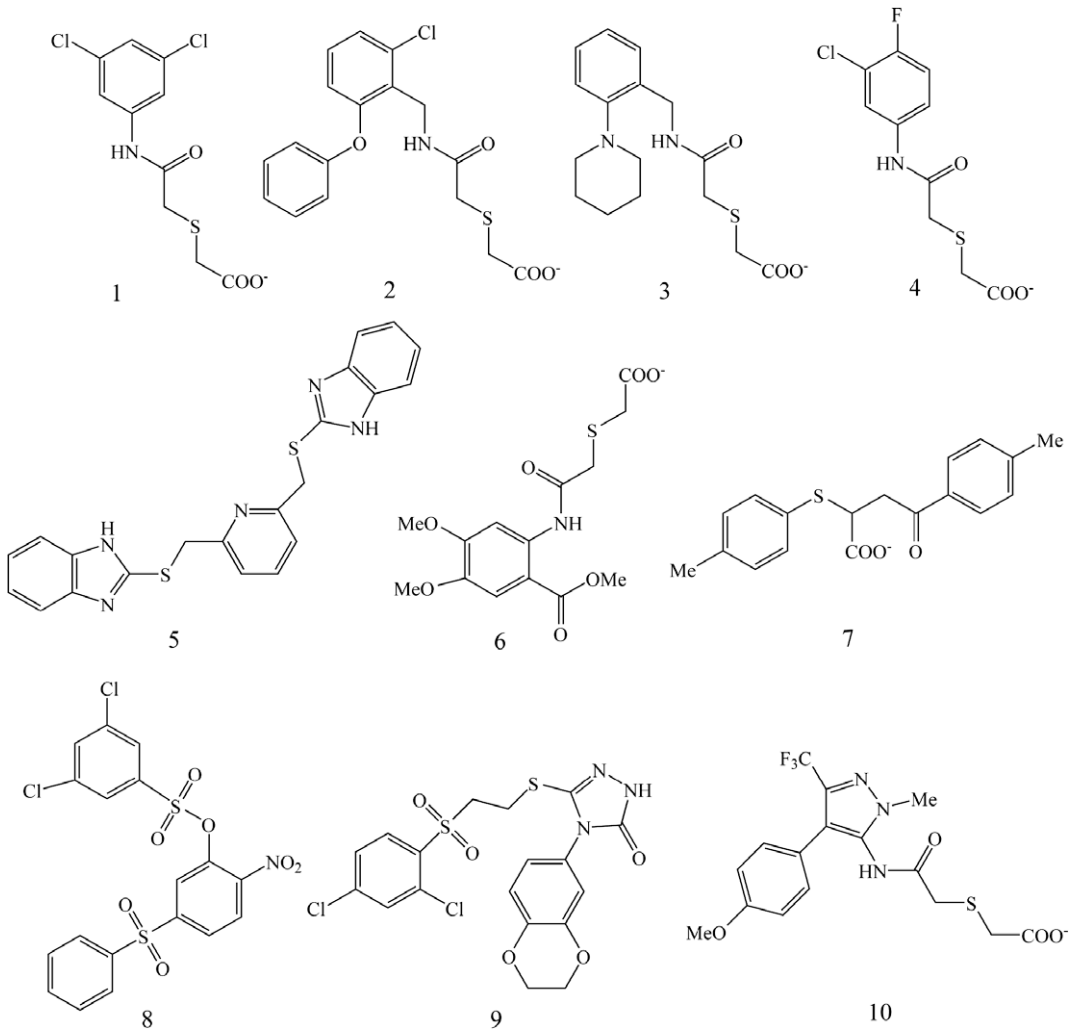

Figure 1. Structures of the discovered active compounds.

Table 1
Inhibition potency of the test compounds against FABP4

Compounds	Inhibition at 100 μM (%)	IC_{50}[a] (μM)
LA	81.4	13.6
OA	66.8	26.1
1	84.9	13.5
2	62.5	42.2
3	58.7	56.3
4	56.2	59.5
5	47.7	>100
6	46.9	>100
7	44.4	>100
8	40.7	>100
9	37.1	>100
10	20.1	>100

[a] Values are means of triplicate experiments with relative standard deviations <3%, the data were analyzed with GraphPad Prism software (Graphpad).

Table 2
Selectivity of the tested FABP4 inhibitors over FABP3

Compounds	FABP4 IC_{50}[a] (μM)	FABP3 IC_{50}[b] (μM)	Selectivity index FABP3/FABP4
LA	1.0	1.0	1.0
1	1.0	>144	>144
2	3.1	1.9	0.6
3	4.1	6.3	1.5
4	4.4	>432	>99

[a] Values are means of triplicate experiments with relative standard deviations <3%, presented as the fold of LA on FABP4.
[b] Values are means of triplicate experiments with relative standard deviations <6%, presented as the fold of LA on FABP3.

FABP4-**2** (complex of FABP4 and **2**), FABP3-**1** (complex of FABP3 and **1**) and FABP3-**2** (complex of FABP3 and **2**). The initial orientations of the ligands were determined from the docking poses of AUTODOCK3.05. The complexes were solvated using a cubic box of TIP3P[23] water molecules extending at least 10 Å away from the boundary of any protein atoms. An appropriate number of counterions were added to neutralize the systems. Each complex/water system was submitted to energy minimization. Afterwards, they were subjected to MD simulations. The SHAKE[24] method was applied to constrain all covalent bonds involving hydrogen atoms. Each simulation was coupled to a 300 K thermal bath at 1.0 atm pressure by applying the algorithm of Berendsen.[25] An integration time step of the MD calculations was 2 fs. In the energy minimizations and MD simulations, periodic boundary conditions were applied in all directions. The analyses of the simulations focused on the production stages. The root-mean-square deviations (RMSDs) of the $C\alpha$ atoms were calculated from the trajectories at 2 ps intervals (Fig. 1S), which showed that the RMSD changed a little after 5000 ps in all four systems, the average values of equilibrated models were 1.38 Å, 1.56 Å, 1.73 Å, 1.5 Å for FABP4-**1**, FABP4-**2**, FABP3-**1** and FABP3-**2**, respectively. After the systems were equilibrated, 20 complex structures were extracted at intervals of 350 ps (from 5000 ps to 12,000 ps), then these structures were aligned, as we found the interaction styles in all the complexes were very similar except FABP4-**1**, which was also stable after 7000 ps, so we picked one of them as an example to analyze the interaction mechanism for each system.

From the binding modes, we found that π–π stack interaction plays vital role in inhibition selectivity. The aromatic ring of **1** could form a π–π stack interaction to the residue Phe16 of FABP4, however, there is no such interaction observed between **1** an FABP3 (Fig. 2A), the aromatic ring was pushed far away from Phe16. This difference might be due to the mutation from Ser53 in FABP4 to Thr53 in FABP3, which pulled the aromatic ring of **1** away from Phe16 through the hydrogen-bond interaction (Fig. 2A). Lacking interaction with Phe16 in FABP3 led to decreasing more than 144-fold of inhibitory activity comparing to FABP4 (Table 2). In contrast, because the diphenyl ether moiety of **2** could form π–π stack interaction to Phe16 in both FABP4 and FABP3 (Fig. 2B), no inhibitory difference was observed for **2**. Therefore, **2** displays similar inhibition potency against both proteins (Table 2). In addition, this π–π stack interaction between ligand and Phe16 is also observed in almost all of the existing crystal structure complexes.[7–11,15,26,27] Thus, a π–π stack interaction to Phe16 might play a critical role for the inhibitory activity in both FABP4 and FABP3. Similar interaction difference was also observed between **3** and **4**, which could be also attributed to the selectivity of **3** and **4**.

The MD simulations also showed that the carboxylates of both **1** and **2** could make polar interactions with Arg126 and Tyr128 (Fig. 3A and B) in the pocket of FABP4, which are critical for the binding of inhibitors as described previously.[7–11] Besides, **2** formed additional hydrogen bonds to Asp76, Gln95 and Arg106 (Fig. 3B).

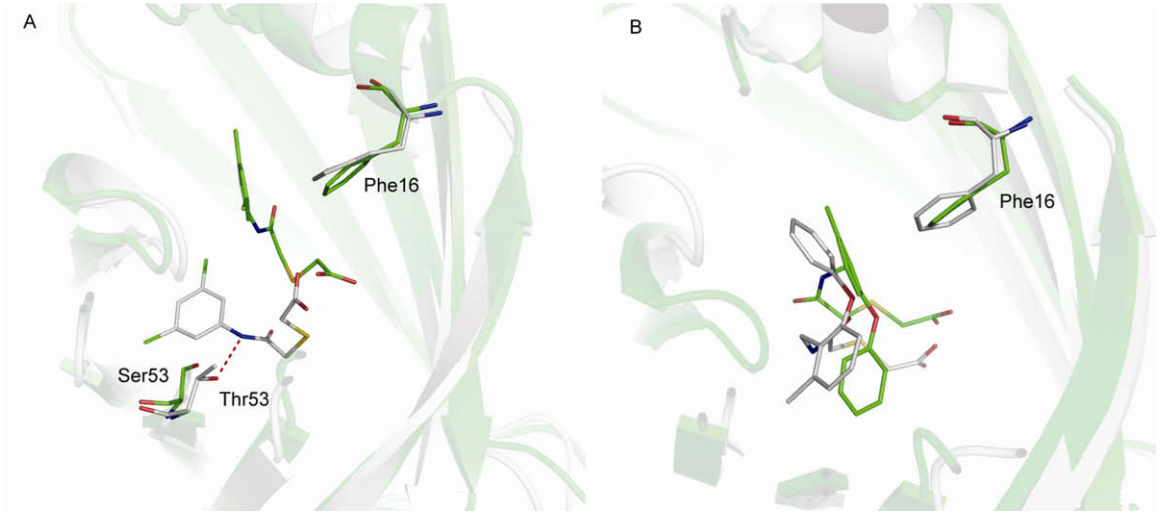

Figure 2. Representatives for the aligned FABP4–ligand complexes (green) and FABP3–ligand complexes (white). (A) π–π stack interaction between **1** and Phe16 of FABP4, hydrogen-bond interactions between **1** and FABP3. (B) π–π stack interaction between **2** and Phe16 of both FABP3 and FABP4. The dashed lines in red represent hydrogen bonds.

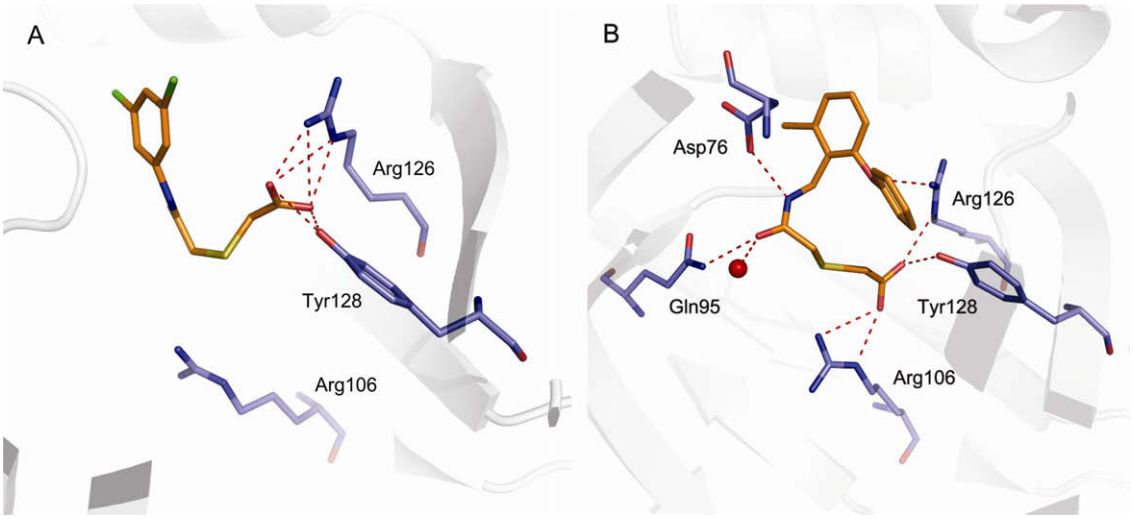

Figure 3. Representatives for the hydrogen-bond interaction models of FABP4-**1** and FABP4-**2**. (A) Hydrogen-bond interactions between FABP4 and **1**. (B) Hydrogen-bond interactions between FABP4 and **2**. The dashed lines in red represent hydrogen bonds. Compounds are shown in orange sticks and residues are shown in slate sticks.

Accordingly, the residues Phe16, Arg106, Arg126 and Tyr128 in both FABP4 and FABP3 are very important to the ligand binding. Arg106, Arg126 and Tyr128 form stable polar interactions with ligands while Phe16 is necessary to form a π–π stack interaction to fix the ligand binding orientations and it could also relate to selectivity between FABP3 and FABP4. Some residues, which are substituted in FABP3, such as Ser53 could be considered in drug design to improve the selectivity of inhibitors over FABP3.

To validate the putative ligand binding modes, three site-directed mutagenesis studies on FABP4 were designed and carried out. One is Phe16Ala of FABP4 for validating the role in π–π stack interaction. The IC_{50} value was determined to be 13.6 μM for LA to inhibit wild-type FABP4, but higher than 700 μM to the mutant. The IC_{50} value was 13.5 μM for **1** to inhibit the wild-type, but around 500 μM to the mutant. However, little change was observed for **2** to inhibit both the wild-type and the mutated FABP4 (Table 3). These values are in well agreement with our MD simulation results (Fig. 2A), which demonstrated again that the π-π stacking interaction between Phe16 and the aromatic moiety of **1** is essential to the binding. Another one is Arg126Ala for breaking the predicted potential polar interactions between the compounds and FABP4. The mutated FABP4 was expressed and inhibitory activities of **1** and **2** were tested. Indeed, the inhibitory activity against mutated FABP4 of both **1** and **2** dramatically decreased (Table 4), indicating the pivotal role of Arg126 for ligand binding as interpreted by our predicted binding modes. The third one is Arg106Ala mutant, which did not show significant difference in the binding affinity of **1** from wide type, but moderately dropped in the binding affinity of **2**, which is the same as our prediction (Table 4). The above results revealed that residue Arg126 is very important for ligand binding, while Phe16 might play more important roles in both affinity and selectivity in some type of ligands such as **1** and **3**.

Table 3
Comparison of the inhibitory activity of the compounds on wild-type and F16A mutated FABP4

Compounds	Wild-type FABP4 IC_{50}[a] (μM)	F16A IC_{50}[a] (μM)	F16A Inhibition at 500 μM[b] (%)	F16A Inhibition at 700 μM[b] (%)
LA	13.6	ND	36.9	39.0
1	13.5	ND	50.7	57.0
2	42.2	76.5	ND	ND

ND: not determined.
[a] Values are means of triplicate experiments with relative standard deviations <3%.
[b] Values are means of triplicate experiments with relative standard deviations <16%.

Table 4
Comparison of the inhibitory activity of the compounds on wild-type and mutated FABP4[a]

Compounds	Ratio of IC_{50} (R106A/wildtype)	Ratio of IC_{50} (R126A/wildtype)
LA	2.0	1.6
1	1.3	11.1
2	3.4	6.6

[a] Values are means of triplicate experiments with relative standard deviations <3%.

In summary, 10 compounds that showed inhibitory potency against FABP4 have been discovered by virtual screening and two of them had high selectivity over FABP3. MD studies and mutation technology have been applied to explore the binding modes of the hits in the pocket of FABP4. From our work, it suggests that residues Arg126 and Tyr128 in FABP4 play essential roles in hydrogen-bond interactions. For instance, Arg126 acts as a hydrogen bond donor to interact with the carboxyl and sulfone groups of the inhibitors. We also noticed that the residues Phe16 and Ser53 play important roles in both affinity and selectivity. Phe16 forms a π–π stack interaction with the aromatic ring of the selective inhibitor **1** via the help of the residue Ser53 which is mutated in FABP3 with Thr53 (Fig. 2A). Therefore, further drug design should strengthen these two specific interaction features.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.095.

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