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The discovery of novel and selective fatty acid binding protein 4 inhibitors by virtual screening and biological evaluation

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ABSTRACT

Adipocyte fatty acid binding protein (AFABP, FABP4) has been proven to be a potential therapeutic target for diabetes, atherosclerosis and inflammation-related diseases. In this study, a series of new scaffolds of small molecule inhibitors of FABP4 were identified by virtual screening and were validated by a bioassay. Fifty selected compounds were tested, which led to the discovery of seven hits. Structural similarity-based searches were then performed based on the hits and led to the identification of one high affinity compound **33b** (K_i = 0.29 ± 0.07 μM, ΔT_m = 8.5 °C). This compound's effective blockade of inflammatory response was further validated by its ability to suppress pro-inflammatory cytokines induced by lipopolysaccharide (LPS) stimulation. Molecular dynamics simulation (MD) and mutagenesis studies validated key residues for its inhibitory potency and thus provide an important clue for the further development of drugs.

1. Introduction

Fatty acid binding proteins (FABPs) are members of the superfamily of lipid-binding proteins (LBPs). FABPs coordinate intracellular lipid storage and trafficking and play a central role as regulators of lipid metabolism. Nine FABPs were identified in the liver (LFABP, FABP1), intestines (IFABP, FABP2), heart (HFABP, FABP3), adipocyte tissue (AFABP, FABP4), epidermis (EFABP, FABP5), ileum (IlFABP, FABP6), brain (BFABP, FABP7), myelin (MFABP, FABP8) and testis (TFABP, FABP9). Among the nine known isoforms, FABP4 is the predominant FABP in adipocytes and macrophages. In addition to its role in trafficking fatty acids within cells, FABP4 is recognized as an adipocytokine that is associated with lipid metabolism and inflammation. Therefore, FABP4 has an impact on diseases such as metabolic syndrome, insulin resistance and atherosclerosis.[1,2]

Several small molecular FABP4 inhibitors[3–7] have been developed with promising potential (Fig. 1). For example, carbazole-based inhibitor **1** and its derivatives can effectively and selectively displace naturally occurring human FABP4 ligands.[5] HTS01037 (**2**) has an inhibitory effect on LPS-induced TNF-α production and reduces blood glucose levels.[4] Biphenyl azole inhibitor BMS309403 (**3**) was found to mitigate the onset of insulin resistance and atherosclerosis in mice.[2,7] Previously, we developed compound **4**, which significantly attenuates the production of pro-inflammatory cytokines induced by LPS simulation.[6] An old uricosuric drug benzbromarone (**5**) was found to inhibit forskolin-stimulated lipolysis in 3T3-L1 cells and reduce blood levels in db/db mice.[3] These findings indicate that FABP4 is a potential drug target and that FABP4 inhibitors may become new therapeutic agents for the treatment of diabetes and atherosclerosis.[8]

In this study, we report the successful application of structure-based virtual screening in the discovery of a series of new scaffold FABP4 inhibitors. The SPECS database, comprised of approximately 200,000 compounds, was screened, and fifty compounds

Abbreviations: FABPs, fatty acid binding proteins; OA, oleic acid; LA, linoleic acid; LPS, lipopolysaccharide; FA, fluorescent displacement assay; TSA, thermal stability shift assay; SBVS, structure-based virtual screening; MD, molecular dynamics simulation.

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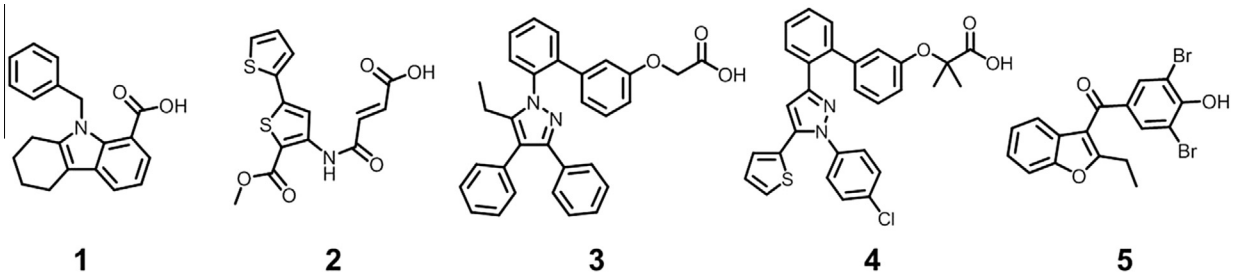

Figure 1. Selected examples of known FABP4 inhibitors as reported in the literature.

were purchased for biological assessment. Seven out of the fifty compounds were shown to be active in fluorescent displacement assay (FA). Further similarity searches based on the hit compounds led to **33b**, which exhibited improved inhibitory activity with a K_i value of 0.29 ± 0.07 μM in FA and a temperature shift of 8.5 °C in the thermal shift assay (TSA). The strong binding capacity of **33b** was further validated by its effective blockade of inflammatory responses, which are determined by the production of pro-inflammatory cytokines induced by LPS stimulation. Molecular dynamics (MD) simulations and mutation studies revealed key residues for the inhibitory effects and provided important clues for further development of novel inhibitors of FABP4.

2. Results and discussion

2.1. Virtual screening led to 7 hits

Structure-based virtual screening (SBVS) has been widely used in drug discovery research.[9–14] Considering both time and enrichment efficiency, a hierarchical virtual screening strategy was taken (Fig. 2) and described in detail in the methods section. Briefly, approximately 200,000 molecules from the SPECS database were downloaded from the ZINC website. After a series of enrichment methods, the Glide XP score was used to rank the compounds. The top 1000 compounds were retained and clustered using Canvas. After post-processing and visual inspection, 50 compounds (Table S1) were selected based on their docking score, interaction characterizations and physicochemical properties.

Candidate compounds were purchased from SPECS and initially screened using the 1,8-ANS fluorescent displacement assay (FA). The results showed that seven of the compounds exhibited more than a 50% inhibition rate (Fig. 3A, Table S2). The K_i of these active compounds was further determined. Compounds **17**, **21**, **27**, **33**, **34**, **52** and **55** exhibited K_i values below 10 μM, showing comparable activity to the endogenous ligand linoleic acid (LA) (K_i = 6.8 ± 3.1 μM) and oleic acid (OA) (K_i = 1.5 ± 0.8 μM). All of the seven active compounds identified in the first round screening contain a carboxylate group (Fig. 3B), which is an important characteristic of the FABP4 endogenous ligands as well as of most of its known inhibitors.

The activity of these new scaffold compounds was then confirmed by the TSA.[15] The TSA is a method to identify ligands that bind to and stabilize proteins by measuring the relative melting temperature shift (ΔT_m). Interactions between FABP4 and ligands were assessed by TSA as previously described.[16] Among the tested compounds, compound **33** demonstrated moderate activity with a temperature shift of 3.5 °C, indicating that it can bind to and stabilize the FABP4 protein. Therefore, compound **33** was selected as a starting point for further study.

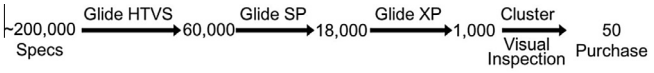

Figure 2. Schematic representation of the virtual screening workflow used for FABP4 inhibitor discovery in this study.

2.2. Similarity searches led to high affinity hits

Compound **33** was characterized as a completely new scaffold, and it demonstrated moderate activity in both the FA and the TSA. Based on the structure of **33**, a similarity search in the ChemDiv database was performed and led to the discovery of an additional 5 active compounds. The binding activity was determined by the FA and the TSA (Fig. 4, Table 1). Among the tested compounds, compound **33b** exhibited the best inhibitory activity with an apparent K_i value of 0.29 ± 0.07 μM and a temperature shift of 8.5 °C. Compound **33c** was slightly less potent than **33b**, with a K_i value of 0.38 ± 0.11 μM and a temperature shift of 6.2 °C. Their inhibitory activity was comparable to that of **3** (K_i = 0.22 ± 0.05 μM, ΔT_m = 10.6 °C) and was far better than the FABP4 endogenous ligands LA (K_i = 6.8 ± 3.1 μM, ΔT_m = 1 °C) and OA (K_i = 1.5 ± 0.8 μM, ΔT_m = 3.3 °C).

2.3. Isothermal titration calorimetry confirmed direct binding

To further assess the activity of **33b**, an isothermal titration calorimetry (ITC) experiment was performed to confirm the direct binding of **33b** to FABP4 (Fig. 5). Titration of high concentrations of FABP4 (500 μM) into the ITC buffer resulted in heat absorption, while the binding with **3** and **33b** resulted in a release of heat. The K_d values were determined based on the binding data that were corrected by subtracting the data from the independent titrations of the protein into the buffer. The K_d value of **33b** was 2.65 ± 0.41 μM, which was comparable to that of **3** (K_d = 2.05 ± 0.49 μM). The results suggested that the binding activities were consistent with the data from the FA and the TSA.

2.4. A Cell-based assay confirmed FABP4 inhibition

Previous studies have demonstrated that the knockdown of FABP4 can effectively reduce inflammatory responses in macrophages.[2,17] The biological effects of **33b** and **33c** were evaluated in a murine macrophage cell line RAW264.7 to see whether they affected the activation of inflammatory responses. The results demonstrated that the expression of two key inflammatory cytokines, TNFα and IL-6, that were stimulated by LPS were significantly attenuated by **33b** and **33c** in a dose-dependent manner (Fig. 6). These data indicated that our compounds significantly inhibited LPS-induced expression of both TNFα and IL-6 in RAW264.7 cells.