

Supporting information

Pimozide, a novel fatty acid binding protein 4 inhibitor, promotes adipogenesis of 3T3-L1 cells by activating PPAR γ

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Supplemental Materials & Methods

Molecular docking simulation

Molecular docking simulation was performed using the three-dimensional crystal structures of substrate free FABP4 (PDB code 1TOU,⁽¹⁾ 1TOW,⁽²⁾ 2NNQ⁽³⁾ and 3FR5⁽⁴⁾) obtained from the Protein Data Bank as our previous described.⁽⁵⁾ The docking simulations were conducted by software AutoDock Vina v.1.0.2. The docking parameters for AutoDock Vina were kept to their default values. The grid box was 20 Å × 14 Å × 12 Å, encompassing the inhibitor binding cavity of FABP4. The binding modes were clustered through the root-mean square deviation (RMSD) among the Cartesian coordinates of the ligand atoms. The docking results were ranked by the binding free energy. The binding modes with lowest binding free energy and the most cluster members were chosen for the optimum docking conformation. The binding results were graphically presented by PyMOL Molecular Graphics System Version 1.3 (Schrödinger, LLC).

Ligand displacement fluorescence-labeled probe assay of FABP4

The binding activities against human FABP4 were evaluated using the 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) displacement assay as described previously.⁽⁶⁾ Briefly, 1,8-ANS (10 μM, Sigma, St. Louis, MO) in phosphate buffered solution (PBS, pH 7.4) was mixed with FABP4 protein (10 μM, final concentration). Compounds at desired concentrations were added and incubated for 3 min at room temperature. The fluorescence signal at 370 nm (excitation)/470 nm (emission) was then determined by SpectraMax[®] i3 Multi-Mode Microplate Reader (Molecular Devices, CA, USA).

Cell cultures

Mouse 3T3-L1 pre-adipocytes were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified 95% air and 5% CO₂ atmosphere. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA). To initiate 3T3-L1 adipocyte differentiations, the standard differentiation cocktail (250 nM dexamethasone, 100 mM 3-isobutyl-1-methylxanthine and 175 nM human insulin, all purchased from Sigma, St. Louis, MO) was added to the medium after 2 days post-confluence. After 2 days, the medium was replaced with fresh complete medium containing insulin. Culture medium was changed every 2 days.⁽⁷⁾ Pimozide (Sigma, St. Louis, MO) or/and GW 9662 (Santa Cruz Biotech, Santa Cruz, CA) was dissolved in DMSO and added to the medium at the beginning of pre-adipocyte differentiations. The DMSO level was less than 0.1% in culture medium, which did not affect pre-adipocyte differentiation. A schematic diagram of drug treatment is summarized in Figure S1.

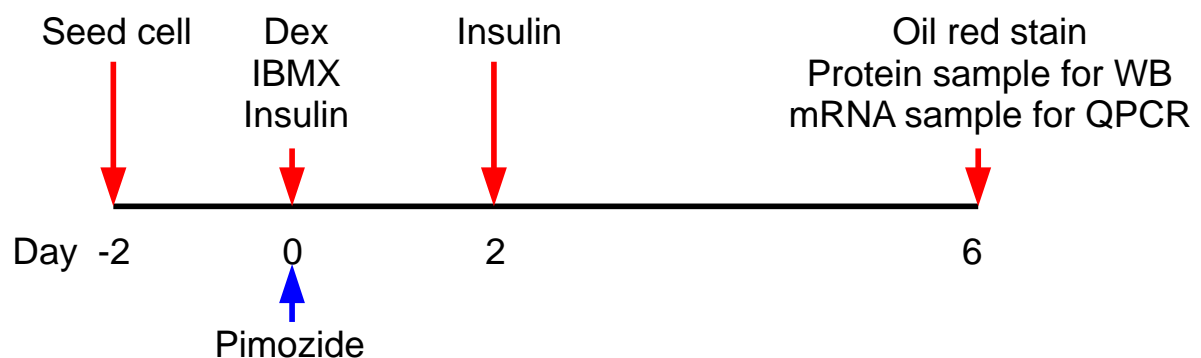


Figure S1. A schematic diagram of drug treatment for the analysis of adipogenesis

Analysis of adipolysis

Confluent pre-adipocytes (day 0) were stimulated by the differentiation cocktail and cultured for 2 days in the absence or in the presence of pimoziide. At day 2, the supernatants were collected and adipolysis was measured using a commercial adipolysis assay kit (Cayman Chemical, Ann Arbor, MI, USA). A schematic diagram of drug treatment is summarized in Figure S2.

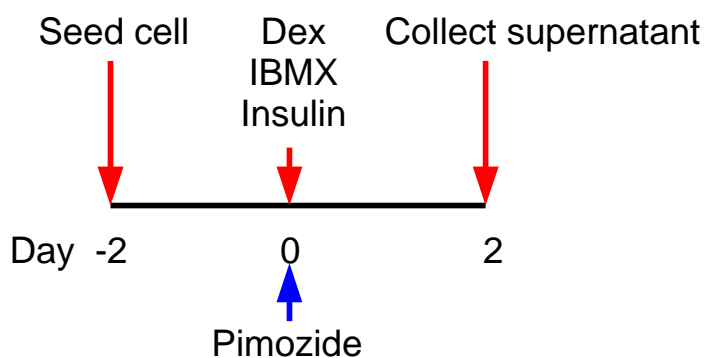


Figure S2. A schematic diagram of drug treatment for the analysis of adipolysis

MTT Assay

To evaluate the cytotoxicity of pimoziide on 3T3-L1, a tetrazolium dye MTT colorimetric assay was performed to determine the cell viability as previously described.⁽⁸⁾ Briefly, 3T3-L1 cells were treated with pimoziide for 24 h followed by incubation with or without isoproterenol (100 nM) for another 24 h. Afterward, and the viability was measured.

Oil Red O Staining

The 3T3-L1 cells were seeded in 24 well plates. The cells were initiated to differentiate using standard differentiation cocktail after 2 days post-confluence, and then treated with pimoziide

at 10 μ M. All the samples were collected at Day 6. Briefly, the cells were washed with PBS and incubated with 4% formalin in PBS for 30 min. The fixative solution was removed and the fixed cells were stained with 0.5% Oil Red O (Sigma, St. Louis, MO) for 20 min. The staining solution was removed and the stained cells were washed with PBS and then photographed. To quantify the degree of lipid accumulation, the cells were incubated with isopropanol to extract the lipid droplet and then the Oil Red staining intensity was measured at the wavelength of 510 nm by a microplate reader (Bio-Rad).⁽⁷⁾

Western blot

Protein samples were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon-P membrane; Millipore, Bedford, MA). The membrane was blocked in a solution of 5% bovine serum albumin, then incubated overnight with primary antibodies against PPAR γ or β -actin (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was washed with blocking buffer twice and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Specific bands were probed with ECL-plus western blotting detection reagent (GE Healthcare Bio-Sciences) and photographed with FujiFilm LAS-3000 (Fujifilm, Tokyo, Japan).

Quantitative real-time PCR

Total RNA was extracted and quantitative real-time PCR was performed as described previously.⁽⁷⁾ The primers for qPCR were summarized in Table S1.

Table S1. Primers used in quantitative real-time PCR

Target gene		Primer sequences
GAPDH	Forward	5'-AGGTCGGTGTGAACGGATTTG-3'
	Reverse	5'-TGTAGACCATGTAGTTGAGGTCA-3'
PPAR γ	Forward	5'-CAAGAATACCAAAGTGCGATCAA-3'
	Reverse	5'-GAGCTGGGTCTTTTCAGAATAATAAG-3'
FABP4	Forward	5'-CAAAATGTGTGATGCCTTTGTG-3'
	Reverse	5'-CTCTTCCTTTGGCTCATGCC-3'
Adiponectin	Forward	5'-TGTTCCCTCTTAATCCTGCCCA-3'
	Reverse	5'-CCAACCTGCACAAGTTCCCTT-3'
C/EBP α	Forward	5'-GACATCAGCGCCTACATCGA-3'
	Reverse	5'-TCGGCTGTGCTGGAAGAG-3'
C/EBP β	Forward	5'-ATTTCTATGAGAAAAGAGGCGTATGT-3'
	Reverse	5'-AAATGTCTTCACTTTAATGCTCGAA-3'

Plasmid and transient transfection

The cDNA of the human FABP4 was isolated from human brain cDNA library by PCR.

Amplified gene was digested with HindIII and KpnI (New England Biolabs, Beverly, MA), and then subcloned into pcDNA3.1 vector. Confluent pre-adipocytes (day 0) were transfected with pcDNA3.1-FABP4 or empty vector using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Statistics

Statistical significance was determined using the One-Way ANOVA (GraphPad Software, CA). The results are presented as the means \pm SEM. The significance was accepted when p-value was <0.05.

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