

Small-molecule inhibitors of FABP4/5 ameliorate dyslipidemia but not insulin resistance in mice with diet-induced obesity

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Abstract Fatty acid binding protein-4 (FABP4) and FABP5 are two closely related FA binding proteins expressed primarily in adipose tissue and/or macrophages. The small-molecule FABP4 inhibitor BMS309403 was previously reported to improve insulin sensitivity in leptin-deficient *Lep^{ob}/Lep^{ob}* (*ob/ob*) mice. However, this compound was not extensively characterized in the more physiologically relevant animal model of mice with diet-induced obesity (DIO). Here, we report the discovery and characterization of a novel series of FABP4/5 dual inhibitors represented by Compounds 1–3. Compared with BMS309403, the compounds had significant in vitro potency toward both FABP4 and FABP5. In cell-based assays, Compounds 2 and 3 were more potent than BMS309403 to inhibit lipolysis in 3T3-L1 adipocytes and in primary human adipocytes. They also inhibited MCP-1 release from THP-1 macrophages as well as from primary human macrophages. When chronically administered to DIO mice, BMS309403 and Compound 3 reduced plasma triglyceride and free FA levels. Compound 3 reduced plasma free FAs at a lower dose level than BMS309403. However, no significant change was observed in insulin, glucose, or glucose tolerance. Our results indicate that the FABP4/5 inhibitors ameliorate dyslipidemia but not insulin resistance in DIO mice.—Lan, H., C. C. Cheng, T. J. Kowalski, L. Pang, L. Shan, C.-C. Chuang, J. Jackson, A. Rojas-Triana, L. Bober, L. Liu, J. Voigt, P. Orth, X. Yang, G. W. Shipps, and J. A. Hedrick. **Small-molecule inhibitors of FABP4/5 ameliorate dyslipidemia but not insulin resistance in mice with diet-induced obesity.** *J. Lipid Res.* 2011. 52: 646–656.

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Fatty acid binding protein-4 (FABP4; adipocyte-FABP, a-FABP, ALBP, aP2) belongs to the family of cytosolic FA binding proteins (1). Members of this protein family exhibit high affinity for small lipophilic ligands and are named according to the tissue from which they were initially identified. FABPs are involved in uptake and metabolism of FAs, maintenance of cellular membrane FA levels, intracellular trafficking of these substrates, modulation of specific enzymes of lipid metabolic pathways, and modulation of cell growth and differentiation. FABP4 is predominantly expressed in adipose tissue and macrophages. Macrophages also express keratinocyte FA binding protein (FABP5, epidermal-FABP: keratinocyte-FABP, e-FABP, k-FABP, mall) (1).

Mouse and human genetic studies support FABP4 and FABP5 as potential drug targets for diabetes and coronary heart disease. Mice deficient in *Fabp4* exhibited a modest increase in insulin sensitivity, but there was no protection from increased weight gain or fatty liver disease (2, 3). This modest phenotype was presumably due to a functional compensation from FABP5, which was upregulated in adipose tissue of *Fabp4*^{-/-} mice (4, 5). Similarly, *Fabp5*^{-/-} mice exhibited a small increase in insulin sensitivity without changes in total body adiposity (6). Although *Fabp4* and *Fabp5* single-knockout mice had only modest phenotypes, the *Fabp4* and *Fabp5* double-knockout mice displayed strong protection from diet-induced obesity (DIO), insulin resistance, type 2 diabetes, and fatty liver disease (7),

Abbreviations: ALIS, Automated Ligand Identification System; 1,8-ANS, 8-anilino-1-naphthalene-sulfonic acid; DIO, diet-induced obesity; FABP, fatty acid binding protein; FP, fluorescence polarization; GTT, glucose tolerance test; HFD, high-fat diet; ITT, insulin tolerance test; *K_i*, inhibition constant; LPS, lipopolysaccharide; PK, pharmacokinetic; SAR, structure-activity relationship; TdF, temperature-dependent fluorescence.

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suggesting a synergistic effect resulting from the dual deletion of FABP4 and FABP5. A functionally significant variation near the human *FABP4* gene locus resulted in decreased *FABP4* expression and was associated with decreased plasma triglyceride level and reduced risk of type 2 diabetes and cardiovascular disease (8). Inhibition of FABP4 or FABP5, or both, may thus be potentially useful for the treatment of dyslipidemia and/or diabetes.

Genetic and epidemiological studies suggest that chemical inhibition of FABP4/5 may be an attractive approach in diabetes drug discovery. Indeed, a selective biphenyl azole inhibitor of FABP4, BMS309403, was identified as binding FABP4 with nM affinity and >100-fold selectivity against FABP5 as well as the heart isoform FABP3 (9). In a ligand displacement assay using 1,8-ANS (8-anilino-1-naphthalene-sulfonic acid) as the probe, the compound displays inhibition constant (K_i) values of <2 nM, 250 nM, and 350 nM for FABP4, FABP5, and FABP3, respectively (10). When administered in vivo, this compound was effective in improving insulin resistance in leptin-deficient *Lep^{ob}/Lep^{ob}* (*ob/ob*) mice and atherosclerosis in *ApoE^{-/-}* mice (11). The effects of this selective FABP4 inhibition were not rigorously characterized in mice with DIO, except for a single panel figure in the supplementary data suggesting that BMS309403 improved glucose tolerance in DIO mice (11).

Several other chemical inhibitors of FABP4 have been published over the last decade. For example, the chemical structures of several tetrahydrocarbazole derivatives of FABP4 inhibitors were reported (12–14), with one compound displaying an IC_{50} value of 600 nM in a fluorescence polarization (FP) assay (15), and another compound in the same series an IC_{50} value of 49 nM in a scintillation proximity assay (16). However, no biological characterization in cell-based or in vivo assays has been reported. Another FABP4 inhibitor, HTS01037, has recently been reported (17). In ligand displacement assays using 1,8-ANS, this compound displayed K_i values of 670 nM, 3.4 μ M, and 9.1 μ M for FABP4, FABP5, and FABP3, respectively. According to the published values, this compound appeared to be \sim 30-fold weaker than BMS309403, yet it bound to FABP4 in X-ray crystallography, and in cell-based assays, HTS01037 inhibited lipolysis in 3T3-L1 adipocytes and reduced lipopolysaccharide (LPS)-stimulated inflammation in mouse macrophages (17). No in vivo characterization has been reported. More recently, a series of small-molecule inhibitors of human FABP4 have been identified through virtual screening (18). In ligand displacement assays using the 1,8-ANS, the most-potent compound displayed IC_{50} values of 13.5 μ M for FABP4 with a selectivity of >100-fold over FABP3. No cellular or in vivo activities have been characterized.

In the present study, we report the discovery of a novel series of potent FABP4/5 inhibitors as represented by Compound 2 and Compound 3. These compounds are triazolopyrimidine derivatives with novel binding modes to the FABP4 protein. We profiled our compounds in vitro and in vivo, together with the other published FABP4/5 inhibitors, especially BMS309403.

EXPERIMENTAL PROCEDURES

Discovery of dual FABP4/5 inhibitors

A large chemical library was screened using the Automated Ligand Identification System (ALIS) platform based on affinity selection-mass spectrometry technology, a robust tool for identifying strong targets quickly from vast mixtures of chemicals (19). Recombinant FABP3, -4, and -5 proteins were prepared from an *Escherichia coli* expression system. ALIS hits were confirmed by a temperature-dependent fluorescence (TdF) assay (see below) to assess their affinity to FABP4 and their selectivity against FABP3. Compounds with an FABP4 TdF K_d value \leq 20 μ M and a selectivity of \geq 10-fold window over FABP3 or showed no binding to FABP3 (defined as $K_d > 25 \mu$ M) were selected for evaluation of their “drug-like” and “lead-like” properties based on widely accepted hit-to-lead criteria (20). The previously reported FABP4-selective inhibitors all had a carboxylic acid moiety in their chemical structures. In this study, we focused our efforts on non-carboxylic acid compounds to differentiate from the other compounds and to achieve superior pharmacokinetic (PK) and cell permeability properties. Desirable hits were further evaluated by a ligand displacement FP assay (see below) to determine their potency toward FABP4 and FABP5. In parallel, we carried out a high-throughput screen of a chemical library base on the FABP4 FP assay. Hits were retrospectively tested with the TdF assays to assess the selectivity against FABP3, and with the FP assays for FABP4/5 dual inhibition using the same criteria as described above. In the next step, we focused our efforts on building SARs (structure-activity relationships) and increasing affinity for FABP4 while maintaining a \geq 10-fold selectivity window over FABP3 in the TdF binding assay and preserving or improving the potency toward FABP5 in the FP assay. Interesting compounds were subjected to cell-based assays to evaluate their ability to inhibit lipolysis in mouse 3T3-L1 adipocytes and MCP-1 secretion from THP-1, a human macrophage cell line. Lead candidates were further evaluated for cocrystallization with recombinant FABP4 protein, and for their ability to improve metabolic parameters in the *ob/ob* or DIO mice.

TdF assays for FABP4 and FABP3

The TdF assay was used to test binding affinity of compounds to recombinant FABP4 or FABP3 proteins using fluorescence-based thermal shift to monitor protein-ligand thermal unfolding (21). The TdF assay was conducted in the 96-well-based CHROMO-4 real-time fluorescence plate reader (BioRad; Hercules, CA). The environmentally sensitive fluorescent dye Sypro Orange (Sigma; St. Louis, MO) was used to monitor the protein folding-unfolding transition. Protein-ligand binding was gauged by the change (or shift) in the unfolding transition temperature (ΔT_m) acquired with protein alone or with protein in the presence of the ligand of interest. Each reaction sample consists of 3 μ M protein (FABP4 or FABP3) and 15, 50, or 100 μ M compound in 2% DMSO incorporated with Sypro Orange dye in 20 μ l reaction buffer (25 mM HEPES, 150 mM NaCl, pH 7.5, and 1 mM DTT). The sample plate was heated from 30°C to 90°C with a thermal ramping rate of 1°C/min. The fluorescence signals were acquired with excitation and emission wavelengths centered at 490 and 560 nm, respectively. Binding affinity (K_d value) was calculated based on the degree of fluorescent shift of the protein with and without compounds.

Ligand displacement FP assay for FABP4 and FABP5

The ligand displacement FP assay was used to determine the in vitro potency of compounds for FABP4 or FABP5 by measuring their ability to displace a fluorescence-labeled probe occupying

the ligand binding pocket of the proteins (15). Compounds were dissolved in DMSO at an initial 10 mM stock concentration. Serial dilutions of compounds by 3-fold, starting at 55 μ M for eight dose points in a final concentration of 2% DMSO, were performed in a 384-well plate. Diluted compounds were mixed with FABP4 or -5 at a final concentration of 0.75 μ M in PBS containing 1 mM DTT and 0.005% Triton X-100, and incubated at room temperature for 30 min. The labeled probe, BODIPY[®] 500/510 C4, C9 (B3824; Invitrogen, Carlsbad, CA), was then added at a final concentration of 100 nM. The reaction mixture was equilibrated for another 10 min. FP signals were read at 535 nm with an EnVision 2103 Multilabel Reader (PerkinElmer; Waltham, MA).

X-ray structure analysis

FABP4 was prepared at 30 mg/ml in 25 mM Tris-HCl, pH 8, and incubated with 3-fold excess of Compound 1 or Compound 2 dissolved in DMSO for 1 h at room temperature. Hanging drops were prepared by mixing 2 μ l of protein solution added to 2 μ l of well solution containing from 8% to 12% polyethylene glycol 6000 buffered with 100 mM Tris-HCl (pH 7.5). Plate-shaped crystals grew within a week and belonged to space group P2₁ with unit cell dimensions of a = 48 Å, b = 72 Å, c = 54 Å, and β = 114 Å. Crystals were incubated with additional 20% glycerol and flash-frozen in liquid nitrogen. X-ray diffraction data were collected on a Rigaku FRE generator equipped with a RAXIS 4²⁺ detector, resulting in data sets of 2.0 for both cocrystals. Diffraction data were processed using the *HKL* package (22) and *CCP4* (23). The structures were solved by molecular replacement using *MOLREP* (24) and the FABP4-linoleate complex (protein data bank ID: 2Q9S). The models were subjected to repeated rounds of building with Coot (25) and refinement with *autoBUSTER* (26).

Adipocyte lipolysis

Mouse 3T3-L1 preadipocytes were maintained in DMEM with 10% FBS. To induce differentiation, cells were plated at a density of 1×10^5 cells in 35 mm² wells of 6-well plates. Two days after cells reached confluence, the medium was changed to induction medium containing DMEM with 10% FBS, 1 μ M dexamethasone (Sigma), 500 μ M 3-isobutyl-L-methylxanthine (Sigma), and 5 μ g/ml insulin (Sigma). After 48 h in induction medium, cells were switched to differentiation medium containing DMEM with 10% FBS plus 1 μ g/ml insulin. Differentiation medium was replaced every 2–3 days. After complete differentiation (usually 7–9 days), cells were washed with PBS and treated with serum-free DMEM with 5 mM glucose and 0.1% BSA, with serial dilutions of compounds dissolved in DMSO (0.4% final concentration). To determine lipolysis, glycerol levels in supernatants were measured using the Free Glycerol Determination Kit (FG0100; Sigma, St. Louis, MO). For isoproterenol-stimulated lipolysis, fully differentiated 3T3-L1 adipocytes grown in 6-well plates (7 days postdifferentiation) were washed three times with KREB buffer containing 5 mM glucose, then treated with compounds diluted in KREB buffer containing 5 mM glucose. After 1 h incubation, isoproterenol was added to the culture to a final concentration of 100 nM. Incubation continued for another 4 h, followed by collection of supernatant to measure glycerol levels. For the lipolysis assay in primary adipocytes, isolated human primary adipocytes were purchased from Zen-Bio, Inc. (Research Triangle Park, NC). Cells were treated with six doses of compounds at 5-fold serial dilutions (25 μ M to 8 nM) in culture medium containing 100 nM isoproterenol for an additional 4 h, and supernatants were likewise collected for measurement of glycerol levels. Niacin at 5-fold lower dose series (5 μ M to 1.6 nM) was used as a positive control.

MCP-1 release from THP-1 macrophage-like cells

THP-1 cells were maintained in RPMI-1640 (American Type Culture Collection) plus 10% FBS in T75 tissue culture flasks at 37°C, 5% CO₂. Cells were differentiated into macrophage-like cells with 40 nM PMA (P8139, Sigma) added to the culture medium for 24 h in a poly-D-lysine-coated 384-well plate (Cellcoat Poly-D-Lysine, 781946; Greiner Bio-One), and each well contained 4×10^4 cells in a 100 μ l medium. After 24 h of differentiation, 100 μ l/well Optimum medium (31985; Invitrogen, Carlsbad, CA) containing 1% lipoprotein-deficient serum (S5519; Sigma) with seven-point serial dilutions of compounds dissolved in DMSO was added to the cultures for 24 h, and supernatants were collected to measure MCP-1 using the Meso-Scale hMCP-1 assay kit (K21AYB-2; Meso-Scale Discovery, Gaithersburg, MD). For the LPS-stimulated MCP-1 assay, THP-1 cells were treated with compounds at four doses diluted down from 25 μ M for 1 h, and then treated with 50 ng/ml LPS for 3 h. Supernatants were collected to measure MCP-1. For assays in human primary macrophages, macrophages were collected from three anonymous healthy human donors (one male, two females) following a standard protocol (27). Informed consents were obtained from the human macrophage donors. Cells were treated with 1, 5, and 25 μ M compounds overnight, and supernatants were collected to measure MCP-1 as described above.

Metabolic studies in vivo

PKs in chow-fed mice was carried out after a single dose at 3, 10, and 30 mg/kg. Both blood and tissue samples were collected at 1 h, 6 h, and 24 h postdosing from subgroups of animals (n = 4 each group) euthanized at the indicated time points. Compound concentrations in the plasma and fat samples were determined using MS. Compounds that were empirically determined to have acceptable PKs were subjected to PK studies in DIO mice prior to chronic dosing studies. PKs in DIO mice was carried out with compounds formulated in 60% high-fat diet (HFD) (Research Diets D12492, with 60% kcal fat; New Brunswick, NJ) at three dose levels (3, 10, and 30 mg/kg) and were administered to DIO mice maintained on the same diet for 3 days (n = 4 each group).

For the metabolic studies in vivo, C57BL/6J mice at 6 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME) and were maintained on a 60% HFD until they reached 12 weeks of age. Compounds were formulated in 60% HFD at three dose levels (3, 10, and 30 mg/kg), and were administered to mice for 8 weeks (n = 15 each group). Rosiglitazone at 5 mg/kg formulated in diet was used as a positive control in this study. Body weight and food intake were monitored weekly, and blood samples were collected for glucose and insulin analyses bi-weekly. Oral glucose tolerance tests (oGTT) were performed after the seventh week of drug administration, and intraperitoneal insulin tolerance tests (ipITT) were performed at the eighth week. For GTT and ITT, blood was collected after 16 h (for GTT) or 3 h (for ITT) of fasting, and basal glucose levels were measured using a glucose oxidase method (Glucometer Elite; Bayer, Elkhart, IN). After this measure, glucose (3 g/kg body weight) or insulin (1 U/kg body weight) was administered by oral gavage or by intraperitoneal injection, respectively. Blood was collected from the tail vein at 30, 60, 90, and 120 min postdose for glucose determination. At the end of the study, blood samples in the postprandial state were collected after terminal bleeding for analyses of metabolic parameters such as triglyceride, free FA, total cholesterol, and selected cytokines using kits from Meso-Scale Discovery or from Wako Chemicals (Richmond, VA). All studies were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility, according

to protocols approved by the legacy Schering-Plough Research Institute Animal Care and Use Committee.

Data analysis

Data were reported as means \pm standard errors of the means (SEM). Depending on data structure, analyses were performed by using the two-tailed Student's *t*-test or analysis of variance (ANOVA), with Dunnett's posthoc test as indicated. *P* values <0.05 were considered statistically significant.

RESULTS

In vitro property of FABP4/5 compounds

Compound 1 was discovered by the ALIS screen (Fig. 1A). The compound displayed a K_d value of 0.86 μ M for FABP4 and 35 μ M for FABP3 in the TdF assay. Subsequent hit-to-lead chemistry efforts resulted in the discovery of Compound 2 and Compound 3, both of which were subjected to extensive profiling in cell-based and in vivo assays. Cocrystal structures for Compound 1 and Compound 2 with recombinant FABP4 were obtained (Fig. 1B). All heteroatoms of the Compound 1 triazolopyrimidine formed hydrogen bonds with the protein and one structural water (Arg 106, Arg 126, Tyr 128; Fig. 1B, left panel). The experimental pKa value of Compound 2 was 4.6, with the compound being insoluble at low pH, indicating that it is neutral at low pH and is soluble when ionized at high pH. This pH profile is similar to that of a carboxylic acid, and suggests that this heterocycle could serve as a bioisostere. The phenyl ring occupies a narrow pocket lined by residues Met 40, Ile 42, Ile 51, Leu 113, and Val 115 and forms an edge-to-face interaction with Tyr 128. The 2-methoxy-5-chloro group points toward the portal region close to residues Phe 16, Tyr 19, Met 20, Val 23, and Val 25. The overlay of the FABP4 complex crystal structure with palmitate is shown in Fig. 1B, middle panel. The ligand occupies the same area as the FA with the carbonyl group and one of the FA carboxylate atoms coinciding, lending further evidence for this heterocyclic as a novel bioisostere. The triazolo-nitrogen, which hydrogen bonds to Arg 106, replaces the water hydrogen bonding to second carboxylate oxygen of the FA. The phenyl group

induces a side-chain movement of Met 40, giving rise to the extended pocket described above. Compound 2 and Compound 3 display binding modes very similar to those of FABP4. The improved potency can be explained by the alleviation of Compound 1 clashes with Tyr 19 and Val 23 in Compound 2 (Fig. 1B, right panel).

TdF K_d (for FABP4 and FABP3) and FP IC_{50} values (for FABP4 and FABP5) of our compounds, together with several representative FABP4 inhibitors published in the literature (11, 12, 17), are summarized in Table 1. In both TdF and FP assays, the FABP4 affinity of our compounds was comparable to that of BMS309403 and Biovitrum 2a, whereas HTS01037 was notably weaker (Fig. 2A), consistent with the published data obtained with the 1,8-ANS FP assays. Importantly, Compound 2 and Compound 3 inhibited both FABP4 and FABP5, but the magnitude of FABP5 inhibition was not as great as that for FABP4 (Table 1 and Fig. 2B).

FABP4/5 compounds inhibited basal and isoproterenol-stimulated lipolysis in 3T3-L1 and primary human adipocytes

Targeted deletion or chemical inhibition of FABP4 was previously shown to decrease lipolysis in adipocytes (17, 28). We asked if our compounds had such an effect. In mouse 3T3-L1 adipocytes, Compound 2 and BMS309403 (10 μ M) inhibited basal lipolysis (represented by reduced glycerol release into the culture medium) to a greater degree than that obtained with 2 μ M rosiglitazone (Fig. 3A). Isoproterenol-stimulated lipolysis in 3T3-L1 cells was apparently reduced by all three compounds. Compound 2 (≥ 10 μ M) and Compound 3 (≥ 10 μ M) appeared more potent than BMS309403 (30 μ M) (Fig. 3B). To test if the anti-lipolysis effect also existed in human cells, we first established that isoproterenol stimulated lipolysis in human primary adipocytes (Fig. 3C), and then observed that both Compound 2 and BMS309403 reduced isoproterenol-stimulated lipolysis in a dose-dependent manner. Compound 2 ($IC_{50} = 22$ μ M) was slightly more potent than BMS309403 ($IC_{50} > 25$ μ M), although both compounds were significantly weaker in activity compared with niacin ($IC_{50} = 3.9$ μ M) (Fig. 3D). The data indicate that Compound 2

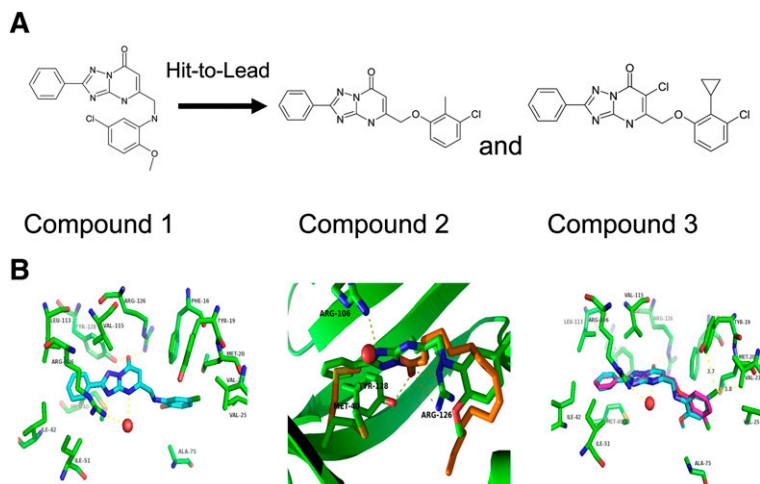


Fig. 1. A: Structures of Compound 1, Compound 2, and Compound 3. B: Left panel, crystal structures of FABP4-Compound 1; middle panel, overlay of FABP4 crystal structures of Compound 1 (green) with palmitate (PDB code 2 hnx); right panel, overlay of FABP4 crystal structures of Compound 1 (green) and Compound 3 (magenta).

TABLE 1. In vitro properties of FABP4 and/or -5 inhibitors

Compound	Reference	TdF K_d		FP IC ₅₀	
		FABP4	FABP3	FABP4	FABP5
Compound 1	–	0.86	μ M >25	1.7 ± 1.1	>25
Compound 2	–	0.01	0.52	0.8 ± 1.3	1.4 ± 1.3
Compound 3	–	0.02	0.56	1.3 ± 1.3	4.3 ± 1.3
BMS309403	(11)	0.004	>25	1.2 ± 1.2	>25
Biovitrum 2a	(12)	0.06	0.87	0.6 ± 1.1	11.1 ± 1.2
HTS01037	(17)	1.89	>25	3.4 ± 1.1	>25

The TdF K_d values represent the mean of three determinations with each value within ~50% of the mean. The FP IC₅₀ values were reported as mean ± standard error of the mean (SEM) from at least three replicated experiments. Values ≥25 μ M were designated as inactive, and no SEM was calculated.

inhibited basal or isoproterenol-stimulated lipolysis in 3T3-L1 and primary human adipocytes more than did BMS309403.

FABP4/5 compounds inhibited basal and LPS-stimulated MCP-1 release from THP-1 cells and primary human macrophages

Chronic inflammation is known to be associated with the pathology of insulin resistance and atherosclerosis, and the improved metabolic profiles of FABP4/5 double-knockout mice were thought to be partially mediated through modulation of inflammatory responses (1). BMS309403 has been previously shown to reduce MCP-1 release from PMA-differentiated THP-1 cells, a human macrophage cell line, at concentrations ≥10 μ M in a FABP4-dependent manner (11). In our study, BMS309403 and Compounds 2 and 3 inhibited basal MCP-1 release from differentiated THP-1 macrophages with similar IC₅₀s and magnitudes of inhibition dose-response curves (Fig. 4A). BMS309403 and Compound 2 at the 25 μ M dose level also inhibited MCP-1 release from LPS-activated THP-1 macrophages. The inhibition was comparable to the activity level of 1 μ M TAK-242, a selective inhibitor of toll-like receptor 4 that suppresses LPS-induced inflammation (29) (Fig. 4B). In order to test whether the ability of FABP4/5 compounds to reduce MCP-1 secretion was present in human primary cells, we isolated macrophages from blood samples of three healthy human donors and treated the cells with three doses of the FABP4/5 compounds. As shown in Fig. 4C, both BMS309403 and Compound 2 at a 25 μ M dose level reduced MCP-1 release from the human primary macrophages. Caution should be used in interpreting this result, inasmuch as the effect, seen only at 25 μ M, may be nonspecific. Taken together, these data suggest that BMS309403 and our compounds inhibited basal and LPS-stimulated MCP-1 secretion from THP-1 cells and primary human macrophages.

FA derivatives are known ligands for both FABP4 and PPAR γ , and PPAR γ activation could lead to similar cellular events in adipocytes and macrophages (30). To examine whether the cell-based activity that we observed was mediated by PPAR γ agonism, we tested these compounds in a PPAR γ transactivation assay. Neither BMS309403, Compound 2, or Compound 3 activated PPAR γ at concentrations up to 10 μ M, whereas in the same assay, the PPAR γ agonist GW1929 displayed an EC₅₀ of ~0.1 μ M,

indicating that the compounds in this study were not PPAR γ ligands.

FABP4/5 compounds ameliorated dyslipidemia but not insulin resistance in DIO mice

BMS309403 was reported to improve insulin resistance in *ob/ob* mice (11). The effect of this compound in DIO mice was disclosed only in a single panel figure (see supplementary Fig. VIII) showing that BMS309403 improved glucose tolerance in DIO mice after dosing at 40 mg/kg for 4 weeks (11). We carried out a study to compare the effect of BMS309403 and Compound 3 on insulin sensitivity and metabolic parameters in DIO mice. The PKs of BMS309403 is poor after oral administration, with plasma drug levels at 0.99 ± 0.57 μ M, 0.23 ± 0.10 μ M, and 0 μ M at 1, 6, and 24 h, respectively, after dosing at 30 mg/kg. Because BMS309403 does not provide a 24 h drug exposure after oral dosing, we chose to carry out the study with drugs formulated in diet. After administration of drugs as diet admixtures at 30 mg/kg in DIO mice for 3 days, BMS309403 and Compound 3 had plasma drug levels at 0.34 ± 0.13 μ M and 16.7 ± 1.3 μ M, respectively. The ratios of compound concentration in epididymal fat pad to those in blood were 1.00 ± 0.41 and 0.09 ± 0.01 for BMS309403 and Compound 2, respectively. All three compounds were heavily protein bound, with <1% fraction existing as free drug in the plasma samples.

We dosed DIO mice at ~12 weeks of age with BMS309403 and Compound 3 at 3, 10, and 30 mg/kg for 8 weeks. Rosiglitazone at 5 mg/kg was used as a positive control. BMS309403 and Compound 3 had minimal effect on body weight or food intake. No significant changes in fasting or nonfasting glucose, insulin, or in glucose or insulin tolerances were observed for either BMS309403 or Compound 3, although rosiglitazone improved both GTT and ITT, as expected (Fig. 5A). Nevertheless, both compounds reduced plasma triglyceride and free FA levels in the blood samples collected at termination (Fig. 5B). Compound 3 reduced plasma free FA levels at all three dose levels, whereas the effect of BMS309403 was only obtained at the 30 mg/kg dose level. No statistically significant changes were observed for BMS309403 or Compound 3 in blood cholesterol or selected adipokines/cytokines (i.e., adiponectin, resistin, leptin, MCP-1, or IL-6). The data indicated that FABP4/5 inhibitors ameliorated dyslipidemia but not insulin resistance in DIO mice.

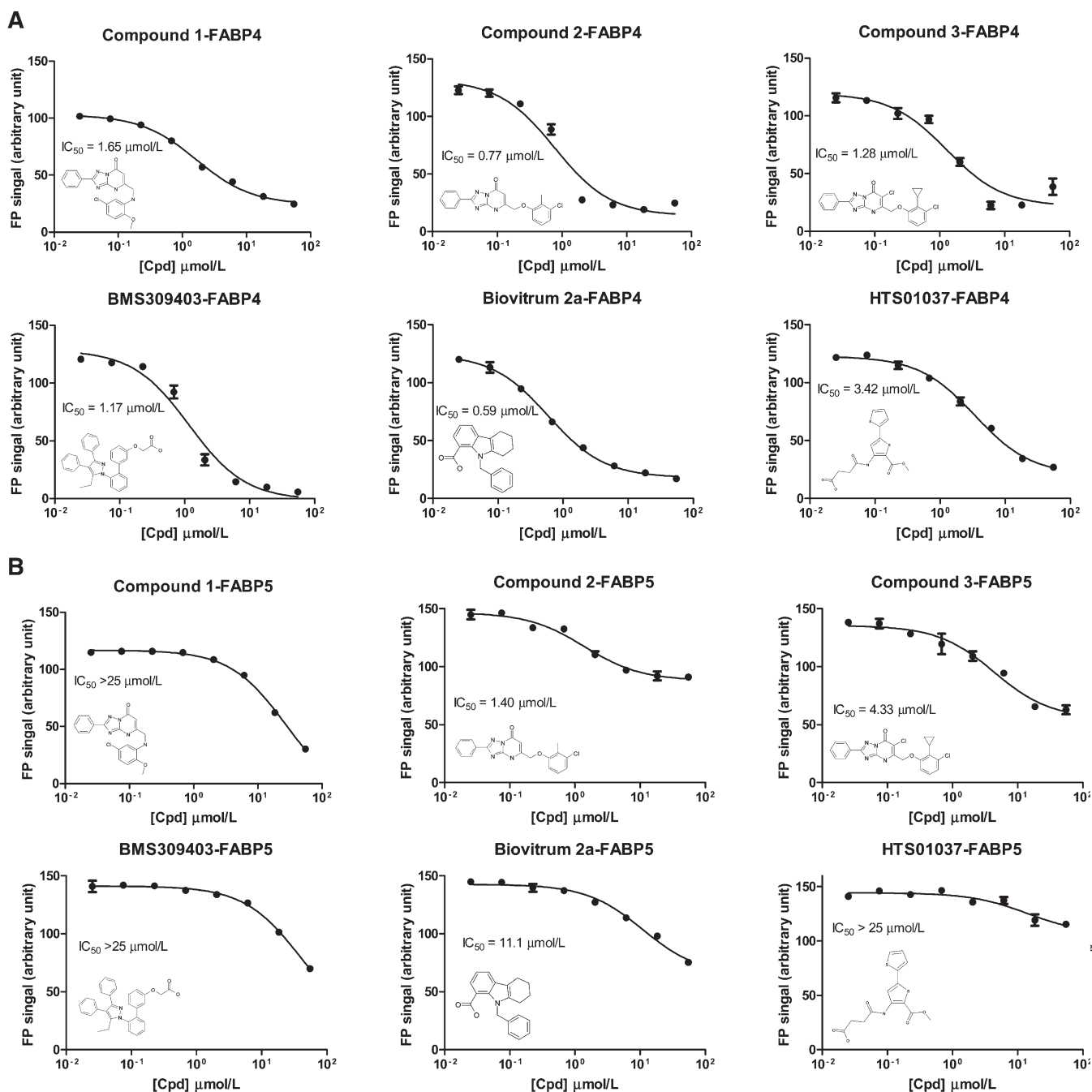


Fig. 2. A: Dose-response curves of compounds in FP assay for FABP4. B: Dose-response curves of compounds in FP assay for FABP5. The IC_{50} values were calculated using the sigmoidal dose-response formula implemented in Prism 5.

DISCUSSION

Recent advances in FABP4 and FABP5 biology, and the discovery of small-molecule FABP4 inhibitors, suggest their potential for treating insulin resistance and type 2 diabetes. Mice with deletion of both FABP4 and FABP5 had strong protection from DIO, insulin resistance, type 2 diabetes, and fatty liver disease (7). The FABP4 inhibitor BMS309403 was reported to improve glucose tolerance in *ob/ob* as well as DIO mice, and atherosclerosis in *ApoE*^{-/-} mice (11). Several other selective FABP4 inhibitors have also been reported with various degrees of in vitro (but not in vivo) characterizations (12, 17, 18). In this study, we present the

discovery of FABP4/5 dual inhibitors represented by Compound 2 and Compound 3. These compounds were not only among the most-potent FABP4 inhibitors, they also had significant in vitro potency toward FABP5. They were slightly more potent than BMS309403 in inhibiting lipolysis in adipocytes, and they were comparable to BMS309403 in terms of the ability to suppress MCP-1 release from macrophages. When chronically administered in vivo, Compound 3 appeared to be more effective in ameliorating dyslipidemia than BMS309403. However, no significant effects on insulin resistance were observed for either BMS309403 or Compound 3 in DIO mice.

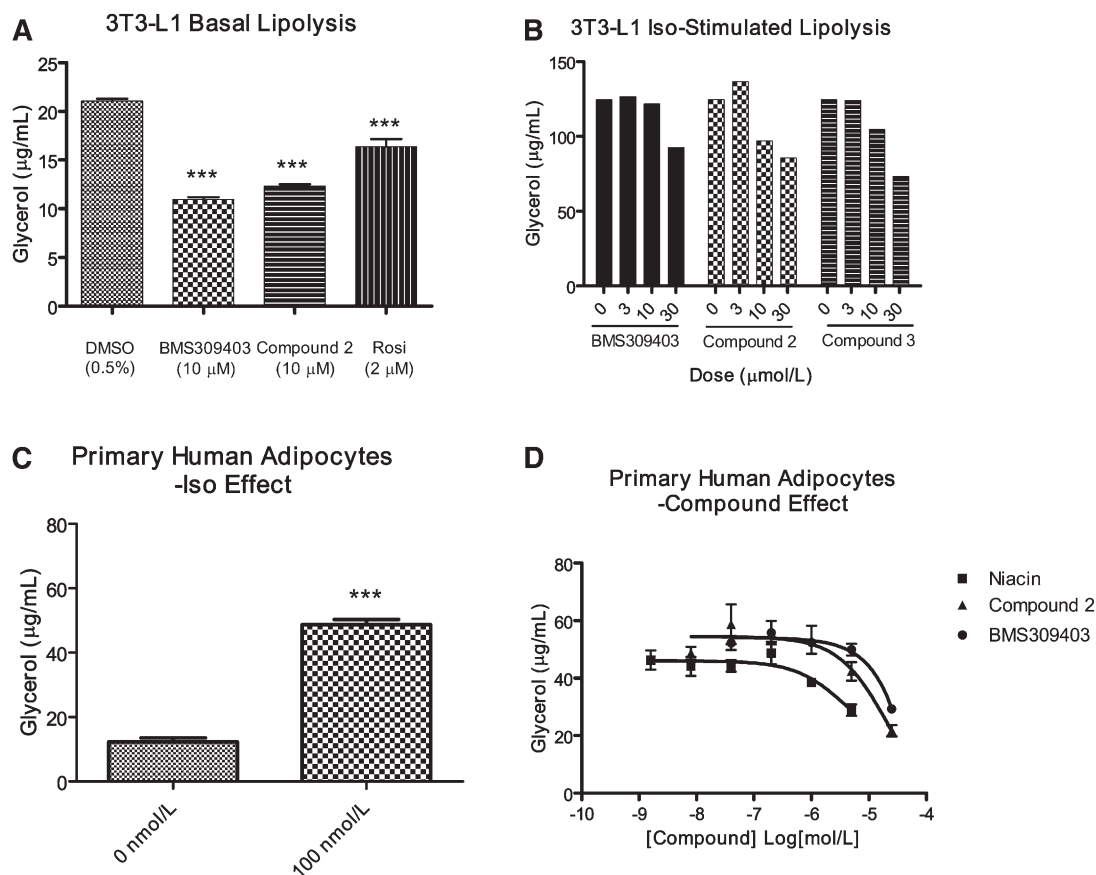


Fig. 3. A: Effect of FABP4/5 compounds on basal lipolysis in 3T3-L1 adipocytes. Rosiglitazone was used as a control. *** $P < 0.001$, one-way ANOVA followed by Dunnett's posthoc tests. B: Effect of FABP4/5 compounds on isoproterenol (Iso)-stimulated lipolysis in 3T3-L1 adipocytes. Treatments were performed in duplicate, so no statistical analysis was performed. C: Effect of 100 nM isoproterenol (Iso) on lipolysis in primary human adipocytes. *** $P < 0.001$, t -test. D: Dose-response curves of niacin, BMS309403, and Compound 2 on isoproterenol (Iso)-stimulated lipolysis in primary human adipocytes.

The lack of an improvement in insulin resistance in DIO mice with either BMS309403 or Compound 3 was unexpected, because BMS309403 had been reported to have an impressive improvement on GTT in both *ob/ob* and DIO mice (11). We do not know the reason behind the discrepancy. The differences between the previous study and our study included doses (40 mg/kg vs. 30 mg/kg), routes of drug administration (oral gavage vs. diet administration), treatment lengths (4 weeks vs. 8 weeks), and ages of mice upon drug treatment (20 weeks vs. 12 weeks). In further attempts to try to reproduce the published data, we conducted three other experiments, dosing BMS309403 at 30–40 mg/kg by either in-diet administration or by oral gavage in either 45% HFD- or 60% HFD-fed mice for 4–6 weeks. In none of these studies were we able to observe an improvement on GTT or ITT with BMS309403. Chemical inhibition of FABP4/5, therefore, does not appear to improve insulin sensitivity as much as seen in the *Fabp4* and *Fabp5* double-knockout mice.

Several factors could account for the inability to improve glucose homeostasis by chemical inhibition of FABP4/5. First, it is possible that a near 100% inhibition of FABP4/5 would be required to elicit an improvement in glucose homeostasis. In support of this speculation, a

recent report showed that >70% germline knockdown of FABP4 by RNA interference in mice did not improve insulin sensitivity in diet-induced obese mice (31). Second, the FABP4 inhibitor has only been reported to improve insulin sensitivity in a single publication, and thus it is not automatically a fully proven concept. It is possible that the primary outcome of pharmacological inhibition of FABP4/5, which is probably less than 100%, lies on lipid rather than glucose metabolism. In humans with a genetic polymorphism in the *FABP4* locus, which reduces but does not abolish FABP4 expression, the most-significant phenotype was a reduction of plasma triglyceride with no change in hemoglobin A1c (8). Consistent with the human genetics data, in our study, BMS309403 and Compound 3 improved lipid parameters but not insulin resistance in DIO mice, suggesting a dichotomous effect on these two parameters. The insulin-sensitizing effect seen in *Fabp4* and *Fabp5* double-knockout mice may be secondary to improved lipid metabolism, or secondary to the reduced body weights of the double-knockout mice (7). Third, the compounds may not be potent enough to elicit a direct effect on insulin sensitivity. Given that FABP4 and -5 are highly abundant proteins in the cytoplasm, the bar to achieving a sufficient degree of pharmaceutical inhibition

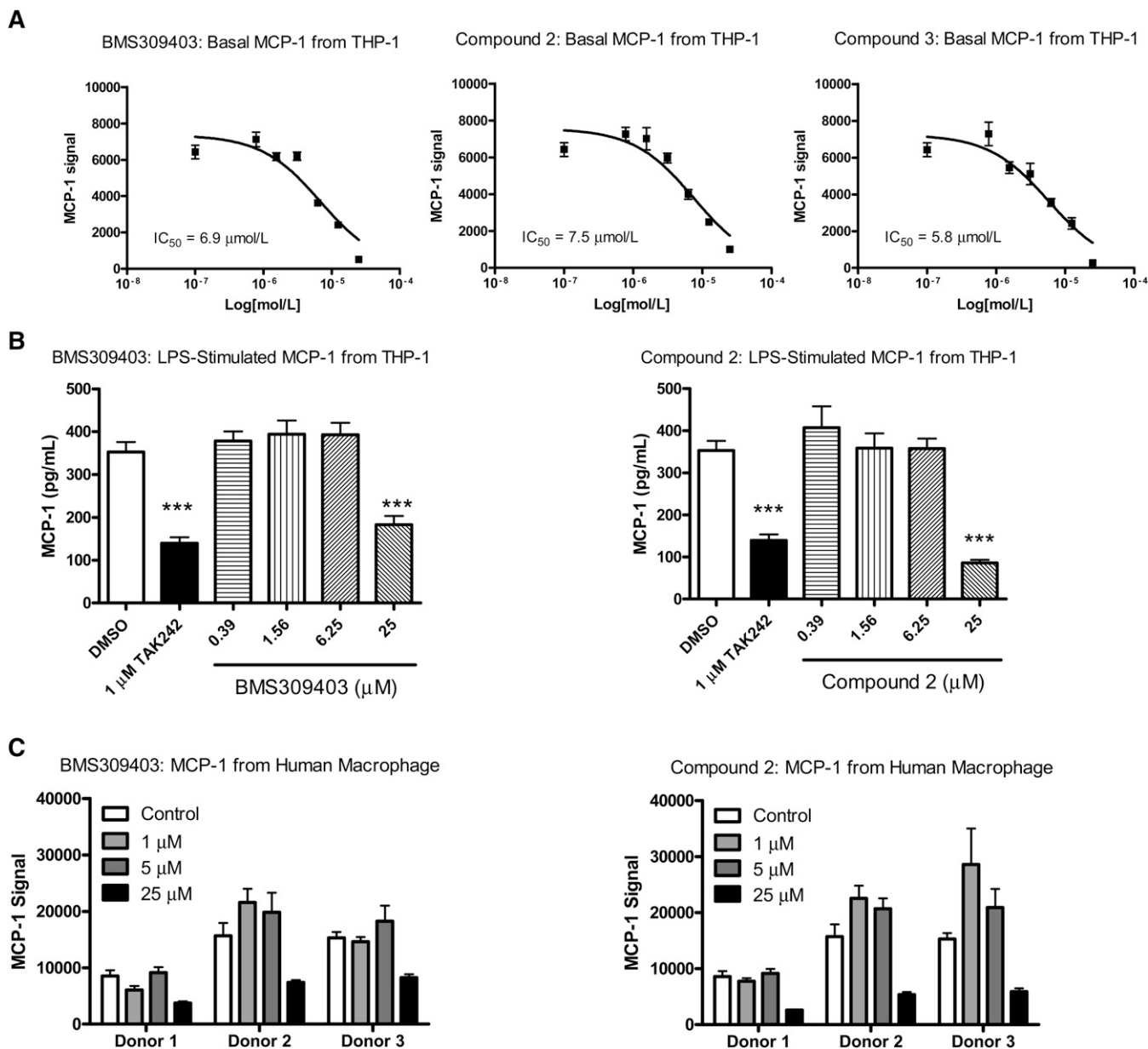


Fig. 4. A: Dose-response of BMS309403, Compound 2 and Compound 3 on basal MCP-1 release from PMA-differentiated THP-1 macrophages. The IC_{50} values were calculated using Sigmoidal dose-response formula implemented in Prism 5. B: Effect of FABP4/5 compounds on LPS-stimulated MCP-1 release from THP-1 macrophage. The TLR-4 inhibitor TAK242 was used as a positive control. *** $P < 0.001$, one-way ANOVA followed by Dunnett's posthoc tests. C: Effect of FABP4/5 compounds on MCP-1 release from primary human macrophage. The error bars represent technical replicates of a single biological sample, so no statistical analysis was performed.

is high. Although BMS309403 and Compound 3 are among the most potent FABP4 inhibitors in vitro (Table 1), drug concentrations engaging the drug target may still be too low in vivo. For example, although the blood concentration of Compound 3 reached $\sim 17 \mu\text{M}$ after ≥ 3 days of in-diet dosing, the fat/plasma distribution ratio for this compound is only $\sim 10\%$, and the free fraction in plasma is $< 1\%$. Consequently, the "free" drug concentration surrounding an adipocyte could be as low as 10 nM. The drug molecules still need to cross the plasma membrane to engage the target protein, and yet additional PK factors could affect drug efficacy in vivo. It is thus plausible that more-potent compounds would be required to elicit an insulin-

sensitizing effect. Finally, compared with the leptin-deficient *ob/ob* mice or the leptin receptor-deficient *db/db* mice, mice with DIO do not develop the same degree of obesity or severity of insulin resistance and hyperglycemia. Thus, more-potent compounds may be required to manifest an effect. Indeed, in a separate study, we were able to observe a minor improvement in ITT in *ob/ob* mice using BMS309403 and Compound 2, dosed at 30 mg/kg in-diet for 8 weeks. However, a decrease in body weight was observed for BMS309403 (but not Compound 2) in this study, which may confound the interpretation of the insulin-sensitizing effect elicited by BMS309403. Taken together, it remains unproven that chemical inhibition of FABP4/5 can

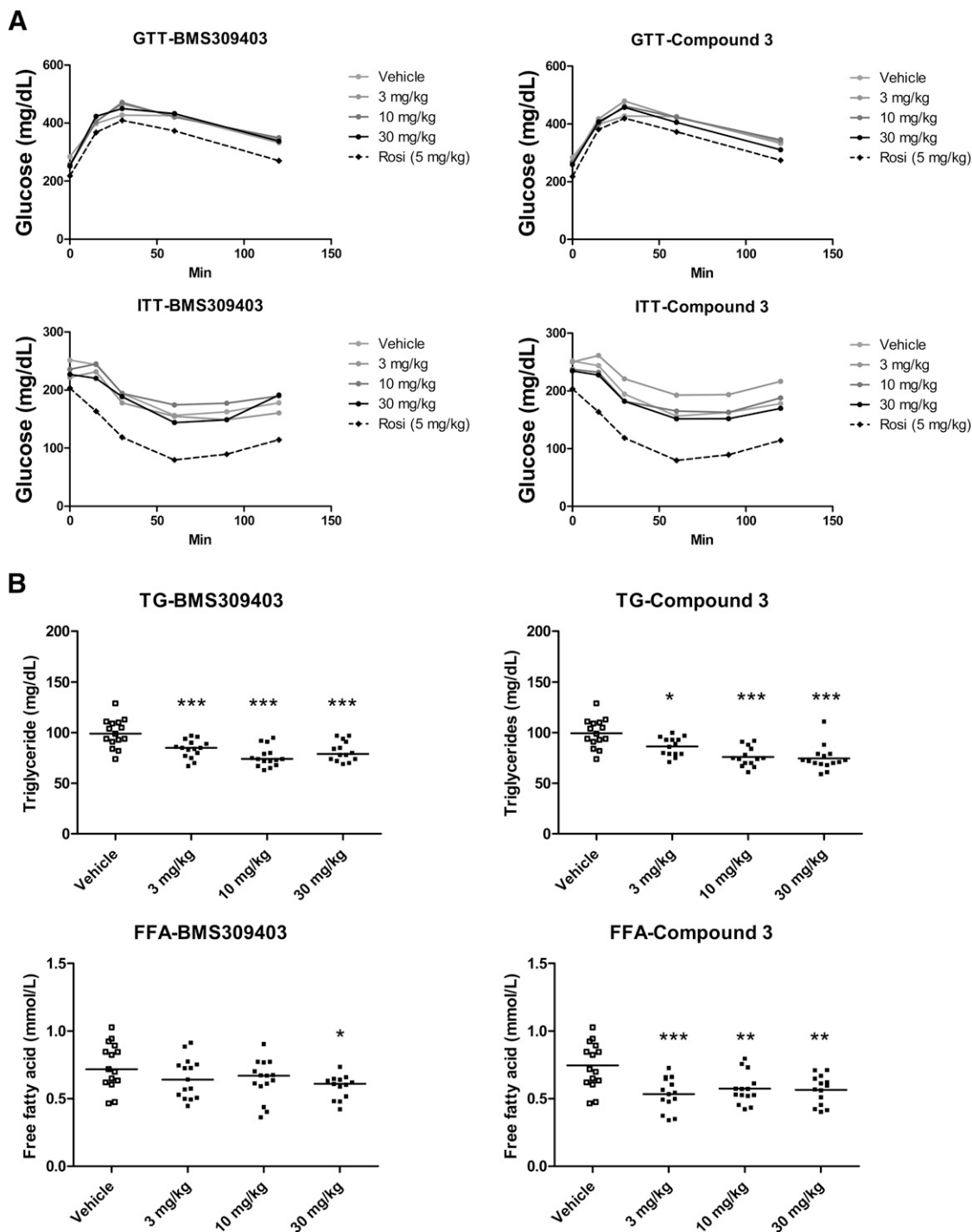



Fig. 5. A: Effect of BMS309403 and Compound 3 in GTT (upper panel) and ITT (lower panel) in DIO mice. Rosiglitazone (rosi) at 5 mg/kg was used as a positive control. B: Effect of BMS309403 and Compound 3 on plasma triglyceride (TG; upper panel) and free fatty acid (FFA; lower panel) levels in mice with DIO.

directly improve insulin resistance, especially in mice with DIO.

A notable challenge in developing the FABP4/5 inhibitors is the lack of straightforward, target-engaged, short-term efficacy readouts to guide the medicinal chemistry efforts. Although the genetics of FABP4/5 deletion have been demonstrated in numerous publications, "little is known about their exact biological functions and mechanisms of action" (1; p. 489). The biochemical signaling

pathway linking FABP4 inhibition to metabolic benefit is not well defined and is still a subject of ongoing research (32, 33). In this study, we used inhibition of lipolysis in adipocytes and inhibition of cytokine release from macrophages as cell-based assays to guide medicinal chemistry efforts. It should be noted that these assays are not necessarily FABP4/5 specific, although FABP4 inhibitors have been shown to inhibit lipolysis in adipocytes (17) and cytokine release from macrophages in an FABP4-depend-

dent manner (11). Caution should thus be exercised when attributing the cell-based assay data to FABP4/5 inhibition. Our compounds did not activate PPAR γ , so we do not think that the in vivo efficacy observed in this study was mediated by PPAR γ . Nonetheless, further study is needed to establish target-specific cell-based assays and short-term in vivo models to guide SARs.

In summary, we discovered a novel series of FABP4/5 inhibitors that improved dyslipidemia in mouse models of DIO. The compounds are among the most-potent and well-characterized FABP4/5 inhibitors reported in the literature. These compounds add to the increasing repositories of FABP4 inhibitors as tools with which to study FABP4/5 biology as well as prototypes for further drug development. 

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