Fatty Acid-binding Proteins Transport N-Acylethanolamines to Nuclear Receptors and Are Targets of Endocannabinoid Transport Inhibitors*

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Background: Transport inhibitors modulate endocannabinoid signaling by inhibiting their uptake through unknown mechanisms.

Results: Effects of transport inhibitors upon endocannabinoid uptake and intracellular trafficking were lost in the absence of fatty acid-binding proteins.

Conclusion: Fatty acid-binding proteins are physiological targets of transport inhibitors.

Significance: These findings identify drug targets for modulating endocannabinoid signaling.

N-Acylethanolamines (NAEs) are bioactive lipids that engage diverse receptor systems. Recently, we identified fatty acid-binding proteins (FABPs) as intracellular NAE carriers. Here, we provide two new functions for FABPs in NAE signaling. We demonstrate that FABPs mediate the nuclear translocation of the NAE oleoylethanolamide, an agonist of nuclear peroxisome proliferator-activated receptor α (PPARα). Antagonism of FABP function through chemical inhibition, dominant-negative approaches, or shRNA-mediated knockdown reduced PPARα activation, confirming a requisite role for FABPs in this process. In addition, we show that NAE analogs, traditionally employed as inhibitors of the putative endocannabinoid transmembrane transporter, target FABPs. Support for the existence of the putative membrane transporter stems primarily from pharmacological inhibition of endocannabinoid uptake by such transport inhibitors, which are widely employed in endocannabinoid research despite lacking a known cellular target(s). Our approach adapted FABP-mediated PPARα signaling and employed in vitro binding, arachidonoyl-[1-14C]ethanolamide ([14C]AEA) uptake, and FABP knockdown to demonstrate that transport inhibitors exert their effects through inhibition of FABPs, thereby providing a molecular rationale for the underlying physiological effects of these compounds. Identification of FABPs as targets of transport inhibitors undermines the central pharmacological support for the existence of an endocannabinoid transmembrane transporter.

N-Acylethanolamines (NAE)2 are members of a functionally diverse family of signaling lipids. While the endocannabinoid AEA activates plasma membrane-localized cannabinoid receptors, OEA and palmitoylethanolamide signal through nuclear peroxisome proliferator-activated receptor α (PPARα) receptors (1–3). Activation of cannabinoid and PPARα receptors produces anti-hyperalgesic, anti-inflammatory, and anorexigenic phenotypes in rodents (2, 3). NAEs are catabolized primarily by FAAH, an intracellular enzyme (4). Pharmacological or genetic ablation of FAAH elevates NAE levels and potentiates NAE-mediated hypoalgesic and anti-inflammatory effects, indicating that modulation of NAE signaling may represent a therapeutic avenue for the treatment of pain disorders (5).

In contrast to NAE signaling and catabolism, intracellular and transmembrane NAE transport remain poorly characterized. Our group recently identified fatty acid-binding proteins (FABP) as intracellular carriers that deliver AEA from the plasma membrane to intracellular FAAH for hydrolysis (6). Chemical inhibition of FABPs reduced AEA uptake and catabolism by ~50%, confirming their importance in mediating intracellular endocannabinoid transport (6). Efficient activation of PPARα receptors by NAEs implicates intracellular trafficking proteins in their delivery to nuclear receptors. However, it is currently not known whether FABPs function in such a capacity. In contrast to intracellular trafficking, endocannabinoid membrane transport remains poorly characterized. Although endocannabinoid internalization was originally attributed to facilitated transport, evidence in support of simple diffusion continues to mount (7–11). To date, a putative endocannabinoid membrane transporter has not been molecularly identified. The strongest, and indirect, evidence to support its existence stems from pharmacological inhibition of endocannabinoid uptake by transport inhibitors structurally related to NAEs (12–15). Although these compounds lack a known cellular target, they continue to be widely employed as selective inhibitors of the putative transporter in numerous experimental settings, ranging from studies of cellular NAE uptake to in vivo physiology (for example, see Refs. 16–20). Elucidation of a target(s) for these compounds would provide mechanistic

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2 The abbreviations used are: NAE, N-acylethanolamines; FABP, fatty acid-binding proteins; PPARα, peroxisome proliferator-activated receptor α; AEA, arachidonoyl ethanolamide; NES, nuclear export signal; FAAH, fatty acid amide hydrolase; OEA, oleoylethanolamide.

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insights into their underlying effects and reduce experimental ambiguities associated with their use.

In the present study, we identify FABPs as the major cellular targets of endocannabinoid transport inhibitors. Furthermore, we show that FABPs mediate activation of nuclear PPARα receptors by OEA, thereby ascribing novel functions to FABPs in endocannabinoid and NAE biology.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—OEA, GW7647 (2-methyl-2-[4-[2-[[[(cyclohexylamino)carbonyl][4-(cyclohexylbutyl)amino]ethyl]phenyl]-thio]-propanoic acid), AEA, arachidonic acid, OMDM1 ((S)-N-octyl-tyrosinol), OMDM2 ((R)-N-octyl-tyrosinol), VDM11 (N-(4-hydroxy-2-methylphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), AM1172 (N-(5Z,8Z,11Z,14Z-eicosatetraenyl-4-hydroxy-benzamide), and AM404 (N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) were from Cayman Chemical (Ann Arbor, MI). 12-NBD stearate (12-NBD oleoyl-tyrosinol), OMDM2 ([(E)-5-[(4-hydroxyphenyl)oxy]-acetic acid) was from Sigma-Aldrich. 53 mCi/mmol)-[14C]AEA (arachidonoyl-[1-14C]ethanolamide) was from PerkinElmer Life Sciences, whereas [14C]Arachidonic acid (1-14C-arachidonic acid, 370 kBq) was from PerkinElmer Life Sciences, whereas [14C]Arachidonic acid, 370 kBq) was from PerkinElmer Life Sciences, whereas [14C]ARA (arachidonoyl-1-[1-14C]ethanolamide, 53 mCi/mmol) was kindly provided by the Drug Supply Program at the National Institute on Drug Abuse.

**Cloning of FABPs**—Mouse FABP3, FABP5, and FABP7 were amplified and subcloned into the eGFP-N1 plasmid (Clontech), generating the corresponding GFP-tagged proteins. Cytoplastically localized FABPs were produced by inserting the NES (residues MASLAAEFRHLQLKEA) from human STAT1 (kindly provided by Nancy Reich, Stony Brook University) into the eGFP-N1 plasmid (Clontech). The supernatants were amplified and subcloned into the eGFP-N1 plasmid (Clontech), generating the corresponding GFP-tagged proteins.

**Cell Culture and Stable Knockdown of FABP5**—HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin/streptomycin, 2 mM 1-glutamine, and 1 mM sodium pyruvate in a humidified incubator containing 95% air and 5% CO2. Transfections were carried out using the GenJet Plus transfection reagent (SignaGen, Rockville, MD) according to the manufacturer's instructions. To knockdown FABP5, shRNAs corresponding to human FABP5 (Open Biosystems, Huntsville, AL) were transfected into HeLa cells. shRNA clone V3LHS_402771 (5'-AGGATGATCATC-CCTTTTGTTA-3') produced the greatest knockdown and was used for all studies. A non-silencing control (Open Biosystems) was transfected into control HeLa cells. RNA interference was confirmed by flow cytometry at the Flow Cytometry Core Facility at Stony Brook University and further expanded.

**PPARα Transactivation**—Cells were co-transfected with plasmids encoding the PPARα ligand binding domain fused to the GAL4 DNA binding domain, 4× upstream activation sequence-luciferase, and β-galactosidase (kindly provided by Jorge Plutzky, Harvard Medical School) as a transfection efficiency control. Twenty-four hours later, cells were incubated for 6 h with PPARα agonists in the presence or absence of inhibitors in DMEM. The cells were subsequently lysed and processed according to the Dual-Light Luciferase and β-Galactosidase Reporter Gene Assay System (Applied Biosystems, Carlsbad, CA). PPARα activation and β-galactosidase activity were quantified using an Optocomp II automated luminometer (MGM Instruments, Hamden, CT). Background luminescence from non-transfected cells was subtracted from all samples. The luciferase signals were normalized to β-galactosidase activity. The degree of PPARα activation was determined by normalizing the luciferase/β-galactosidase ratios in cells incubated with ligands to those obtained in cells lacking ligands.

**Purification of FABPs**—Human FABP3 and FABP7 were inserted into the pET28a (EMD Chemicals, Gibbstown, NJ) according to the manufacturer’s instructions. To knockdown FABP5, shRNAs corresponding to human FABP7 were inserted into the pET28a (EMD Chemicals, Gibbstown, NJ) producing the corresponding GFP-tagged proteins. All proteins were expressed in BL21(DE3) Escherichia coli cells using the T7 expression system (Invitrogen). Cells were grown until A500 reached 0.6–1.0 and were induced by addition of 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. After a 20-h incubation at 15°C, the cells were pelleted by centrifugation at 5,000 × g at 4°C and resuspended in 3 volumes of ice-cold buffer A (1× PBS, 150 mM NaCl, pH 8.5). The cells were lysed by sonication on ice, followed by a 30-min centrifugation at 15,000 × g at 4°C. FABP3 and FABP7 were purified using the IMPACT purification system (New England Biolabs, Ipswich, MA). The supernatants were loaded onto chitin columns (New England Biolabs). The columns were washed with buffer B (20 mM Tris-HCl, 150 mM NaCl, pH 7.0) and on-column self-cleavage was performed by incubating the columns with buffer C (20 mM Tris, 250 mM NaCl, 50 mM dithiothreitol, pH 7.0) for 20 h at 4°C, resulting in the release of untagged FABPs. FABP5 was purified by loading onto nickel-nitrilotriacetic acid columns (Qiagen, Valencia, CA). After mixing the supernatant with the nickel-nitrilotriacetic acid-agarose for 10 min at 4°C, the samples were loaded on columns, washed, and eluted with buffer B containing 250 mM imidazole. Eluted FABPs were pooled, concentrated, and loaded onto an XK 16/70 Sepharac S-100 column (GE Healthcare Life Sciences) that had been equilibrated with buffer A. The proteins were purified using the AKTAPrime plus system (GE Healthcare Life Sciences) with the flow rate set to 0.2 ml/min. FABP-containing fractions were collected and Coomassie staining confirmed >90% purity. FABPs were subsequently delipidated by incubation with Lipidex-5000 (Sigma) for 1 h at 37°C with occasional mixing. FABPs were eluted with buffer A and stored at −80°C until use.

**Binding of Ligands to FABPs**—Purified FABPs (3 μM) were incubated with 0.5 mM NBD-stearate in 30 mM Tris-HCl, 100 mM NaCl buffer (pH 7.4) in the presence or absence of competitors. Increasing concentrations of competitors (0.01–20 μM) were added to the tubes and the loss of fluorescence intensity was measured with a JASCO FP-6200 spectrofluorometer with respective excitation and emission wavelengths of 466 and 520–560 nm. Slit widths were set to 10 and 5 nm for the excitation and emission monochromators, respectively. Fluores-
cence in tubes lacking FABPs was subtracted from all samples. The EC\textsubscript{50} values for all compounds were plotted using GraphPad Prism. The K\textsubscript{d} of each ligand was determined using the following equation: $K_d = EC_{50}/1 + ([\text{NBD-stearate}]/K_d)$. The K\textsubscript{d} of NBD-stearate for FABP3, FABP5, and FABP7 were determined by incubating the FABPs with increasing concentrations of NBD-stearate. The K\textsubscript{d} values were obtained from the resulting saturating curves using one site binding analyses in GraphPad Prism. The K\textsubscript{d} of NBD-stearate for FABP3, FABP5, and FABP7 was 0.18, 0.16, and 0.22 μM, respectively.

**Immunolocalization of Proteins**—HeLa cells were fixed and mounted onto slides as previously described (6). For experiments examining endogenous FABP5 expression, Triton X-100-permeabilized cells were incubated with rabbit anti-FABP5 (1:500) (BioVendor R&D, Candler, NC) followed by donkey anti-rabbit 594 (1:800) (Molecular Probes) antibodies. The images were acquired using a Zeiss LSM 510 META NLO Two-Photon Laser Scanning Microscope.

**Western Blotting**—Western blot experiments were performed exactly as previously described (6). Blots were probed with rabbit anti-GFP (1:2000) (Molecular Probes), mouse anti-β-actin (1:20000) (Abcam, Cambridge, MA), or rabbit anti-β-actin (1:2000) (BioVendor R&D, Candler, NC) followed by donkey anti-rabbit 594 (1:800) (Molecular Probes) antibodies. Western blot experiments were performed with rabbit anti-GFP (1:2000) (Molecular Probes), mouse anti-β-actin (1:20000) (Abcam, Cambridge, MA), or rabbit anti-β-actin (1:2000) (BioVendor R&D, Candler, NC) followed by donkey anti-rabbit 594 (1:800) (Molecular Probes) antibodies (Molecular Probes) and developed using the Immun-star HRP substrate (Bio-Rad) and exposed to film.

**FAAH Enzyme Assays**—FAAH activity assays were performed as previously described (6). Briefly, cell homogenates were incubated with 100 μM AEA + 0.1 μCi of [3\textsuperscript{14}C]AEA in Tris-HCl (pH 9) containing 0.1% BSA. Reactions were stopped by addition of 2 volumes of 1:1 chloroform:methanol and the phases were separated by centrifugation. The methanol phase was quantified using a Beckman LS 6500 scintillation counter.

**Cellular Uptake of AEA**—The uptake experiments were performed exactly as described (6). For transport inhibitor studies, HeLa cells were preincubated with vehicle or inhibitors for 15 min and subsequently incubated for 5 min with 100 nM [3\textsuperscript{14}C]AEA in the presence or absence of inhibitors. AEA uptake was quantified as described (6).

**Uptake of Arachidonic Acid**—Vector- or FABP5 shRNA-expressing HeLa cells were incubated for 5 min with 100 nM [3\textsuperscript{14}C]arachidonic acid in the presence of vehicle (0.5% ethanol) or 10 μM VDM11 in media containing 0.15% BSA. The reactions were stopped by addition of ice-cold DMEM + 0.15% BSA. The cells were rapidly placed on ice and washed with DMEM + 0.15% BSA. The cells were subsequently scraped with PBS containing 2 mM EDTA, extracted with 2 volumes of chloroform:methanol (1:1), and the phases were separated by centrifugation. The chloroform phase containing [3\textsuperscript{14}C]arachidonic acid was subjected to liquid scintillation counting. Arachidonic acid uptake at 4°C was subtracted from all samples.

**Reverse Transcription-Polymerase Chain Reaction Analysis of FABP Expression**—Total RNA was extracted from HeLa cells using the RNeasy mini kit (Qiagen). cDNA synthesis was carried out using the Superscript III first strand synthesis kit (Invitrogen). The cDNAs were subjected to polymerase chain reaction using primers specific for FABPs or β-actin. The following primers were used: FABP1, 5′-ATCGTGCAATGGGAAACCAACTATGATTGTTGTA-3′ (forward) and 5′-CAATGCTATGATTTGTGTA-3′ (reverse); FABP2, 5′-TGCAATTACACAAAGAGGA-3′ (forward) and 5′-TATACATTGCTGTGCATTG-3′ (reverse); FABP3, 5′-CAACATACGAGAAGATG-3′ (forward) and 5′-GTGGTGGATGTGACTAG-3′ (reverse); FABP4, 5′-CAAGGCAGCAGTTGTGTA-3′ (forward) and 5′-CATGAGCAGCTTCACCACCA-3′ (reverse); FABP5, 5′-AATGGCCAAGCCAGATTGTA-3′ (forward) and 5′-CATGACACACTCCACCAC-3′ (reverse); FABP6, 5′-GCCGCCGAAAACTCAAGATCGT-3′ (forward) and 5′-TGCTGGAGACCTCCACCAGC-3′ (reverse); FABP7, 5′-GAGATGTCATCAGCAAGAA-3′ (forward) and 5′-CACATTTCATCCGCTACAC-3′ (reverse); FABP9, 5′-ATGAAAGAAGTTGGAGTAAAAGATGTTGTA-3′ (forward) and 5′-TCTTGTGATTTGTTGTCCT-3′ (reverse); FABP12, 5′-GTGACCATCTGACAGTGTTG-3′ (forward) and 5′-CACAGCCTTCCACCACCC-3′ (reverse) and 5′-CACAGTACTTTCCACCAGCA-3′ (reverse); and β-actin, 5′-AGATGACCAGCAGTATGTTGTA-3′ (forward) and 5′-CACAGCCTTCCACCACCA-3′ (reverse). The cycling conditions were as follows: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s for a total of 35 cycles. The products were visualized on an agarose gel.

**Quantitative Real-time Polymerase Chain Reaction**—cDNAs were generated from control and shRNA-expressing HeLa cells using the StepOne Plus Real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA) as described previously (21). The following primers were used: human FABP5, 5′-TCACAGGCAATGGGAAACCAACTATGATTGTTGTA-3′ (forward) and 5′-CTGTCCCAAGGTCTGTTGTA-3′ (reverse) and 5′-CATGAGCAGCTTCACCACCA-3′ (reverse). The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 60 s. Amplification levels were determined by using the ΔΔC\textsubscript{T} method with β-actin serving as the housekeeping control as described (21).

**Statistical Analyses**—Results are expressed as mean ± S.E. of at least three independent experiments performed in triplicate. Statistical significance was evaluated using two-tailed unpaired t tests against controls.

**RESULTS**

**FABP Mediate Nuclear Translocation and PPARα Activation by NAESs**—FABPs are small intracellular proteins that distribute between the cytoplasm and nucleus (22, 23). This localization pattern makes FABPs ideally suited for ligand delivery to nuclear receptors, including PPARs (22, 23). Similar to fatty acids and their metabolites, NAEs activate nuclear PPARα receptors (2, 3), prompting us to speculate that FABPs may shuttle NAEs into the nucleus. We employed HeLa cells because they lack FAAH (24, 25) and have been previously employed to examine PPARα signaling by NAEs (2, 3). Furthermore, HeLa cells express one predominant FABP subtype, FABP5 (Fig. 1A), which potentiates AEA uptake into cells (6).
PPARα activation was monitored by transfecting cells with a PPARα reporter system (see “Experimental Procedures” and Ref. 26). The synthetic PPARα agonist GW7647 and OEA both activated PPARα receptors, with GW7647 being more efficacious (Fig. 1B). Although OEA activated PPARα with a half-maximal concentration (EC₅₀) of ~0.7 μM (Fig. 1C), we employed 10 μM OEA in all subsequent experiments to produce robust receptor activation for the inhibition studies. Treatment of cells with the FABP inhibitor BMS309403 (6, 27) reduced robust receptor activation for the inhibition studies. Treatment with the FABP inhibitor BMS309403 (6, 27) reduced receptor activation by 50 nM GW7647 or 10 μM OEA by 55–60% (Fig. 1D), confirming FABP involvement in OEA trafficking to PPARα receptors. Overexpression of FABP3, FABP5, or FABP7, which interact with OEA with varying affinities (Table 1), did not enhance PPARα signaling by OEA (Fig. 1D), indicating that endogenous FABP5 is present in sufficient levels to permit maximal PPARα activation.

To validate FABP-NAE interactions in intact cells, we constructed a dominant-negative FABP by fusing FABP5 to a nuclear export signal (NES), thereby restricting FABP5 expression to the cytoplasm (Fig. 2A). By sequestering ligands in the cytoplasm, this fusion protein is expected to reduce PPARα activation. Indeed, PPARα signaling by GW7647 and OEA was significantly reduced in cells expressing NES-FABP5 (Fig. 2B), confirming that FABP5 is capable of binding synthetic ligands and endogenous NAEs in cells. shRNA-mediated knockdown was employed to further substantiate the role of FABPs in nuclear ligand delivery. HeLa cells were stably transfected with shRNAs targeting FABP5, resulting in a >85% knockdown at the mRNA and protein levels (Fig. 2C–E). Immunofluorescence analysis of endogenously expressed FABP5 in vector- and shRNA-transfected cells further confirmed the knockdown (Fig. 2F). Compared with control cells, PPARα activation by GW7647 and OEA was reduced by ~45–60% in shRNA-expressing cells (Fig. 2G), confirming FABP involvement in OEA trafficking to PPARα receptors. PPARα signaling was restored to control levels upon overexpression of mouse FABP5 in shRNA-expressing cells, confirming the specificity of the knockdown (Fig. 2H and I). Similar effects were observed following transfection of FABP3 or FABP7 (Fig. 2F), indicating that full PPARα activity can be restored by reintroduction of other FABPs into cells. Collectively, our results establish FABPs as shuttles that mediate NAE delivery to PPARα receptors.

**FABPs Are Targets of Endocannabinoid Transport Inhibitors**—The structural similarities between endocannabinoid transport inhibitors and NAEs (Fig. 3) prompted us to speculate that FABPs may serve as cellular targets for these compounds. We employed three approaches to confirm this hypothesis: 1) in vitro binding of transport inhibitors to FABPs, 2) inhibition of FABP-mediated PPARα signaling by transport inhibitors, and 3) inhibition of AEA uptake by these compounds.

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**TABLE 1**

| Compound | FABP5 | FABP3 | FABP7 |
|----------|-------|-------|-------|
| GW7647   | 0.70 ± 0.23 | ND | ND |
| BMS309403 | 0.89 ± 0.31 | ND | ND |
| Arachidonic acid | 0.12 ± 0.01 | 0.03 ± 0.01 | 0.09 ± 0.01 |
| AEA      | 1.26 ± 0.18 | 3.07 ± 0.28 | 0.80 ± 0.06 |
| OEA      | 2.22 ± 0.34 | 4.69 ± 0.43 | 0.35 ± 0.04 |
| OMDM1    | 2.67 ± 0.18 | ND | ND |
| OMDM2    | 3.85 ± 0.25 | ND | ND |
| VDM11    | 1.75 ± 0.21 | ND | ND |
| AM1172   | 0.49 ± 0.09 | ND | ND |
| AM404    | 0.39 ± 0.08 | ND | ND |

* ND, not determined.
The prototypical transport inhibitors, OMDM1, OMDM2, VDM11, AM1172, and AM404 (12, 14, 15, 28), which reduce AEA uptake across multiple cell types, were employed. These compounds interacted with FABP5 in vitro with affinities ranging from ~0.4 to ~4 μM (Table 1). In general, inhibitors containing an arachidonoyl chain (VDM11, AM1172, and AM404) bound to FABP5 with greater affinities than those possessing an oleoyl chain (OMDM1 and OMDM2). A similar trend was observed for AEA and OEA, with AEA binding to FABP5 with ~2-fold higher affinity than OEA (Table 1). However, all of these compounds bound to FABP5 with lower affinities compared with arachidonic acid, which displayed a $K_i$ of 0.12 μM.

The comparable affinities of transport inhibitors and NAEs for FABP5 indicate that these compounds may target FABPs in cells. We employed PPARα signaling, whose dependence upon FABPs (see above) makes it ideally suited to examine the effects of FABP knockdown on PPARα activation. We observed a reduction in FABP5 mRNA and protein levels following shRNA-mediated knockdown of FABP5, as assessed by quantitative real-time PCR and Western blot analysis. PPARα activation by GW7647 or OEA was attenuated in FABP5 knockdown cells, consistent with the proposed role of FABP5 in modulating PPARα signaling. These findings suggest that FABP5 may play a direct role in regulating PPARα activation, potentially through the sequestration or transport of endocannabinoids in cells.
Rates of AEA hydrolysis were similar in control- and FABP5 down in HeLa cells reduced AEA uptake by previous study employing FABP inhibitors (6), FABP5 knock- AEA uptake and hydrolysis (24, 29). In agreement with our FABP5 shRNA-expressing cells with FAAH, whose activity mechanism for endocannabinoid uptake that is targeted by upon AEA uptake. HeLa cells were employed as they possess a of transport inhibitors. We validated this approach by demon- strating that BMS309403 fails to reduce GW7647-mediated PPARα activation in cells expressing NES-FABP5 or upon FABP5 knockdown (Fig. 4, A and B). The inability of BMS309403 to further inhibit PPARα signaling in NES-FABP5 cells may stem from residual FABP-independent (and BMS309403-insensitive) nuclear import of GW7647 upon FABP inhibition by BMS309403. Following FABP5 knockdown, PPARα signaling was likewise refractory to inhibition by BMS309403, confirming the selectivity of this compound for FABPs. The inhibitory effects of the transport inhibitors, OMDM1, OMDM2, VDM11, AM1172, and AM404 were subsequently examined in vector-, NES-FABP5-, and FABP5 shRNA-expressing HeLa cells. All of the inhibitors reduced PPARα activation in control cells by ~40–60% (Fig. 4C). OMDM1 and OMDM2 were the least potent inhibitors, whereas VDM11 and AM1172 were the most efficacious. The inhibitory effects of all inhibitors were attenuated in cells expressing NES-FABP5 (Fig. 4D) and upon FABP5 knockdown (Fig. 4E), confirming that FABP5 is a major cellular target of endocannabinoid transport inhibitors. We subsequently examined the effects of transport inhibitors upon AEA uptake. HeLa cells were employed as they possess a mechanism for endocannabinoid uptake that is targeted by transport inhibitors (24, 25). We confirmed that FABPs mediate AEA uptake in HeLa cells by transfecting control- and FABP5 shRNA-expressing cells with FAAH, whose activity maintains an inward concentration gradient that promotes AEA uptake and hydrolysis (24, 29). In agreement with our previous study employing FABP inhibitors (6), FABP5 knockdown in HeLa cells reduced AEA uptake by ~50% (Fig. 5A). Rates of AEA hydrolysis were similar in control- and FABP5 shRNA-expressing cell homogenates (Fig. 5B), ruling out the possibility that differential FAAH activity may account for reduced AEA uptake in FABP5 knockdown cells. To gain mechanistic insights into AEA uptake inhibition by transport inhibitors, we examined AEA uptake in control- and FABP5 shRNA-expressing cells. As expected, AEA uptake was reduced by ~25–50% in FAAH-transfected HeLa cells incubated with OMDM1, OMDM2, VDM11, AM1172, or AM404 (Fig. 5C). VDM11 was the most potent inhibitor, whereas OMDM2 was the least efficacious. It is important to note that VDM11 and AM404 are FAAH substrates and can inhibit its activity, thereby collapsing the AEA gradient maintained by FAAH and reducing AEA uptake independently of FABPs (7, 30). To elucidate whether transport inhibitors reduced AEA uptake by targeting FAAH, we compared the proportion of cellular AEA that was hydrolyzed following uptake in the presence or absence of transport inhibitors. Intracellular AEA hydrolysis remained unchanged in the presence of transport inhibitors (Fig. 5D), indicating that these compounds target a carrier upstream of FAAH, such as FABP5. To confirm this, we examined the effects of transport inhibitors upon AEA uptake following FABP5 knockdown. Strikingly, these compounds failed to reduce AEA uptake in FABP5 shRNA cells (Fig. 5E), in agreement with the PPARα approach above. Last, we sought to demonstrate that transport inhibitors block other processes mediated by FABPs, such as fatty acid transport. Indeed, incubation of HeLa cells with 10 μM VDM11 significantly inhibited arachidonic acid uptake (Fig. 5F). Similar to AEA, arachidonic acid transport was reduced by FABP5 knockdown and was insensitive to further inhibition by VDM11 (Fig. 5F). Collectively, these results confirm that FABPs, rather than a putative membrane transporter, are the main cellular targets of endocannabinoid transport inhibitors. DISCUSSION The hydrophobic properties of bioactive lipids necessitate the presence of intracellular carriers to facilitate ligand delivery to sites of signaling and inactivation. NAEs belong to a family of lipids that activate diverse receptor systems, including nuclear PPARα receptors (2, 3). Our current results indicate that FABPs are essential for efficient intranuclear NAE trafficking and engagement of PPARα receptors. FABPs also mediate endocan- bianoid hydrolysis by FAAH (6), indicating that they may carry out context-dependent functions within the endocannabinoid system. For example, the paracrine nature of NAE signaling (31) likely mandates a complementary cellular pattern of PPARα and FAAH expression, with FABPs undertaking signaling and meta- bolic trafficking functions in the respective cells. Subtype-specific interactions between FABPs and PPAR receptors have been recently reported. For example, FABP3 preferentially delivers ligands to PPARα, whereas FABP5 enhances activation of PPARβ/δ (22, 23). Selective coupling between FABPs and PPARs occurs under limiting ligand conditions (23) and may not be relevant for NAE signaling in vivo. In support of this, overexpression of FABP3 did not elevate PPARα activation by OEA and its overexpression in FABP5 knockdown cells restored PPARα signaling to wild-type levels. Therefore, the presence of FABPs capable of binding NAEs,
regardless of subtype, is sufficient for full PPARα activation. However, we cannot rule out the possibility that the lower affinity of OEA for FABP3 may limit PPARα potentiation. The current study expands the repertoire of ligands known to interact with FABPs. Prior reports have identified endogenous fatty acids and their derivatives, retinoic acid, and certain xenobiotics as ligands for FABPs (22, 32, 33). Our prior (6) and current study establish uncharged fatty acid amides as physiological ligands for FABPs. NAEs and structurally related transport inhibitors interact with FABPs with nanomolar to low micromolar affinities (Table 1). Such affinities are considerably lower than those of the more abundant fatty acids (34, 35), indicating that NAEs may not be the preferred endogenous ligands for FABPs. However, cellular expression of FABPs is robust enough (see Fig. 2F) to permit physiological interactions with moderate affinity lipids, including NAEs. NAEs displayed the highest affinities for FABP7 and lowest for FABP3 (Table 1), supporting our recent finding that overexpression of FABP7 and FABP5, but not FABP3, potentiated AEA uptake in cells (6). Accordingly, because of its short time scale, endocannabinoid uptake may favor higher affinity carriers such as FABP5 and FABP7. In contrast, in the current manuscript overexpression regardless of subtype, is sufficient for full PPARα activation. However, we cannot rule out the possibility that the lower affinity of OEA for FABP3 may limit PPARα potentiation.

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![Figure 4](image1.png)  
**Figure 4. FABPs are cellular targets of transport inhibitors.** A and B, effect of 20 μM BS309403 upon PPARα activation by 50 nM GW7647 in control HeLa cells and cells expressing (A) NES-FABP5 or (B) FABP5 shRNA. *, p < 0.05 and **, p < 0.01, ns indicates no significant difference (n = 3). C–E, effect of endocannabinoid transport inhibitors upon PPARα activation by 50 nM GW7647 in (C) control, (D) NES-FABP, or (E) FABP5 shRNA-expressing HeLa cells. OMDM1, OMDM2, VDM11, AM1172, and AM404 reduced PPARα signaling in control cells (black bars) but were without effect in NES-FABP5 and shRNA-expressing cells. *, p < 0.05 and **, p < 0.01 (n = 4).

![Figure 5](image2.png)  
**Figure 5. FABP5 is the main target of transport inhibitors in HeLa cells.** A, [14C]AEA uptake was reduced in FABP5 shRNA-expressing HeLa cells compared with controls. The cells were transfected with FAAH to permit analysis of AEA transport and hydrolysis as described under “Experimental Procedures.” **, p < 0.01 (n = 5). B, similar (p > 0.05) levels of [14C]AEA hydrolysis in homogenates of control and FABP5 shRNA-expressing HeLa cells transfected with FAAH (n = 3). C, treatment of HeLa cells expressing FAAH with 10 μM OMDM1, OMDM2, VDM11, AM1172, or AM404 for 5 min reduced [14C]AEA uptake, *, p < 0.05 and **, p < 0.01 (n = 3). D, transport inhibitors did not reduce the proportion of [14C]AEA hydrolysis relative to uptake in intact FAAH-expressing HeLa cells (n = 3). E, transport inhibitors failed to reduce [14C]AEA uptake in FAAH-transfected, FABP5 shRNA-expressing HeLa cells (n = 3). F, uptake of [14C]arachidonic acid was reduced in the presence of 10 μM VDM11 and in cells expressing FABP5 shRNA. [14C]Arachidonic acid uptake was similar in vehicle-treated and VDM11-treated FABP5 shRNA cells. *, p < 0.05, ns indicates no significant difference (n = 3).
of these FABPs failed to potentiate PPARα signaling, indicating that high affinity carriers are dispensable for efficient receptor activation, probably a consequence of the longer time scale of these experiments. In support of this model, introduction of these FABPs into FABP5 knockout cells restored PPARα activation to wild-type levels (Fig. 2f), confirming that lower affinity carriers are capable of delivering ligands to PPARs.

Our data also reveal unexpectedly strong binding of the synthetic transport inhibitor AM404 to FABP5. These results are pharmacologically relevant because the in vivo biosynthesis of AM404 from acetaminophen by FAAH was recently demonstrated (36, 37). Our data support a model for FABP-mediated trafficking of AM404 from its intracellular site of biosynthesis to plasma membrane-localized receptors (36). Compared with AM404, the closely related VDM11 displayed ~4-fold lower affinity for FABP5. This difference in affinity likely stems from unfavorable steric interactions between the additional methyl group in the phenyl ring of VDM11 (Fig. 3) and residues within the FABP binding pocket. Collectively, our data support the current model that FABPs possess promiscuous binding pockets that accommodate a broad range of lipophilic ligands.

Efficient intracellular endocannabinoid transport requires soluble protein carriers. We recently identified FABPs as intracellular chaperones for AEA (6), whereas Oddi et al. (38) ascribed a similar function to heat shock protein 70. Therefore, intracellular NAE trafficking may operate via an orchestrated network of carrier proteins. In contrast, endocannabinoid membrane transport remains a topic of significant controversy. Endocannabinoid membrane transport was hypothesized to occur via distinct mechanisms, including simple diffusion, facilitated diffusion, and endocytosis (39, 40). Because a putative endocannabinoid membrane transporter has not been cloned, the facilitated diffusion model is predicated upon its purported saturability, temperature dependence, and inhibition by structural analogs of NAEs (12). Saturation and temperature dependence analyses of endocannabinoid uptake suffer from inherent technical limitations (7, 8, 41–43) and cannot be applied to support the facilitated diffusion model. Therefore, the strongest indirect support for the existence of a membrane transporter stems from studies demonstrating inhibition of AEA uptake by transport inhibitors (12–15).

In light of recent reports demonstrating simple diffusion of AEA followed by FABP-mediated intracellular trafficking (6, 7, 9, 10), we hypothesized that transport inhibitors may reduce AEA uptake by targeting intracellular AEA carriers, thereby mimicking inhibition of a putative membrane transporter. In this study, we identified FABPs as the main cellular targets of transport inhibitors, confirming that the efficacy of these compounds stems from inhibition of proteins distinct from their intended target. Our study focused upon FABP5 as it is widely expressed across multiple cell types and mammalian tissues (6, 22, 44) and its inhibition likely underlies the efficacy of transport inhibitors in many cells. For example, AEA uptake is reduced by transport inhibitors in neurons and HaCaT keratinocytes, which express FABP5 (15, 22, 45–47). Although our study focused upon FABP5, it is noteworthy that FABP3 and FABP7 bind to NAEs with relatively similar affinities (Table 1), and a similar trend may exist for transport inhibitors. In such a case, these compounds may exert their effects by targeting multiple FABPs within a cell. During the revision of this manuscript, a report was published demonstrating that a catalytically silent variant of FAAH interacts with transport inhibitors (48). These findings expand the repertoire of known intracellular endocannabinoid carriers that are targeted by these compounds. Taken together, our results indicate that inhibition of endocannabinoid uptake by transport inhibitors cannot be used as criteria, nor does it serve as evidence for facilitated endocannabinoid transport. As such, there is currently no compelling evidence to support the existence of an endocannabinoid membrane transporter.

Our current study identifies FABPs as the primary targets of endocannabinoid transport inhibitors, providing a molecular rationale for the observed effects of these compounds in vivo that include regulation of nociception, inflammation, neuroprotection, and neuronal signaling (16, 18–20, 49–51). However, a subset of physiological responses modulated by these compounds are inconsistent with FABP (or by extension FAAH) inhibition (18–20). For example, AM404 and VDM11 attenuate retrograde endocannabinoid signaling in striatal slice preparations presumably by interfering with endocannabinoid release, effects attributed to inhibition of the putative endocannabinoid membrane transporter (18, 20). In contrast, FABP inhibition would be expected to potentiate endocannabinoid signaling by attenuating its intracellular inactivation. We propose two alternative scenarios to account for such effects of “transport inhibitors”: 1) that in addition to inhibiting FABPs, transport inhibitors target a putative trans-synaptic protein that mediates retrograde endocannabinoid transport and/or 2) that these compounds engage off-target receptor systems. With regard to the latter, it is known that AM404 activates transient receptor potential vanilloid receptor 1 (52), sodium channels (53, 54), and calcium channels (55), and some of its physiological effects likely stem from such off-target interactions (56). VDM11 induces hypomotility in rats (57) and inhibits FAAH (7, 30). Another transport inhibitor not employed in the current study, UCM707, is an agonist at cannabinoid receptor 2 (58). Such inherent nonspecificity of transport inhibitors indicates that more selective reagents will be required to unambiguously probe endocannabinoid inactivation.

In conclusion, our study ascribes novel functions to FABPs in endocannabinoid/NAE biology. As proteins that cycle between the cytosol and nucleus, FABPs regulate the availability of NAEs at nuclear receptors and modulate NAE signaling. Our current model predicts that FABPs serve as intracellular conduits for diverse bioactive lipids, thereby permitting efficient signaling and their prompt metabolism. Emerging evidence implicates FABPs in numerous physiological processes, with documented roles in systemic metabolism, atherosclerosis, and diabetes (27, 59). The importance of FABPs in such processes is only beginning to be understood.
Cellular Targets of Endocannabinoid Transport Inhibitors

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