High-throughput screening for fatty acid uptake inhibitors in humanized yeast identifies atypical antipsychotic drugs that cause dyslipidemias

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Abstract Fatty acids are implicated in the development of dyslipidemias, leading to type 2 diabetes and cardiovascular disease. We used a standardized small compound library to screen humanized yeast to identify compounds that inhibit fatty acid transport protein (FATP)-mediated fatty acid uptake into cells. This screening procedure used live yeast cells expressing human FATP2 to identify small compounds that reduced the import of a fluorescent fatty acid analog, 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (C1-BODIPY-C12). The library used consisted of 2,080 compounds with known biological activities. Of these, 1.8% reduced cell-associated C1-BODIPY-C12 fluorescence and were selected as potential inhibitors of human FATP2-mediated fatty acid uptake. Based on secondary screens, 28 compounds were selected as potential fatty acid uptake inhibitors. Some compounds fell into four groups with similar structural features. The largest group was structurally related to a family of tricyclic, phenothiazine-derived drugs used to treat schizophrenia and related psychiatric disorders, which are also known to cause metabolic side effects, including hypertriglyceridemia. Potential hit compounds were studied for specificity of interaction with human FATP and efficacy in human Caco-2 cells. This study validates this screening system as useful to assess the impact of drugs in preclinical screening for fatty acid uptake.—Li, H., P. N. Black, A. Chokshi, A. Sandoval-Alvarez, R. Vatsyayan, W. Sealls, and C. C. DiRusso. High-throughput screening for fatty acid uptake inhibitors in humanized yeast identifies atypical antipsychotic drugs that cause dyslipidemias. J. Lipid Res. 2008. 49: 230–244.

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During the past 20 years, obesity among adults and children has increased significantly in the United States. Being overweight or obese increases the risk for the development of adverse health conditions, including hyper-
chain acyl-coenzyme A synthetases (ACSLs) (22–24). The involvement of both FATP and ACSL is consistent with the model of vectorial acylation, in which fatty acid uptake is coupled to esterification with CoA (25–28). Our own work exploiting the genetically tractable yeast Saccharomyces cerevisiae has demonstrated that a membrane-bound FATP homolog, Fat1p, and an ACSL, either Faalp or Faadp, interact physically as well as functionally to catalyze import and activation (reviewed in Ref. 29). Fat1p and ACSL are both essential when cells are grown under hypoxic conditions, which render yeast auxotrophic for long-chain unsaturated fatty acids (30). Likewise, specific interactions have also been demonstrated between FATP1 and ACSL1 to potentiate fatty acid uptake in murine adipocytes (28). A number of studies have shown that fatty acid transport, particularly for protonated fatty acids, may also occur by diffusion or flip-flop between the two faces of the membrane (31–37). Using adipocytes, it was demonstrated that the association and flip-flop of oleate across the plasma membrane occurred very rapidly (33). A recent review of the diffusional model of fatty acid transport suggests that flip-flop is tied to the metabolic conversion to acyl-CoA, consistent with our model of vectorial acylation (32). Together, these reports provide compelling evidence for targeting fatty acid transport to limit the cellular uptake of fatty acids and thus lipotoxicity.

Previously, we developed a live cell high-throughput screening (HTS) assay using a fat1ΔfaalΔ yeast strain to identify chemical compounds that will inhibit fatty acid uptake (P. N. Black and C. C. DiRusso, United States Patent 7,070,944) (38). In this system, transport is a function of mouse FATP2 expressed from a yeast promoter and long-chain fatty acid activation is provided by the endogenous ACSL Faa4p. We demonstrated that this HTS assay is rapid to execute, inexpensive to implement, and has appropriate sensitivity for HTS. In the present work, we conducted a pilot study using a 2,080 compound library from MicroSource Discovery Systems, Inc. (Gaylordsville, CT), called SpectrumPlus® to improve and validate this live cell HTS method. We also devised secondary screens to eliminate false-positives in the yeast system and further evaluated the potential hit compounds using the human Caco-2 cell line. The members of the largest family of structurally related compounds identified in this screen are derivatives of a tricyclic phenothiazine core from which atypical antipsychotic drugs have been developed. Among these are chlorpromazine and the related compound clozapine. Many of the drugs in this class cause adverse side effects, including weight gain, hypertriglycerideridemia, hyperglycemia, and ketoacidois, which in severe cases have led to patient death (reviewed in Ref. 39). Consequently, several of these drugs have received black box warning labels. The mechanism by which these drugs cause these clinical symptoms is unknown. Our identification of these compounds as inhibitors of fatty acid uptake leads us to suggest that the metabolic dysregulation associated with the administration of these drugs is caused in whole or in part by reduced fatty acid uptake, resulting in the disruption of normal cellular lipid trafficking.

**Chemical compound library**

The SpectrumPlus compound library (2,080 compounds) was obtained from MicroSource Discovery Systems, Inc. The library includes five subsets of compounds: 1) Genes Plus, composed of 960 compounds that represent new and classical therapeutic agents as well as established experimental inhibitors and receptor agonists; 2) Pure Natural Products Collection, a unique collection of 720 diversified pure natural products and their derivatives, including simple and complex oxygen heterocycles, alkaloids, sequiterpenes, diterpenes, pentacyclic triterpenes, sterols, and many other diverse representatives; 3) Agro Plate, containing 80 compounds representing classical and experimental pesticides, herbicides, and purported endocrine disruptors; 4) Cancer Plate, including 80 cytotoxic agents, antiproliferative agents, immune suppressants, and other experimental and therapeutic agents; and 5) Spectrum Plus Plate, containing 240 biologically active and structurally diverse compounds. The compounds are supplied as 10 mM solutions in DMSO and were diluted in PBS to a final concentration of 80 μM for screening in yeast using a Caliper RapidPlate 96/384 Dispenser (Caliper Life Sciences, Hopkinton, MA).

**Reagents and cell culture**

Yeast extract, yeast peptone, and yeast nitrogen base without amino acids and dextrose were obtained from Difco (Detroit, MI). Complete amino acid supplement and single amino acids were from Sigma (St. Louis, MO). Fatty acid-free bovine serum albumin (FAF BSA) and other chemical reagents were also from Sigma, unless stated otherwise. Corning® white with clear flat-bottom 96-well and 384-well microplates were used for screening of yeast. For Caco-2 cells, tissue culture-treated 96-well transparent with clear flat-bottom polystyrene microplates were from Fisher Scientific (Pittsburgh, PA). The fluorescent long-chain fatty acid analog, 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (C12-BODIPY-C₁₂), was obtained from the Molecular Probes Division of Invitrogen.

S. cerevisiae strain LS2086 containing deletions within the FAT1 and FAA1 genes (fat1Δfaa1Δ; MATa ura3-52 his3A200 ade2-101 lya2-801 leu2-3,112 faa1Δ::HIS3 fat1Δ::G418) (26) was used for the primary screen. For most experiments, yeast minimal medium with dextrose (YNBD) contained 0.67% yeast nitrogen base (YNB), 2% dextrose, adenine (20 mg/l), uracil (20 mg/l), and amino acids as required [arginine, tryptophan, methionine, histidine, and tyrosine (20 mg/l); lysine (30 mg/l); and leucine (100 mg/l)]. When a rich medium was required (e.g., toxicity studies in yeast), yeast complete media with adenine (YPD) was used. Growth in liquid culture and on plates was at 30°C.

Caco-2 cells were maintained in Earl’s minimal essential medium with 20% FBS in a 95% air/5% CO₂ atmosphere at 37°C, as described previously (40). For growth and differentiation, the BD Biosciences Intestinal Epithelium Differentiation Media Pack was used. Cells were plated in basal seeding medium at a density of 2.5 × 10⁴ cells/cm² on a collagen-coated black-clear 96-well plate (BD Biosciences). Enterostim Medium was added to each well after removal of the basal seeding medium 24 h later. Both media contained mito-serum extender. After another 24 h, cells were washed once with complete balanced Hanks’ solution containing Ca²⁺ and Mg²⁺ without phenol red before the C₁₂-BODIPY-C₁₂ uptake assay.

**Cloning and expression of human FATP2 in yeast**

For expression of the human FATP2 in yeast, the cDNA encoding the protein was amplified using PCR from a cDNA within
NIH Image Clone LD. 30348317 using forward primer 5'-gcataag-cccgtgatgtctccctgcatcaca-3' and reverse primer 5'-cgagac-ccagtaagggatctcctggttttac-3'. The amplified DNA was cloned into the yeast expression vector pDB121 to give pDB126. In this construct, the expression of targeted human FATP2 is under the control of the GAL10 promoter (41). The human FATP2 plasmid and pRS416-GAL4-ER-VP16 encoding a synthetic transcrip- tional activator, which is a protein fusion between the Gal4 DNA binding domain, a β-estradiol-responsive regulatory do- main, and a VP16 RNA polymerase activation domain (42), were cotransformed into strain LS2086 for HTS assays. Expression of human FATP2 was induced by the growth of yeast cells over- night in selective medium containing 10 nM β-estradiol. The level of human FATP2 protein was routinely measured using an antibody directed toward a T7 epitope fused to the C termi- nus of human FATP2 in Western blots of cell extracts.

**HTS**

For screening of the 2,080 compound MicroSource library, a Biomek® FX laboratory automated workstation (Beckman Coulter, Inc., Fullerton, CA) was used. LS2086 yeast cells expressing hu- man FATP2 (pDB126/fat1faa1A) or transformed with the empty vector pDB121 (negative control cells) were pregrown in YNBD without leucine and uracil (YNBD –leu –ura); the cells were sub- sequently subcultured to absorbance at 600 nm = 0.02 in the same medium containing 10 nM β-estradiol to induce expression and incubated overnight at 30°C with shaking. Mid-log-phase yeast cells [0.8–1.2 optical density at 600 nm (OD600)] were harvested and resuspended in PBS at a cell density of 6 × 10^7/ml before dis- pensing to a 384-well assay plate (22.5 µl/well). Wells in the first two rows of each plate received the negative control cells, and all other wells received cells expressing human FATP2. Compounds (25 µl) were then added to a final concentration of 80 µM. After 2 h of incubation at 30°C, the C1-BODIPY-C12 uptake assay was performed. To each well, a mixture of C1-BODIPY-C12 (final concentration, 1.25 µM), FAF BSA (final concentration, 0.75 µM), and trypan blue (final concentration, 2.1 mM) was added to give a final volume of 100 µl. Trypan blue quenched the extracellular fluo- rescence, and only cell-associated fluorescence was measured after 30 min on a Bio-Tek Synergy HT multidetection microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) with filter sets of 485 ± 20 nm excitation and 528 ± 20 nm emission. On each plate, two rows each of negative control cells and positive control cells were incubated with 0.8% DMSO in PBS and assayed si-multaneously to calculate a Z' factor for each plate. The results presented are the values obtained for two screening experiments (see Tables 1, 3–7 below).

**Determination of inhibition constant**

Ligand competition curves were fit by nonlinear least-squares regression using a one-site competition model and Prism soft- ware (GraphPad Software, Inc., San Diego, CA) to determine the concentration of added competitor that reduced C1-BODIPY-C12 fluorescence readout by 50% (IC50). Inhibition constant (Ki) values were calculated from the IC50 by the equation of Cheng and Prusoff (43): \[ K_i = IC_{50}/(1 + [\text{Radioligand}]/K_d) \], where radioligand is the concentration of C1-BODIPY-C12 and Kd is the apparent affinity of C1-BODIPY-C12 for the yeast cells expressing human FATP2 or Caco-2 cells. The apparent Kd was deter- mined by measuring uptake activity in the presence of different C1-BODIPY-C12 concentrations.

**Toxicity assays**

Wild-type yeast strain YB332 was used to evaluate the com- pound toxicity. Cells were grown in YPDA overnight and sub- cultured into the same medium at 0.1 OD600 in the presence of different concentrations of compound as indicated. Optical density was measured every hour during 8 h of growth. To test viability after treatment, cells were grown and treated with compound for 2 h as usual to test uptake inhibition but were har- vested by centrifugation, washed three times with PBS, serially diluted, and plated on YPDA. After growth for 2 days at 30°C, colony numbers were counted and scored to give the number of colony-forming units per milliliter.

To evaluate potential toxic effects on Caco-2 cells, we used the MTT [for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (44). For these experiments, cells were treated with compound exactly as in the uptake assay using a 96-well format. At the end of the incubation period with test compound, the medium was removed and cells were treated with 100 µl of MEM containing 1.2 mM MTT for 3 h at 37°C. The reac- tion was terminated and cells were lysed by the addition of 5% SDS and 0.005 N HCl with incubation at 37°C for 1 h. The final absorb- ance was read at 570 nm to detect the formation of the purple formazan product. Cell viability was determined relative to a stan- dard curve prepared using untreated cells.

**Lipid analyses**

To identify lipids labeled with C1-BODIPY-C12, LS2086 yeast cells (pDB126/fat1faa1A) were grown in 50 ml of YNBD (–leu, –ura) containing 10 nM β-estradiol to 1.0 OD600 at 30°C. The culture was then concentrated by centrifugation and resus- pended in PBS to a cell density of 6 × 10^7/ml. To half of the cell suspension, perphenazine (in PBS) was added to a final concentration of 40 µM; a control flask received PBS alone. Incubation with the compound continued for 2 h, then C1- BODIPY-C12 (final concentration, 1.25 µM) in FAF BSA (final concentration, 0.75 µM) was added for 30 min to evaluate up- take. Metabolism was stopped by the addition of 0.1 volume of 6.4 M perchloric acid. Lipids were extracted essentially as de- tailed previously (45). After extraction, the dried lipids were resuspended in 50 µl of chloroform, and aliquots (5 µl) were spotted to Whatman Partisil LK5 TLC plates (250 µm, 150 Å silica). To resolve phospholipids, the solvent was a mixture of 25 ml of methyl acetate, 25 ml of 2-propanol, and 7 ml of 0.25% KCl (solvent I); to resolve neutral lipids, the solvent consisted of three parts cyclohexane to two parts ethyl acetate (solvent II). All solvents were of HPLC grade. Lipids were visualized under UV light.

**Visualization of C1-BODIPY-C12 uptake into Caco-2 cells**

Caco-2 cells were cultured as detailed above on four-well tissue culture-treated microscope slides (BD Falcon; BD Biosciences); two wells were used as controls and two wells were used for com- pound treatment. Treatment of cells with 80 µM perphenazine for 1 h and uptake of C1-BODIPY-C12 were essentially as detailed for HTS with the following modifications. After treatment with compound or MEM (control), medium was removed and cells were washed with MEM. C1-BODIPY-C12 (final concentration, 5 µM in 5 µl FAF BSA) was added for 3 min without trypan blue. Cells were then immediately rinsed with MEM, and Elvanol mounting medium was applied to each well. Cells were imaged using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a 63× objective using fluorescence modes. The instrument settings for
brightness and contrast were optimized to ensure that the confocal microscope was set for its full dynamic range relative to untreated cells on the same slide; the same settings were used for all subsequent image collections. An argon laser source was used for imaging, with excitation at 488 nm and emission at 505 nm.

**Data analysis**

Values in arbitrary units of the fluorescent signals from each HTS plate were exported to Excel (Microsoft Corp., Redmond, WA) spreadsheet templates, and the assay quality-control Z-factor was calculated (46). ChemTree software (Golden Helix, Inc., Bozeman, MT) was used for additional statistical analysis and to study structure-activity relationships.

**RESULTS**

**Library screening**

In previous work, we developed a live cell, high-throughput method for the selection of small chemical modulators of fatty acid uptake dependent upon a murine FATP expressed in a *S. cerevisiae* strain deficient in the long-chain fatty acid transporter Fat1p and the major ACSL Faa1p (fat1Δfaa1Δ) (38). In this study, this strain was used to express the human FATP2 under the control of the *GAL10* promoter. Fatty acid uptake was measured as an increase in cell-associated fluorescence attributable to the accretion of the fluorescent fatty acid analog C1-BODIPY-C12 through a human FATP2-dependent process. External fluorescence was quenched by the vital dye trypan blue. We used this system to screen a 2,080 chemical compound library, SpectrumPlus (MicroSource), to select for compounds that altered fatty acid uptake. This library consists of compounds that are structurally diverse, including known drugs, experimental actives, and pure natural products. The collection is advantageous in that the compounds included have been chosen for diversified structures containing unique structural scaffolds. Additionally, most compounds included in the set have known pharmacological and toxicological profiles that can be used to determine whether the hit compound or structur-

**TABLE 1. Compounds that reduced cell-associated fluorescence in humanized yeast**

| Compound Identifier | Name                  | Percentage Transport Inhibition | Comment                  |
|---------------------|-----------------------|--------------------------------|--------------------------|
| 01500138            | Benzethonium chloride | 100, 100                        |                          |
| 01500184            | Chlorpromazine        | 100, 100                        |                          |
| 01500315            | Gentian violet        | 100, 100                        | Quenching agent          |
| 01503610            | Benzalkonium chloride | 100, 100                        |                          |
| 01503253            | Methylbenzethonium chloride | 100, 100       |                          |
| 01503227            | Perhexiline maleate   | 100, 100                        |                          |
| 01503223            | Pararosaniline pamoate | 100, 100                        | Quenching agent          |
| 02900006            | Quinolinamine HCl     | 100, 100                        |                          |
| 01500575            | Thiordizaine HCl      | 100, 100                        |                          |
| 01505294            | Altmoriptan           | 100, 100                        |                          |
| 00100325            | Digitonin             | 100, 100                        | Membrane disruptor       |
| 01503934            | Perphenazine          | 91, 100                         |                          |
| 01503200            | Centrinomium bromide  | 100, 96                         |                          |
| 01504079            | Tomatine              | 97, 98                          | Antimicrobial agent      |
| 01503936            | Percizaine            | 72, 96                          |                          |
| 01503637            | Methiothepin maleate  | 59, 100                         |                          |
| 01500127            | Anthrallin            | 81, 82                          |                          |
| 00310035            | Sanguinarine sulfate  | 53, 64                          | Antibiotic/antifungal agent |
| 00201664            | Celastrol             | 75, 97                          | Quenching agent          |
| 01503207            | Cyclobenzaprine HCl   | 80, 86                          |                          |
| 01505205            | Olmesartan medoxomil  | 80, 98                          |                          |
| 01502237            | Harmol hydrochloride  | 89, 83                          |                          |
| 0150473             | Phenazopyridine hydrochloride | 53, 51                          |                          |
| 01500994            | Flufoxazine HCl       | 54, 99                          |                          |
| 01504017            | Sinapiside A          | 55, 92                          | Antifungal agent         |
| 01503239            | Hyacynthione          | 62, 69                          |                          |
| 01500898            | Emodin                | 74, 60                          |                          |
| 01500685            | Clozapine             | 66, 50                          |                          |
| 00300058            | Juglone               | 53, 47                          | Antifungal agent         |
| 01504119            | Rhodomyrtokin B       | 64, 40                          |                          |
| 0005556             | Chrysobolin           | 34, 42                          |                          |
| 02111118            | Diacetyldeisovaleryl-rhodomyrtokin | 45, 60                        |                          |
| 0150759             | Quinalizarin          | 70, 54                          |                          |
| 01500602            | Gossypol              | 40, 44                          | Quenching agent          |
| 01500510            | Promethazine          | 44, 66                          |                          |
| 01500509            | Promazine             | 42, 46                          |                          |
| 01500505            | Prochlorperazine edisylate | 45, 47                          |                          |
| 00200022            | Aklavin HCl           | 44, 69                          |                          |
| 01504074            | Embelin               | 44, 58                          |                          |

*Percentage inhibition calculated from the ratio of fluorescence in arbitrary fluorescence units (AFU) for cells with compound compared with positive control cells with 0.8% DMSO alone. Results are from individual samples in two high-throughput screening trials (trial 1, trial 2).
then fluorescence was measured. The Z

terminates the vector (pDB121) after incubation with the fatty

take assay. Data points are from one trial.

ally similar compounds can be safely and effectively pre-

scribed for additional medicinal uses.

In our HTS, library compounds were tested at 80 μM on 384-well plates with yeast cells expressing the human FATP2. The fatty acid uptake activity of the cells was determined by comparing the cell-associated fluorescence of cells expressing human FATP2 with that of cells carrying the vector (pDB121) after incubation with the fatty acid analog C1-BODIPY-C12 (38). The Z’ factors were calculated from values obtained from positive and negative controls (two lanes of each per plate) and ranged from 0.61 to 0.80, indicating a robust assay with good discrimination between the positive and negative controls (47). Two screening trials were performed, and there was excellent consistency between the two experiments. Potential inhibitors were identified using a cutoff value of 3 SD from the mean of the positive and negative controls (Table 1). As expected, most compounds (98%) had no effect on C1-BODIPY-C12 uptake (Fig. 1). Four compounds enhanced the fluorescence signal, and 39 compounds were identified as potential inhibitors, as they reduced the cell-associated fluorescence compared with that of the cells incubated with vehicle alone. Of the 39 compounds, 22 (1.06%) reduced fluorescence by 80–100% and 17 (0.82%) gave intermediate levels (40–79% compared with positive controls).

Secondary screens eliminate autofluorescent and quenching compounds

This primary screening does not distinguish between effectors that alter apparent fluorescence readings by non-specific mechanisms, apart from those that interact directly with FATP. Nonspecific effectors might include those that increase apparent fluorescence as a result of intrinsic properties specific to the compound or those compounds that act as quenching agents. Additionally, some compounds may disrupt membrane integrity and facilitate the internalization of trypan blue, which in turn quenches the imported fluorescent ligand. Therefore, all compounds identified as hits in the primary screen were subsequently tested for autofluorescence, the ability to quench C1-BODIPY-C12 fluorescence, and the ability to disrupt membrane integrity (Table 2).

Two compounds identified in the primary screen, acriflavinium hydrochloride and calcein, yielded high intrinsic fluorescence that was not quenched by trypan blue, suggesting that these compounds did not enhance fatty acid uptake but increased fluorescence by nonspecific mechanisms (Fig. 1, Table 2). Sixteen of the 39 compounds that were hits from the primary screening were colored. These colored compounds might cause an inner filter effect, reducing the fluorescence signal of C1-BODIPY-C12 as a result of overlap of their absorption spectrum with the emission spectrum of the BODIPY fluorophore. To test this concern, we determined the absorp-

### Table 2. Tests to eliminate compounds with nonspecific effects, including autofluorescence, quenching, and membrane disruption

| Compound Identifier | Compound Name               | AFU Compound Alone | AFU Compound with C1-BODIPY-C12 | AFU before Compound | AFU after Compound | Mechanism                  |
|---------------------|------------------------------|--------------------|---------------------------------|--------------------|------------------|--------------------------|
| 01500618            | Acriflavinium chloride       | 43,824             | 60,413                          | 882                | 2,821            | Autofluorescence          |
| 01503223            | Pararosaniline pamoate       | 100                | 219                             | 861                | 1,054            | Quenching                 |
| 00100325            | Digitorin                    | 108                | 28,371                          | 802                | 420              | Membrane disruption       |
| 01503934            | Perphenazine                 | 99                 | 24,628                          | 863                | 816              | Unknown, potential hit    |
| Positive controls   | NR                           | NR                 | NR                              | 1,092              | 1,145            |                          |
| Negative controls   | NR                           | NR                 | NR                              | 240                | 286              |                          |

C1-BODIPY-C12, 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid; NR, not relevant.

**AFU of 80 μM compound with filter sets of 485 ± 20 nm excitation and 528 ± 20 nm emission as used to measure cell-associated C1-BODIPY-C12.**

**Average AFU for 5 μM C1-BODIPY-C12 alone was 24,361.**

Yeast cells expressing human fatty acid transport protein 2 were preloaded with 5 μM C1-BODIPY-C12 under standard assay conditions and then fluorescence was measured.

The same cell samples described in footnote e were incubated with 80 μM compound and incubated for 2 h at 30°C, then fluorescence was measured again.

Yeast cells expressing human FATP2 as described in footnote e with 0.8% DMSO added and no compound.

Yeast cells with vector incubated with 0.8% DMSO added and no compound.
tion spectrum of these compounds from 300 to 800 nm and found two structurally related compounds, gentian violet (purple) and pararosaniline pamoate (red), that have absorption maxima at 590 and 550 nm, respectively. Therefore, they most likely quench C1-BODIPY-C12 fluorescence (emission at 510–625 nm). As expected, when these compounds were added directly to 5 μM C1-BODIPY-C12 in the absence of cells, the fluorescence was reduced significantly (Table 2). These results showed that both compounds acted as quenching agents to cause the apparent reduction in cell-associated C1-BODIPY-C12 fluorescence. Another compound, celastrol (orange), also modestly quenched the BODIPY fluorescence.

Compounds that act as membrane permeants will decrease cell-associated fluorescence because they will allow the vital dye trypan blue to enter the cells. Among these, the common detergent digitonin (48) is found in the SpectrumPlus library and was identified by its ability to reduce fluorescence to background levels. To eliminate compounds that disrupt membrane integrity, cells were preloaded with C1-BODIPY-C12 in the presence of trypan blue for 30 min; each compound was added and cells were incubated for 2 h, then we revaluated cell-associated fluorescence. A reduction in fluorescence intensity after the addition of compound compared with fluorescence intensity before the addition of compound was interpreted as indicative of a loss of membrane integrity. Among the 39 compounds, only the cationic detergent digitonin acted as a strong membrane-permeabilizing agent using this test (Tables 1, 2). The SpectrumPlus library also contains at least one other cationic detergent, polymixin B sulfate. This compound weakly inhibited fatty acid uptake (~30% inhibition compared with positive controls) and was not included in the selected list of hits from our primary screen. Other compounds with known detergent-like properties, including centrimonium bromide (described below), did not affect fluorescence readings of cells preloaded with C1-BODIPY-C12.

**Structural analysis of hit compounds in yeast**

After eliminating the autofluorescent compounds and quenching and permeabilizing agents, we compared the structures of the remaining compounds to identify similar structural cores using ChemTree software. Using a multiple tree clustering algorithm, ChemTree automatically groups compounds that act according to similar quantitative structure-activity relationship mechanisms. Four groups of compounds that inhibited C1-BODIPY-C12 uptake were thus identified as having similar structural scaffolds. Members of the first set are structurally related to the natural ligands of FATP, fatty acids, in that each has a long aliphatic hydrocarbon chain, including centrimonium bromide and benzalkonium chloride (Table 3). However, the hydrocarbon chain alone was not sufficient for the inhibi-

| Compound Structure | Compound Name | Percentage Inhibition |
|--------------------|---------------|-----------------------|
| ![Benzalkonium chloride](image) | Benzalkonium chloride [ammonium, alkyl(dimethylphenylmethyl)-, chloride] | 100, 100 |
| ![Centrimonium bromide](image) | Centrimonium bromide (hexadecyl-trimethyl-ammonium, bromide) | 100, 96 |
| ![Embelin](image) | Embelin (2,5-dihydroxy-3-undecyl-cyclohexa-2,5-diene-1,4-dione) | 44, 57 |
| ![Batyl alcohol](image) | Batyl alcohol [3-(octadecyloxy)-1,2-propanediol] | 0, 0 |
| ![Avocadine](image) | Avocadine [(2,4-dihydroxyheptadec-16-ynyl) acetate] | 0, 0 |

*Percentage inhibition compared with positive controls without compound. Results are for two experiments.*
bition of C1-BODIPY-C12 uptake, because at least five other compounds with this structural motif in the SpectrumPlus collection were inactive: batyl alcohol, roccellic acid, vocadyne acetate, avocadyne acetate, and caperatic acid. One compound, embelin, had intermediate activity. The inactive compounds have the common characteristic of being highly charged, with multiple hydroxyl groups on the terminal carbon of the chain that may preclude interaction with the transporter.

The largest number of hits that fell into one structural class was related to the phenazothiazines (Table 4). Five of these were highly active in preventing uptake,
because cell-associated fluorescence remained at background levels. These included perphenazine, perciazine, chlorpromazine, thioridazine, and flufenazine. Three derivatives had intermediate activity (promethazine, promazine, and prochlorperazine edisylate) and two were completely inactive (isothiopendyl HCl and triflupromazine).

To further assess the effects of this class of compounds on fatty acid uptake and metabolism, we compared C₁-BODIPY-C₁₂ incorporation into lipids of yeast cells expressing human FATP2 after treatment with PBS (control) or with perphenazine (Fig. 2). As expected, C₁-BODIPY-C₁₂ was incorporated into both phospholipids and neutral lipids of control cells, and treatment with perphenazine substantially reduced the labeling of essentially all lipid classes.

A third group of related hit compounds contained a tricyclic core motif differing from the phenothiazines in that the central ring has seven members rather than six (Table 5). The structures of these compounds are very similar, and subtle variations contribute to substantial differences in activity. Three compounds strongly inhibited uptake at 80 μM: clomipramine, methiothepin maleate, and cyclobenzaprine. Two were slightly active (clozapine and imipramine), whereas amitriptyline and loratadine were inactive.

The final set of compounds, although more structurally diverse within the group, were related in that all were heterocyclic and highly oxygenated (Table 6). These varied in the number of rings, from the simplest two-ring compound juglone to the five-ring structure of aklavin.

**Toxic compounds are eliminated by evaluating yeast growth**

We previously demonstrated that heat-killed cells become permeable to the fatty acid analog and the quenching agent trypan blue, resulting in low fluorescence readings using the C₁-BODIPY-C₁₂ assay (38). This led us to question whether the mechanism of apparent inhibition of transport was merely attributable to cell death and associated membrane dysfunction. To test compound toxicity, wild-type yeast cells (YB332) were grown in YPDA to mid-log phase and then diluted to 0.1 OD₆₀₀. Serial dilutions of a test compound were added to medium, and growth of the yeast cells was monitored relative to cells incubated without the addition of compound. Figure 3 shows the growth curves of YB332 in the presence of PBS (control) or 100 μM centromonium bromide, anthralin, or perphenazine. At 100 μM, perphenazine seemed to inhibit the growth of yeast cells. Therefore, we further tested the viability of yeast cells in the presence of this putative inhibitory compound by plating cells on solid medium after treatment with compound for 2 h to mimic the conditions used in our HTS experiments. Using this method, >95% of the cells were viable. Therefore, we do not believe that inhibition by perphenazine was merely the result of cell death.

**Evaluation of hit compounds in Caco-2 cells**

Caco-2 cells are derived from a human colon adenocarcinoma (49). In culture, these cells spontaneously differentiate into polarized, columnar cells that are representative of the small intestine (50). When grown on plastic, they form domes typical of normal and transporting epithelium (51, 52). This cell line has been used extensively as a model for transport studies (50, 53). Previous work has shown that fatty acid uptake in Caco-2 cells is saturable, decreased by trypsin treatment of cells, and can be competed by natural fatty acids (54). These cells express higher levels of both FATP2 and FATP4 than other FATP isoforms (P. N. Black, P. Fraisl, and C. C. DiRusso, unpublished data). To further evaluate hit compounds identified from our screening of yeast cells, we performed C₁-BODIPY-C₁₂ uptake inhibition experiments in 96-well format using Caco-2 cells. We initially tested the hit compounds in Caco-2 cells at 100 and 10 μM (Table 7). At 100 μM, most of these compounds inhibited Caco-2 uptake of C₁-BODIPY-C₁₂ to varying extents. Seven compounds reduced uptake to essentially control levels, whereas most had intermediate activity. When the concentration of compound was de-

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**Fig. 2.** Perphenazine inhibits the incorporation of the fluorescent fatty acid analog into various lipids. Lipids were isolated from yeast cells expressing human FATP2 after treatment with PBS (−) or perphenazine (+) and C₁-BODIPY-C₁₂ as detailed in the text. A: Solvent system I was used to distinguish phospholipids from neutral lipids. B: Solvent system II was used to separate neutral lipids. Images shown are representative of two experiments. TLC plates were scanned using a phosphorimaging system. Positions of commercial standards are labeled as follows: NL, neutral lipids; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; TLN, triolean; CE, cholesteryl ester; C, cholesterol.
creased to 10 μM, only three compounds inhibited uptake by 25–40% of control levels (Table 7).

We next evaluated the concentration-dependent inhibition of centrimonium bromide, perphenazine, and anthralin on Caco-2 cells by comparison with yeast (Fig. 4). The results shown in Fig. 4B indicate that the Ki values of centrimonium bromide and perphenazine for Caco-2 cells was higher than that of yeast cells (Fig. 4A). For anthralin, we were unable to determine the Ki in Caco-2 cells, because at 200 μM the uptake was still ~70% of the control value. As was the case in yeast, C1-BODIPY-C12 was incorporated into Caco-2 cells, labeled various cellular lipids, and did not show preferential compartmentalization (Fig. 5). Treatment with a representative compound, perphenazine, substantially reduced incorporation without causing cell death; at 80 μM perphenazine, there was 94 ± 9% cell survival, as estimated using the MTT cytotoxicity assay (five experiments).

### Table 5. Compounds related to cyclobenzaprine

| Compound Structure | Compound Name | Percentage Inhibition |
|--------------------|---------------|-----------------------|
| ![Clomipramine](image) | [3-chloro-5-(3-(dimethylamino)propyl)-10,11-dihydro-5H-dibenz(b,f)azepine] | 100, 100 |
| ![Methiothepin maleate](image) | [(+−)-1-(10,11-dihydro-8-(methylthio)dibenzo(b,f)thiepin-10-yl)-4-methylpiperazine] | 59, 100 |
| ![Cyclobenzaprine](image) | [1-propanamine, 3-(5H-dibenzo(a,d)cyclohepten-5-ylidene)-N,N-dimethyl- (9CI)] | 80, 86 |
| ![Clozapine](image) | [8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenz(b,e) (1,4)diazepine] | 66, 50 |
| ![Imipramine](image) | [1-(3-dimethylaminopropyl)-4,5-dihydro-2,3,6,7-dibenzazepine] | 0, 26 |
| ![Amitriptyline HCl](image) | [10,11-dihydro-5-(γ-dimethylaminopropylidene)-5H-dibenzo(a,d)cycloheptene] | 0, 0 |
| ![Loratadine](image) | [ethyl 4-(8-chloro-5,6-dihydro-11H-benzo(5,6)cyclohepta(1,2-b)pyridin-11-ylidene)-1-piperidinecarboxylate] | 0, 0 |

*See Table 3 for details.*
DISCUSSION

Validation of the screening method

In this study, we tested a HTS system for use in identifying fatty acid uptake inhibitors. The target of this HTS, the human fatty acid uptake system, is likely to be involved in the development and progression of many disease states that result from excessive intracellular accumulation of free fatty acids. We chose to use *S. cerevisiae* cells expressing the human FATPs for our primary screening because

| Compound Structure | Compound Name | Percentage Inhibition |
|-------------------|---------------|-----------------------|
| ![Anthralin](image) | Anthralin (1,8,9-trihydroxyanthracene) | 81, 82 |
| ![Emodin](image)  | Emodin (1,3,8-trihydroxy-6-methyl-9,10-anthracenedione) | 74, 60 |
| ![Juglone](image) | Juglone (5-hydroxy-1,4-naphthoquinone) | 53, 47 |
| ![Chrysanobin](image) | Chrysanobin (6-methylnanthracene-1,2,8-triol) | 34, 42 |
| ![Quinazalin](image) | Quinazalin (1,2,5,8-tetrahydroxyanthracene-9,10-dione) | 70, 34 |
| ![Rutilantinone](image) | Rutilantinone [1-naphthacenecarboxylic acid, 1,2,3,4,6,11-hexahydro-2-ethyl-2,4,5,7,10-pentahydroxy-6,11-dioxo-, methyl ester, (1R,2R,4S)-] | 3, 54 |
| ![Aklavin](image) | Aklavin [methyl (1S,2S)-4-(4-dimethylamino-5-hydroxy-6-methyl-oxan-2-yl)oxy-2-ethyl-2,5,7-trihydroxy-6,11-dioxo-3,4-dihydro-1H-tetracene-1-carboxylate hydrochloride] | 44, 69 |
| ![Mitoxanthrone](image) | Mitoxanthrone [1,4-dihydroxy-5,8-bis(2-((2-hydroxyethyl)amino)ethylamino)-9,10-anthracenedione] | 19, 39 |

*a See Table 3 for details.*
1) the biochemistry and molecular genetics of fatty acid uptake in yeast are well understood and analogous to those of mammalian cell systems; 2) phenotypic tests for fatty acid uptake and metabolism are readily available; 3) yeast cells are small in size, grow rapidly, and are resistant to cell lysis upon automated manipulation; and 4) the yeast HTS system is simple and inexpensive to implement. Our strategy was to carry out the HTS and initial screening for lead compounds using the humanized yeast system and then to analyze these compounds for their inhibition of fatty acid uptake in human cells. We validated this approach by screening a 2,080 compound library and identifying groups of compounds with similar chemical structures that inhibited fatty acid uptake in both yeast cells and human Caco-2 cells.

Hit compounds similar in structure to long-chain fatty acids

BODIPY-labeled fatty acids have been used to monitor fatty acid uptake (22, 26, 27) and have been shown to be biosynthetically incorporated into lipids of cells (55; this work) and cultured organs (56). The length of C1-BODIPY-C12 is approximately equivalent to that of an 18-carbon fatty acid. Our laboratory also demonstrated that the uptake of this fluorescent fatty acid analog is effectively competed by the natural fatty acid oleate in yeast (38). In the present study, of 2,080 compounds screened, four groups of structurally related compounds were identified as hits as a result of inhibition of C1-BODIPY-C12 uptake. Among these were two ionic detergents, centrimonium bro-

![Fig. 3. Growth of yeast in the presence of selected hit compounds. Wild-type yeast was cultured in the presence of 100 μM compound as indicated; cells with an equivalent volume of 0.8% DMSO in PBS were included as a control. Growth was monitored at optical density at 600 nm over 8 h as shown. CTAB, centrimonium bromide.](image)

TABLE 7. Inhibition of fatty acid uptake in Caco-2 cells by compounds identified using humanized yeast

| Identifier | Compound Name               | Percentage Inhibitiona (Experiment 1, Experiment 2) |
|------------|------------------------------|----------------------------------------------------|
| 01500138   | Benzethonium chloride        | 91, 96, 14, 14 Quenching agent                     |
| 01500184   | Chlorpromazine               | 43, 68, 9, 9                                        |
| 01500315   | Gentian violet               | 95, 98, 52, 53                                      |
| 01503610   | Benzalkonium chloride        | 88, 94, 7, 8                                       |
| 01503253   | Methylbenzethonium chloride  | 90, 95, 52, 53                                      |
| 01503277   | Perhexiline maleate         | 95, 98, 9, 9                                       |
| 01503293   | Pararosaniline pamoate       | 57, 76, 14, 14                                      |
| 02900061   | Clomipramine HCl            | 27, 58, 5, 5                                       |
| 01500575   | Thioridazine HCl            | 42, 67, 12, 12                                      |
| 01505204   | Almitriptan                  | 10, 49, 10, 10                                      |
| 00100392   | Diginison                    | 79, 97, 18, 18                                      |
| 01503934   | Perphenazine                 | 53, 74, 14, 14                                      |
| 01503200   | Centrimonium bromide        | 39, 65, 15, 15                                      |
| 01504079   | Tomatine                     | 85, 92, 9, 10                                      |
| 01503936   | Percizine                    | 11, 49, 9, 8                                       |
| 01503637   | Methiothepin maleate        | 11, 50, 6, 5                                       |
| 01500127   | Anthralin                    | 0, 42, 15, 16                                      |
| 00310085   | Sanguinarine sulfate         | 30, 60, 13, 13                                      |
| 00201664   | Celastrol                    | 77, 88, 19, 19                                      |
| 01503207   | Cyclobenzaprine HCl         | 2, 44, 15, 15                                      |
| 01505205   | Olmesartan medoxomil        | 7, 47, 18, 18                                      |
| 01502237   | Harmol hydrochloride         | 10, 48, 6, 6                                       |
| 01508473   | Phenazopyridine hydrochloride| 73, 87, 40, 41                                      |
| 01506994   | Flufenazine HCl             | 41, 67, 14, 15                                      |
| 01504017   | Sapindoside A               | 92, 63, 0, 0                                       |
| 01503239   | Hyacanthione                 | 38, 65, 16, 15                                      |
| 01508086   | Emolin                       | 62, 79, 31, 31                                      |
| 00300038   | Juglone                      | 9, 48, 0, 0                                        |
| 01504119   | Rhodomyrtin B                | 44, 68, 15, 15                                      |
| 0300556    | Chrysarobin                  | 17, 53, 11, 11                                      |
| 00211118   | Diacetyldeisovaleryl-rhodomyrtin| 46, 69, 19, 19                                 |
| 01506602   | Gossypol                     | 72, 80, 23, 24                                      |

aPercentage inhibition calculated from the ratio of fluorescence in AFU for cells with compound compared with positive control cells with DMSO alone. Results given are averages of triplicate samples for each of two experiments.
mid and benzalkonium chloride, which are similar to fatty acids in having a long hydrocarbon chain. These compounds may act as nonspecific detergents or may mimic fatty acids by interacting with FATP specifically, for example, by occupying the fatty acid binding site on the protein. We noted that these two compounds have critical micelle concentrations in the same range as cholic acid and deoxycholic acid (1–4 mM), which did not reduce apparent uptake.

Fig. 4. Concentration dependence of the inhibition of uptake by centrimonium bromide (CTAB), perphenazine, and anthralin. The 100% of control values indicates values for cells incubated with PBS (yeast) or Hanks’ balanced salt solution (Caco-2). A: Yeast cells expressing human FATP2 were incubated with compounds at 30°C for 2 h before uptake assay. B: Caco-2 cells were incubated with different concentrations of the compounds at 37°C for 1 h before uptake assay. * Anthralin at 200 μM kills Caco-2 cells. Data points are means of three experiments assayed in triplicate. Error bars indicate SEM. Ki, inhibition constant.

Fig. 5. Perphenazine blocks the incorporation of C1-BODIPY-C12 into Caco-2 cells. Caco-2 cells were treated for 1 h with vehicle (MEM) alone (A) or 80 μM perphenazine followed by C1-BODIPY-C12 for 3 min (B). Cells were imaged under fluorescence using confocal microscopy as detailed in the text.
fatty acid uptake, so nonspecific detergent effects do not adequately explain the inhibition of uptake by centrimo-
nium bromide and benzalkonium chloride. Filipin, nystatin, and β-escin, three other compounds that disrupt cell wall
and/or membrane architecture, also had no effect on up-
take in our primary screening assay. Therefore, at the con-
centration used in these experiments (80 μM), it does not
appear that these compounds disrupt FATP2-mediated fatty
acid transport by nonspecific actions.

**Some atypical antipsychotic drugs also inhibit uptake**

The other two groups of compounds all have a three-ring
structure similar to the BODIPY moiety of C1-BODIPY-C12
(Tables 4, 5). One concern is that these compounds are
merely competitive inhibitors of the fluorescent fatty acid
analog and not the natural ligands. However, this is most
likely not the case, because C1-BODIPY-C12 is taken up in
a FATP-specific manner and can be metabolized upon
import. Furthermore, the native ligand (oleate) effectively
competes with C1-BODIPY-C12 (38). Thus, it is presumed
the fluorescent ligand binds to the transporter in a man-
ner analogous to the natural ligand. Compounds identified
in this screen within these two structural classes fall
into a family with well-known pharmacological properties
that include antipsychotic drugs, which act by inhibition
of neuroreceptors, including the dopamine receptor
(39). Hints at a role for these compounds in fatty acid
and lipid metabolism come from studies showing that
these compounds are associated with hypertriglyceride-
mia, cholestatic liver, and insulin resistance (57, 58). A
recent gene expression array study demonstrated a corre-
lation between alterations in gene expression result-
ning from treatment of cells in culture with pheno-
thiazine drugs and expression changes induced by treat-
ment of cells with arachidonic acid (59). Together,
these data strongly suggest that these drugs may affect
lipid homeostasis in whole or in part through the in-
hibition of fatty acid uptake into cells. On the other hand,
although these adverse side effects might seem to elimi-
nate this class of compounds from consideration as
potential fatty acid uptake inhibitors, they may prove
useful in other ways. First, they will be valuable to
understand the mechanisms causing the unwanted side
effects of the drugs now in clinical use. Second, they
potentially could be modified/rationally designed to
interact more specifically with neuroreceptors rather
than the FATP proteins and visa versa. Third, they are ex-
pected to be informative regarding mechanisms of
FATP activity.

**Few antifungal drugs in the library altered transport activity**

Several compounds identified as hits were the anti-
fungal agents tomatine, juglone, and sapinoside A. These
compounds might act by killing the cell or inhibiting met-
abolic processes, including uptake. Because our primary
screening assay requires only a 2 h incubation period with
the compound, and because the addition of compound be-
fore C1-BODIPY-C12 uptake did not result in reduced cell-
associated fluorescence, we do not believe that these
compounds are merely killing the cells. Cell death also re-
results in increased membrane permeability. Metabolic poi-
soning might have the same effect in reducing membrane
function, but this mechanism is more difficult to disprove
with the types of assays available. We also note that the
SpectrumPlus collection contains numerous antifungal
agents that had no effect on C1-BODIPY-C12 uptake by
our method. Among these were fluconazole, griseofluvan,
and clotrimazole.

In summary, we have designed multiple systems to iden-
tify and evaluate fatty acid uptake inhibitors. Primary
screening and initial specificity evaluation used yeast cells
that express a human FATP family member. Secondary
screening has been developed in human cells (Caco-2)
to verify and further evaluate the specificity of the lead
compounds for inhibition of fatty acid uptake. This up-
take assay has some aspects in common with another
assay recently reported by Liao et al. (60). However, our
method differs in that any cell type (from yeast to human
cells) can be used with a high degree of sensitivity and
reproducibility using an inexpensive quenching agent.
In the future, we hope to use this method to identify
compounds that discriminate between different classes of
fatty acids or different FATP family members. Apart from
identifying fatty acid uptake inhibitors, this system will
also be of value in identifying fatty acid uptake activators.
Additionally, although we have targeted human FATP-
mediated fatty acid uptake in genetically defined yeast,
modifications could be made in this system to target
other participants in fatty acid transport, metabolism,
and trafficking operative in living cells. Another antici-
pated use of this screening system is in determining
whether or not lead drugs interfere with fatty acid up-
take and metabolism before they enter whole animal or
human trials. In this regard, this inexpensive, rapid, live
cell assay should prove an adaptable secondary screen for
this purpose.

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