

Novel antagonists of the thioesterase domain of human fatty acid synthase

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Abstract

Fatty acid synthase (FAS) is up-regulated in a wide range of cancers and has been recently identified as a potential therapeutic target. Indeed, previous research has shown that inhibition of FAS with active site-modifying agents can block tumor cell proliferation, elicit tumor cell death, and prevent tumor growth in animal models. Here, we use a high-throughput fluorogenic screen and identify a novel pharmacophore, 5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione, which inhibits the thioesterase domain of FAS. The novel antagonists are competitive inhibitors of the thioesterase domain, inhibit *de novo* fatty acid synthesis, and elicit FAS-dependent tumor cell death. This set of novel FAS antagonists provides an important pharmacologic lead for further development of anticancer therapeutics. [Mol Cancer Ther 2007;6(7):2120–6]

Introduction

Fatty acid synthase (FAS) is the sole enzyme in mammals responsible for cellular synthesis of palmitate, the precursor for most fatty acids. FAS has six separate enzymatic pockets that act sequentially to condense acetyl CoA and malonyl CoA, ultimately generating palmitate linked to the acyl carrier protein. Palmitate is liberated from the enzyme by the COOH-terminal thioesterase domain via a serine hydrolase mechanism. FAS activity has little effect in normal cells because they take up and use dietary lipids. However, tumor cells behave differently. FAS is up-regulated in several tumor types, including those of the breast (1–3), prostate (4–6), and ovaries (7–9), and it has been linked to poor prognosis in these cancers. This linkage apparently results from the fact that tumor cells must *de novo* synthesize fatty acids. This difference in FAS dependence between normal and tumor cells could lead to a favorable therapeutic index for drugs that target FAS.

Covalent inhibitors of FAS, such as orlistat (Xenical®), c75, and cerulenin, block tumor cell proliferation *in vitro*

and decrease the growth of tumor xenografts in mice (10, 11). Equally as important, recent evidence shows that the pharmacologic inhibition of FAS sensitizes tumor cells to chemotherapeutic agents, such as Taxol (12) and Herceptin (13). Unfortunately, cerulenin and c75 are known to have significant affinity for other protein targets (14, 15), and orlistat is poorly soluble. Consequently, these compounds are not ideal for deployment as anticancer drugs. Given that FAS holds so much promise as a drugable target and the paucity of suitable inhibitors, we sought to identify an alternative class of FAS antagonists that are suitable leads for drug design.

Here, we report on the identification of new classes of antagonists of the thioesterase domain of FAS. More than 37,000 compounds were screened to identify a 5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione-based pharmacophore that inhibits the FAS thioesterase. The most potent of these compounds blocks the activity of the FAS holoenzyme and is cytotoxic to the lipogenic MB-MDA-435 breast cancer cells¹ but much less toxic to the nonlipogenic breast epithelial cells (16, 17). A comprehensive search of public databases and literature revealed no known connection between the pharmacophore and FAS (or any other serine hydrolase). Therefore, these compounds represent a novel class of serine hydrolase inhibitors.

Materials and Methods

Expression and Purification of the FAS Thioesterase

Expression of the recombinant thioesterase domain of FAS using pTrcHis-TOPO vector (Invitrogen Corp.) was described previously (10). Large-scale expression and purification was done by Invitrogen.

Biochemical Compound Screening

The primary screen of 36,500 compounds from the DIVERSet Collection (ChemBridge) and the secondary screen of 515 structurally related compounds (ChemBridge) were done in 96-well Fluorotrac 200 plates (Greiner) using 4-methylumbelliferyl heptanoate (4-MUH; Sigma) as a fluorogenic substrate (18, 19). The optimal substrate concentration was 120 $\mu\text{mol/L}$ 4-MUH or approximately $5 \times K_m$. Briefly, reaction mixtures contained FAS thioesterase in buffer A [45 μL ; 100 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.05% Brij 35] or buffer A alone. Controls included protein solution plus vehicle (DMSO) to determine untreated enzyme activity and buffer A plus DMSO to quantify background hydrolysis of the fluorogenic substrate. Compounds (5 μL) or a 10% (v/v) DMSO solution (control) was added to yield final concentrations of approximately 12 $\mu\text{mol/L}$ (for primary screen) and 1 $\mu\text{mol/L}$ (for secondary screen). The plates were incubated

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¹ Recent studies have generated questions about the origin of this cell line.

at 37°C for 30 min before adding 4-MUH in 5 μ L DMSO/buffer A (1:1). Final DMSO concentration was 5.5% (v/v). Plates were incubated at 37°C for 60 min and assayed at 360/435 nm. Compounds that inhibited enzymatic activity >40% (for primary screen) or >50% (for secondary screen) were examined further. Compounds that were examined in great detail required the purchase of additional dry material and were assayed for identity to that in the parent library and the reported structure by liquid chromatography-mass spectrometry (data not shown).

Concentration-Response Curves to Determine IC₅₀ Values

To establish the potency of each compound, they were tested at concentrations 0.16 to 10 μ mol/L. Data points were collected in triplicate and each experiment was done at least twice independently. Reaction volumes contained 2 μ L of each dilution of lead antagonist or vehicle (DMSO) with 45 μ L of 500 nmol/L FAS thioesterase in buffer A or buffer A alone. Plates were preincubated for 30 min at 37°C before adding 5 μ L 120 μ mol/L 4-MUH in 1:1 DMSO/buffer A. Final DMSO concentration was 8.7% (v/v). Fluorescence was monitored every 5 min for 40 to 60 min to generate concentration-response curves from which IC₅₀ values were determined. The inhibition constants (K_i) were calculated using the equation of Cheng and Prusoff (20).

Kinetic Characterization of Inhibitors

To characterize the mechanism of action of selected compounds, their ability to block the turnover of 4-MUH (10–60 μ mol/L) was measured in the presence of 500 nmol/L FAS thioesterase across a concentration range. The reaction mixtures contained 2 μ L lead antagonist or vehicle (DMSO) with 45 μ L of 500 nmol/L FAS thioesterase in buffer A or buffer A alone. The final DMSO was 8.7% (v/v). Plates were preincubated for 30 min at 37°C before adding 10 to 60 μ mol/L 4-MUH in DMSO/buffer A (1:1). The formation of fluorescent product was monitored in 5-min intervals for 40 to 60 min. Linear regression curves were analyzed using GraphPad Prism (Biosoft).

Cell Culture

MDA-MB-435 breast cancer cells (11, 21) were used for biological testing of the lead antagonists. MDA-MB-435 cells undergo cell cycle arrest and apoptosis when FAS is inhibited, thereby providing a model platform. Cells were maintained in minimal Eagle's medium, Earle's salts (Irvine Scientific) supplemented with 10% fetal bovine serum (Irvine Scientific), 2 mmol/L L-glutamine (Invitrogen), minimal Eagle's medium vitamins (Invitrogen), nonessential amino acids (Irvine Scientific), and antibiotics (Omega Scientific) at 37°C and 5% CO₂. The MCF-10A cell line is a well-characterized nonlipogenic cell line and was therefore used as a model for normal levels of FAS dependence (17, 22). Cells were maintained in the complete medium described above supplemented with 100 ng/mL recombinant human epidermal growth factor (Sigma) at 37°C and 5% CO₂. MDA-MB-435 cells were generously provided by Dr. Janet Price (University of Texas, Austin, TX). All other cell lines were purchased from the American Type Culture Collection.

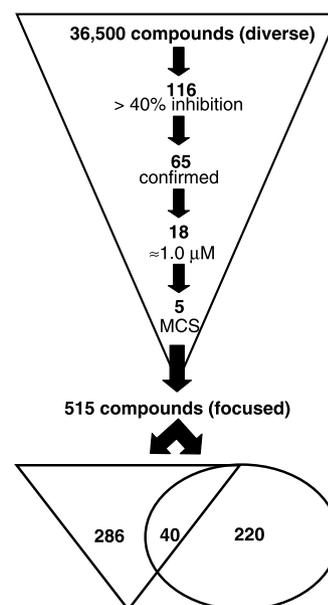


Figure 1. Screening strategy for antagonists of human FAS. The biochemical assay (triangles) was used to screen more than 36,500 drug-like compounds each at a concentration of 12 μ mol/L. Turnover of the 4-MUH substrate by the thioesterase yields fluorescence on liberation of the 4-methylumbelliferone. Five compounds contained the maximum common substructure (MCS), which is a putative pharmacophore for FAS thioesterase. A focused library of 515 compounds was purchased based on the pharmacophore. A secondary screen was done against this focused library at \sim 1 μ mol/L. A parallel cytotoxicity screen using human foreskin fibroblasts (*ova1*) was also done. Compounds (\sim 195) yielding viabilities >90% tend to correlate with precipitating compounds and were dropped from the study. In addition, compounds (\sim 100) that left <10% viable cells after treatment were dropped due to unacceptable toxicity. Forty compounds with suitable activity, solubility, and toxicity were studied in greater detail.

Measuring Fatty Acid Synthesis by the FAS Holoenzyme

Fatty acid synthesis by the FAS holoenzyme in cell lysates was measured by incorporation of [¹⁴C]malonyl-CoA (Amersham). MDA-MB-435 cells (5×10^6 total) were lysed by sonication in buffer B [20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 1 mmol/L DTT]. Each reaction contained 20 μ g total cellular protein and 5 or 20 μ mol/L of inhibitor or vehicle as a control. The final DMSO concentration was 1.5% (v/v). Samples were incubated on ice for 30 min before addition of reaction mixture (130 μ L; 115 mmol/L KCl, 192.2 μ mol/L acetyl-CoA, 576.9 μ mol/L NADPH) and [¹⁴C]malonyl-CoA (5 μ L; 0.1 μ Ci). Samples were incubated at room temperature for 15 min and fatty acids were extracted with chloroform/methanol (1:1). The chloroform fractions were dried and reextracted with hydrated butanol/water (1:1). The butanol fractions were reduced to 300 μ L and added to EcoLume (ICN Biomedicals) scintillation fluid (4 mL). Labeled fatty acids were detected by scintillation. All samples were prepared in duplicate and the experiment was done at least twice.

Measuring Cytotoxicity

For cytotoxicity experiments, MB-MDA-435 and MCF10-A cells were plated in 96-well plates at 3×10^3 per well in 200 μL appropriate complete MEM and incubated overnight at 37°C and 5% CO_2 . Cells were treated with lead antagonists (0.78–100 $\mu\text{mol/L}$) or vehicle in triplicate, with a final percentage of DMSO not exceeding 1% (v/v). After 48 h, the medium was aspirated and replaced with complete MEM containing 333 $\mu\text{g/mL}$ 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and 25 $\mu\text{mol/L}$ phenazine methosulfate using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega). Plates were incubated for 2 h and absorbance was assayed at 490 nm. Background levels of formazan formation were measured in medium alone. IC₅₀ values were derived by fitting triplicate data points over the concentration range with the “dose-

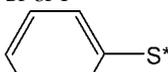
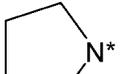
response–inhibition” function of GraphPad Prism. Each experiment was done at least twice.

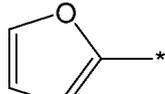
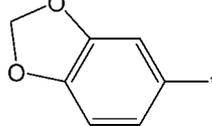
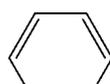
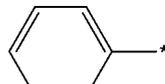
Results

A Chemically Diverse Compound Library Contains Antagonists of the FAS Thioesterase Domain

The activity of the recombinant thioesterase was assessed by its ability to cleave 4-MUH, which is hydrolyzed to the fluorescent 4-methylumbelliferone (18, 19). We initially screened a library of 36,500 drug-like compounds. The primary screen was conducted at a concentration of $\sim 12 \mu\text{mol/L}$ of each compound, revealing 116 compounds that blocked $>40\%$ of the thioesterase activity (Fig. 1). These compounds were retested to confirm activity. Eighteen compounds were identified with an apparent K_i of $<1.0 \mu\text{mol/L}$, 5 of which contain the

Table 1. Structure-activity trends within the focused library

Structural element	Frequency [†]	Active (%) [†]
$R_1 =$ Br or I	35	94
	17	88
	304	74
CH ₃	25	24
H	27	19
NO ₂	19	0
N(CH ₃) ₂ or N(CH ₂ CH ₃) ₂	18	0
	22	0
	20	0
	12	0

MCS		
Structural element	Frequency [†]	Active (%) [†]
$R_2 =$ CH ₃	24	79
	15	67
	13	62
H	51	61
	323	57
	28	50

NOTE: Although the carbon backbone is drawn here for clarity, the individual E/Z configurations have not been determined.

*Indicates point of connectivity.

[†]Active is defined as having $\geq 50\%$ inhibition at 1 $\mu\text{mol/L}$.

[‡]Substitutions with frequency <10 are excluded.

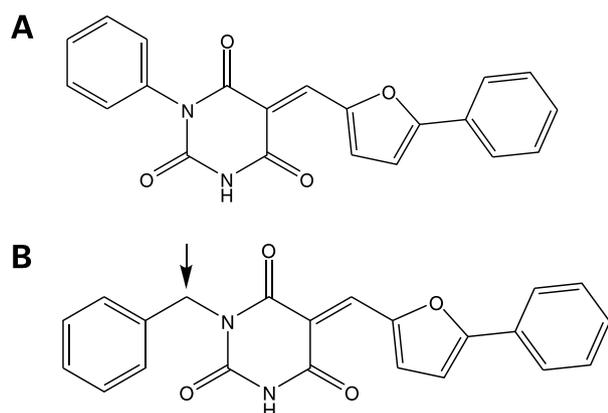


Figure 2. Two cyclic systems represent compounds with favorable structural properties. The structures of 40 compounds that were active in the biochemical assay and had suitable general cytotoxicity were analyzed using Meqi software to ascertain structural elements that correlate with activity. Two cyclic systems were prominent in that set; they differ only by methylene (arrow). Absolute E/Z configurations have not been determined.

5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione-based pharmacophore. This pharmacophore had not previously been reported as a serine hydrolase inhibitor and represents the basis for promising lead compounds as inhibitors of FAS.

Structure-Activity Trends Emerge from Studies with the Focused Library

A focused library containing 515 derivatives of the 5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione pharmacophore was characterized. All of the compounds contain this maximum common substructure, with varied substitutions at R_1 and either at a single or at both amines in the pyrimidine ring. A secondary screen against this focused library was conducted at a concentration of $\sim 1 \mu\text{mol/L}$ of each compound using the fluorogenic assay described above. Under these conditions, just over half (286) of the compounds inhibited FAS thioesterase activity by at least 50% (Fig. 1). Interestingly, substitutions at R_1 , β to the furan ring, had the most dramatic effect on activity (Table 1). Generally, substitutions at this site with high electron density, such as bromine, iodine, or phenyl rings, exhibit potent activity. In contrast, substitutions with relatively low electron density, such as a methyl group and alkyl amines (both cyclic and acyclic), or no substitution at all, show little or no activity despite substitutions at other sites. Substitutions at R_2 , those at an amine on the pyrimidine ring, seem to make less of a contribution to binding because a range of chemically diverse substitutions fails to affect activity. This suggests that this portion of the compound does not interact directly with the protein. However, a bulky disubstituted pyrimidine ring has deleterious effects on binding because, of the 42 compounds in the library that are substituted at both amines, only 6 are active; all of them are *N-N'*-dimethyl. This may indicate steric hindrance with the positioning of the inhibitor into its binding pocket.

A parallel screen was done to determine general toxicity of the compounds on human foreskin fibroblasts at a concentration of $100 \mu\text{mol/L}$ (data not shown). Compounds (~ 195) yielding viabilities $>90\%$ tend to correlate with precipitating compounds and were dropped from the study. In addition, compounds (~ 100) that left $<10\%$ viable cells after treatment were dropped due to unacceptable toxicity. The compounds with suitable activity against the FAS thioesterase and minimal toxicity were then analyzed

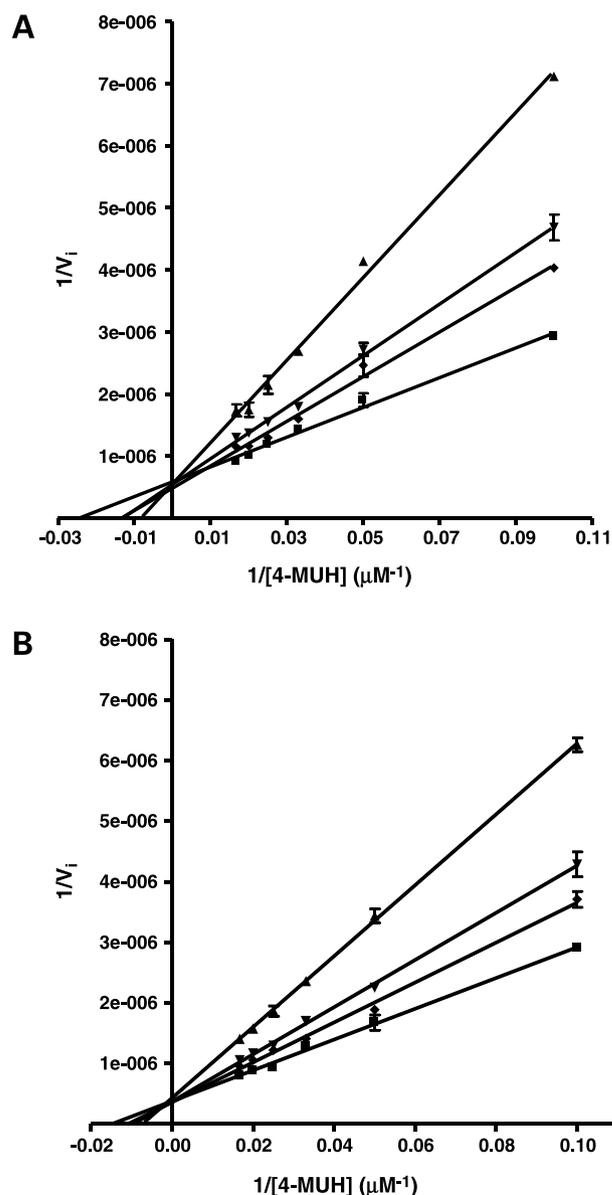


Figure 3. The lead antagonists are competitive inhibitors of the thioesterase domain. Kinetic characterization of recombinant thioesterase (500 nmol/L) activity following treatment with DMSO (\blacksquare) or selected compounds from each class: A (compound 1; Table 2) and B (compound 4; Table 2) at $0.25 \mu\text{mol/L}$ (\blacklozenge), $0.50 \mu\text{mol/L}$ (\blacktriangledown), and $1 \mu\text{mol/L}$ (\blacktriangle). Competitive inhibition is indicated by the intersection of the linear regression lines at $1/V_{\text{max}}$. All treatments were done in triplicate. Bars, SD.

using Meqi software² to extract structural characteristics that correlate with activity. Two classes of compounds, substituted on one or both phenyl rings, were well represented in this population (Fig. 2). The most promising compounds from each class were studied further.

The Lead Antagonists Are Potent, Competitive Inhibitors of the FAS Thioesterase Domain

Kinetic analysis was used to determine the K_i value for selected lead antagonists and to assess the general mechanism of their inhibition of the FAS thioesterase domain. K_i values were determined by concentration-response curves and analyzed with the equation of Cheng and Prusoff (20) and/or by Lineweaver-Burke plots. The representative double reciprocal plots of the V_i and 4-MUH data revealed that compounds from both class A and B are competitive inhibitors as indicated by linear regression plot lines intersecting at $1/V_{max}$ for the untreated controls (Fig. 3A and B). The corresponding data for orlistat, a known FAS inhibitor, are included for comparison. These analyses were done using GraphPad Prism software. As expected from the screening data, the K_i values are submicromolar in this assay and are comparable with that of orlistat.

The Lead Antagonists Inhibit the FAS Holoenzyme

To test inhibitory activity against full-length FAS, we measured the effect of the compounds on *de novo* fatty acid synthesis in cell lysates, where the FAS holoenzyme remains active. The incorporation of [¹⁴C]malonyl CoA, a precursor of palmitate, into fatty acids was measured according to published methods (23). Treatment of cell lysates with 20 $\mu\text{mol/L}$ of selected compounds abrogated fatty acid biosynthesis by >90% for the most potent compounds within 15 min (Fig. 4). Treatment with 5 $\mu\text{mol/L}$ of the same compounds inhibited *de novo* fatty acid synthesis an average of 52%. These numbers roughly correlate with the corresponding cytotoxicity IC_{50} values in MB-MDA-435 cells and confirm activity against the FAS holoenzyme. Interestingly, compound 4, a congener of cyclic system B, showed no significant difference in the amount of inhibition when the 5 $\mu\text{mol/L}$ and the 20 $\mu\text{mol/L}$ concentrations were compared.

The Lead Antagonists of FAS Are More Cytotoxic to MDA-MB-435 Cancer Cells than to Nonlipogenic Cells

Lead antagonists were tested for their ability to kill MDA-MB-435 cells, which are FAS dependent, or MCF-10A cells, which are immortal but not highly lipogenic (17, 22). Concentration-response curves of cell viability were generated for selected compounds to calculate IC_{50} values (Table 2). The IC_{50} values for the MD-MBA 435 cells are generally 2- to 5-fold lower than those observed for the MCF-10A cells and roughly correspond to the concentrations required for 50% inhibition of fatty acid biosynthesis (Fig. 4). A few lead antagonists (such as compound 4; Table 2) exhibited no apparent toxicity toward MCF-10A cells at concentrations up to 50 $\mu\text{mol/L}$. The data for the

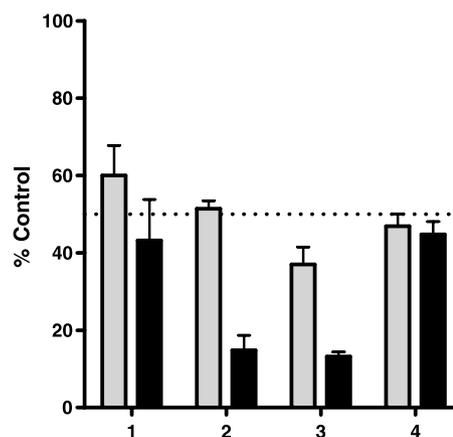


Figure 4. Lead antagonists inhibit *de novo* fatty acid biosynthesis. *In vitro* FAS activity was measured as the incorporation of [¹⁴C]malonyl-CoA over 15 min following preincubation of MB-MDA-435 cell lysates with compounds 1 to 4 (see Table 2) at 5 $\mu\text{mol/L}$ (gray columns) or 20 $\mu\text{mol/L}$ (black columns). *De novo* fatty acids were extracted and quantified by scintillation. Treatments were done in duplicate. Columns, mean of two experiments ($n = 4$); bars, SE.

known FAS inhibitor, orlistat, are in good agreement with that of the lead antagonists; orlistat has a 3-fold lower IC_{50} in the MD-MBA-435 cells than in the MCF-10A cells. Altogether, these observations are generally consistent with the idea that the cytotoxic effects of the compounds are a result of the inhibition of FAS in whole cells. Nevertheless, the possibility that the compounds react with additional cellular targets cannot be excluded.

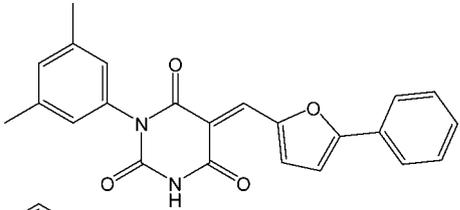
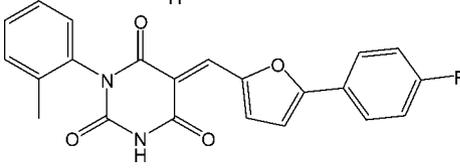
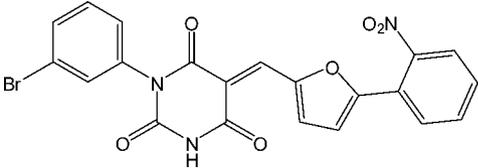
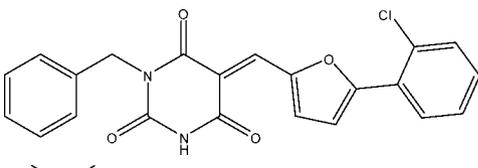
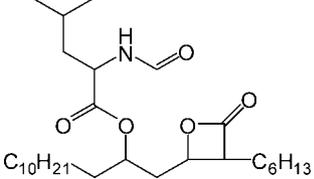
Discussion

The objective of this study was to identify novel antagonists of the thioesterase domain of human FAS that might be useful for further drug development. We screened more than 36,500 drug-like compounds and identified a 5-(furan-2-ylmethylene) pyrimidine-2,4,6-trione-based pharmacophore. Derivatives of this pharmacophore (a) are competitive inhibitors of the recombinant thioesterase, (b) inhibit the FAS holoenzyme and block fatty acid synthesis, and (c) elicit breast cancer cell death at concentrations significantly lower than those toxic to nonlipogenic breast epithelial cells. Based on these observations, these compounds represent a unique class of FAS antagonists that could be further developed as antineoplastic agents.

The compounds described here fulfill the Lipinski rule-of-five analysis, a guideline used to identify drug-like molecules for preclinical development (24). Lead compounds with high cLogP values are less likely to be successful drug candidates due to poor systemic absorption and membrane permeability. The FAS inhibitor orlistat ($\text{cLogP} = 8.609$), for example, is highly insoluble under physiologic conditions, with current use limited to the gut (25). The compounds presented in this study have $\text{cLogP} \approx 5$, suggesting the potential for both aqueous solubility and suitable membrane permeability. Therefore, we believe that

² <http://www.pannanugget.com>

Table 2. Structures, K_i values, and cytotoxicity of selected lead antagonists

Structure	K_i ($\mu\text{mol/L}$)	MB-MDA-435 IC_{50} ($\mu\text{mol/L}$)	MCF-10A IC_{50} ($\mu\text{mol/L}$)
	0.38 ± 0.1	3.47 ± 0.8	24.3 ± 3.0
	0.91 ± 0.2	4.1 ± 0.5	8.0 ± 2.3
	0.88 ± 0.10	8.9 ± 3.0	29.0 ± 14.6
	0.85 ± 0.16	9.5 ± 2.8	>50
	0.30 ± 0.09	20.0 ± 0.2	72.1 ± 1.6

NOTE: All data points were done in triplicate. IC_{50} values are the mean \pm SE of at least two experiments ($n = 2$). Individual E/Z configurations have not been determined for compounds 1 to 4. Compound 5 is orlistat, a known inhibitor of FAS thioesterase, which contains a β -lactone moiety.

the identified compounds represent a promising pharmacophore for development of drugs targeting FAS.

Both the initial screen for FAS thioesterase antagonists and the subsequent kinetic characterizations were done using 4-MUH as a mimic of the natural substrate. We found that the lead antagonists inhibited 4-MUH turnover by thioesterase and also blocked fatty acid synthesis by full-length FAS as measured via incorporation of a radiolabel. Therefore, the simplest interpretation of our findings is that the 4-methylumbelliferone substrate is a reasonable mimic of the natural substrate and that the lead antagonists we have identified can antagonize the thioesterase in near physiologic conditions. However, a small percentage of the lead antagonists, compound 4 in particular, showed no significant difference in FAS inhibition when the 5 $\mu\text{mol/L}$ and the 20 $\mu\text{mol/L}$ concentrations were compared. Future efforts to obtain cocrystal structures should provide insight into the inhibitory mechanisms of both classes (cyclic systems A and B). The position of the inhibitors in the large

palmitate binding pocket and the difference in binding, if any, between the two classes will be of particular interest and will guide future synthetic efforts.

Our findings also show that the lead antagonists are competitive antagonists of the thioesterase, meaning that they bind to the unoccupied enzyme and reduce turnover of substrate. FAS is a multidomain enzyme and contains an ACP to which the elongating fatty acid alkyl chain is bound during biosynthesis. The resulting palmitoyl-ACP complex is just 48 Å from the thioesterase active site (26) where it is hydrolyzed to free palmitate. Hence, the "effective concentration" of native substrate for the thioesterase is high. Competitive inhibitors must meet a high hurdle to compete with endogenous substrate. Given the substantial potency of the lead compounds even under these challenging conditions, it is likely that optimization of these lead inhibitors will yield compounds suitable for testing *in vivo*.

The barbituric acid moiety found in the pharmacophore is common to pharmaceutically important compounds,

such as phenobarbital and pentobarbital. Given the similarity in chemical structure between these drugs and the thioesterase antagonists, we tested phenobarbital and the core barbiturate moiety for the ability to inhibit the FAS thioesterase and found both to be without effect in the fluorogenic assay at concentrations up to 100 $\mu\text{mol/L}$ (data not shown). Additionally, the FAS thioesterase lacks any structural homology to the γ -aminobutyric acid-mediated chloride channel family of proteins targeted by phenobarbital and pentobarbital (27–29). Modeling of pentobarbital binding to chloride channels reveals steric hindrance of 5'-methylbutyl side chains with amino acids protruding from the ion channel (30–32). It is tempting to speculate that the bulky ring structures at positions R_1 and R_2 (see Table 1) found in cyclic systems A and B would likewise inhibit binding to the targets of current clinical barbiturates, which would render central nervous system side effects negligible.

Overall, the compounds identified here are promising leads that exhibit pharmacologic advantages over previously described FAS inhibitors. These compounds block fatty acid synthesis, exhibit selective cytotoxicity against FAS-dependent breast cancer cells, and satisfy the Lipinski rule-of-five analysis. Thus, our current study builds a solid framework for further optimization and preclinical testing of compounds containing the 5-(furan-2-ylmethylene)pyrimidine-2,4,6-trione pharmacophore.

Acknowledgments

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