Discovery of Inhibitors for the Ether Lipid-Generating Enzyme AGPS as Anti-Cancer Agents

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Supporting Information

ABSTRACT: Dysregulated ether lipid metabolism is an important hallmark of cancer cells. Previous studies have reported that lowering ether lipid levels by genetic ablation of the ether lipid-generating enzyme alkyl-glycerone phosphate synthase (AGPS) lowers key structural and oncogenic ether lipid levels and alters fatty acid, glycerophospholipid, and eicosanoid metabolism to impair cancer pathogenicity, indicating that AGPS may be a potential therapeutic target for cancer. In this study, we have performed a small-molecule screen to identify candidate AGPS inhibitors. We have identified several lead AGPS inhibitors and have structurally characterized their interactions with the enzyme and show that these inhibitors bind to distinct portions of the active site. We further show that the lead AGPS inhibitor 1a selectively lowers ether lipid levels in several types of human cancer cells and impairs their cellular survival and migration. We provide here the first report of in situ-active pharmacological tools for inhibiting AGPS, which may provide chemical scaffolds for future AGPS inhibitor development for cancer therapy.

Cancer cells possess fundamentally altered lipid metabolism that underlies cancer pathogenicity, including heightened de novo lipogenesis and lipolysis as well as aberrant incorporation of exogenous lipids. This altered lipid metabolism is required for cell proliferation, tumor growth, invasiveness, and metastasis. Lipids have diverse roles in driving cancer pathogenicity by contributing to cell membrane structure, formation of lipid rafts for oncogenic signaling, lipid signaling molecules that promote proliferation and tumor growth, and lipid-mediated post-translational modification of proteins.

Tumors also possess heightened levels of a particular lipid class, known as ether lipids, compared to normal tissues, and ether lipid levels have been correlated with proliferative capacity and tumorigenic potential of cancer cells. One or more ether linkages, rather than an ester linkage, on the glycerol backbone characterize ether lipids. While the precise roles of intracellular and circulating ether lipids are not yet clear, their particular physicochemical properties contribute to their biological importance in cellular structure, membrane fusion and vesicle formation, free radical scavenging, storage of lipid second messengers, and lipid signaling molecules. Ether lipid synthesis occurs in peroxisomes and begins with the esterification of dihydroxyacetone phosphate (DHAP) with a long-chain fatty acyl-CoA ester by the enzyme DHAP acyltransferase (DHAPAT) and subsequent replacement of the fatty acyl chain by a fatty alcohol to form alkyl-DHAP by alkyl-glycerone phosphate synthase (AGPS; Figure 1).

We recently demonstrated that the critical AGPS enzyme is heightened in aggressive cancer cells and primary human breast tumors and that its genetic ablation significantly impairs cancer aggressiveness and tumorigenesis. Metabolomic profiling revealed that AGPS knockdown in breast cancer cells lowers the levels of several ether lipid species, arachidonic acid, and arachidonic acid-derived prostaglandins. Quite intriguingly, the pathogenic impairments conferred by AGPS knockdown in cancer cells are due to the specific depletion of the oncogenic signaling lipid lysoosphatidic acid ether (LPAe) and prostaglandins. These studies indicated that AGPS may serve as an attractive therapeutic target for combating malignant human cancers, through altering the landscape of oncogenic signaling lipids that drive cancer aggressiveness.

Here, we have performed a small-molecule screen to identify AGPS inhibitors. We have identified several lead compounds whose inhibitory properties were investigated by biochemical and structural studies. One of the inhibitors is demonstrated to

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lower ether lipids and impair cancer pathogenicity in different types of human cancer cells. We put forth the discovery of the first AGPS inhibitors, which we hope will open the door for developing a new therapeutic strategy for targeting aggressive and metastatic tumors.

**RESULTS AND DISCUSSION**

**Identification of AGPS Inhibitors by thermoFAD-based Library Screening.** AGPS is a flavoenzyme that catalyzes the formation of alkyl-glycerone phosphate by displacing the acyl group of acyl-glycerone phosphate with a fatty alcohol. The enzyme is located in the peroxisomes. Inhibition of *Cavia porcellus* AGPS activity was assessed by a radioactivity assay using 100 μM palmitoyl-DHAP, 96 μM [1-14C]hexadecanol, and 180 μM inhibitor and detecting the formation of [1-14C]hexadecyl-DHAP as a function of time. The controls were performed using AGPS alone and the catalytically inactive AGPS mutant T578F. Measurements were performed at least in triplicate.

**Figure 1.** AGPS functional role and inhibition of AGPS activity by lead inhibitors. (A) AGPS catalyzes the formation of alkyl-glycerone phosphate by displacing the acyl group of acyl-glycerone phosphate with a fatty alcohol. The enzyme is located in the peroxisomes. (B) Inhibition of *Cavia porcellus* AGPS activity was assessed by a radioactivity assay using 100 μM palmitoyl-DHAP, 96 μM [1-14C]hexadecanol, and 180 μM inhibitor and detecting the formation of [1-14C]hexadecyl-DHAP as a function of time. The controls were performed using AGPS alone and the catalytically inactive AGPS mutant T578F.

**Table 1. Structure and Effect on AGPS Thermal Stability of the Identified Inhibitors**

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<tr>
<th>Inhibitor</th>
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<tr>
<td>ZINC-69435460*</td>
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<tr>
<td>1a*</td>
<td><img src="image2" alt="Structure" /></td>
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<tr>
<td>1e</td>
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<td>Antimycin A*</td>
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*ZINC-69435460 (3-(2-fluorophenyl)-N-(1-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-5-yl)ethyl)butanamide) was purchased from Molport in its double racemic mixture. The S,R enantiomer gave the best fitting to the electron density map (Supplementary Figure S1a) and, therefore, can be tentatively assigned as the enantiomer preferentially bound by the enzyme. 1a was synthesized as racemic mixture (see Supplementary Information). The S enantiomer gave the best fitting to the electron density map (Figure 2a) and can be tentatively assigned as the enantiomer preferentially bound by the enzyme. Commercial antimycin A from Sigma-Aldrich is a mixture of Antimycin A1 (R = C₆H₁₃), A2 (R = C₅H₁₁), A3 (R = C₄H₉), and A4 (R = C₃H₇).
S-(4-ethyl)butanamide (ZINC-69435460) and the antifungal agent antimycin A (from Prestwick Chemical Library).

We next wanted to confirm whether these lead compounds inhibited AGPS activity. We used a radioactivity-based enzymatic assay, using palmitoyl-DHAP and [1-14C]-inhibited AGPS activity. We used a radioactivity-based detection, but our data show that the two lead compounds of the lipid substrates and the low sensitivity of radioactive inhibition and isothermal calorimetry but were unsuccessful.


Three-dimensional Structure of AGPS Bound to the Inhibitors ZINC-69435460 and Antimycin A. To explore the binding mechanisms between AGPS and the identified inhibitors, the crystal structures of AGPS in complex with ZINC-69435460 and antimycin A were determined by X-ray crystallography at 2.0−2.2 Å resolution, enabling a detailed view of the inhibitor binding (Table 2, Figure 2, Supporting Information Figure S1A).

In both cases, inhibitors reside in the V-shaped active site, whose vertex is the putative substrate cavity, in front of the FAD. Most importantly, both inhibitors establish specific interactions with the protein residues, but their binding mode and location in the active site are significantly different (Figure 3). The two molecules can therefore be considered as distinct lead compounds with regard to their mode of binding and locate two, only partially overlapping, druggable sites within the protein.

ZINC-69435460 (Table 1) binding causes localized rearrangements in the side chains of residues in the active site to enable inhibitor binding (Supporting Information Figure S1A). The dihydrobenzimidazole moiety occupies the hydrophilic DHAP site, making π-stacking interactions on the FAD isoalloxazine ring and preventing the access to the reactive site N5 atom of the flavin (Figure 2B, Supporting Information Figure S1A). On the other hand, the more hydrophilic amide substituent points with the fluorophenyl ring toward the aliphatic tunnel that hosts the acyl chain of the substrate. Binding is mediated by hydrophobic interactions in the substrate acyl-chain tunnel and by H-bonds with several residues, including His616 and His617, which belong to an active-site loop, directly involved in catalysis (so-called HHH loop).

Antimycin A shows a different mode of binding in that this compound resides on an outer segment of the active site without any direct contact with the flavin (Figure 2C).

Table 2. Crystal Data Collection and Refinement Statistics

<table>
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alkyl chain pointing toward (but not in direct contact with) the flavin. Conversely, the butanoate substituent of the inhibitor is extended along the substrate tunnel, in the direction of the so-called "gating helix" (Figures 2A,C and 3B). Finally, the 3-formylamino salicylic acid portion interacts with a positive charged region at the entrance of the active site, interlacing H-bonds with residues surrounding the catalytic site. The binding does not cause dramatic changes in the protein conformation, but as for ZINC-69435460, small changes in side chains orientation do occur, especially with regard to two Arg (Arg419 and Arg566) residues that H-bond to the inhibitor (Figure 2C).

In essence, ZINC-69435460 binds deeper into the active site in direct contact with FAD, whereas antimycin A acts as a plug that obstructs access to the hydrophobic active-site tunnel (Figure 3).

Structure–Activity Relationships Surrounding ZINC-69435460 Derivatives. The investigation of antimycin A was insightful with regard to its mode of binding and to uncover druggable sites in the protein. However, as it is a known inhibitor of cytochrome C reductase, it is not an attractive lead compound for further investigations. Along this line, we focused our efforts on the synthesis and evaluation of several structurally related analogs of ZINC-69435460. All molecules were tested in ThermoFAD and, in the case of a positive signal,
also in inhibition assays. With only two exceptions (see below), all compounds showed no strong interactions as judged from the absence of a detectable shift in the melting temperature (Supporting Information Table S1). These results highlighted the specificity of inhibitor recognition by AGPS as all three inhibitor moieties, fluorophenyl, butanamido linker, and dihydrobenzimidazole ring, are involved in specific polar and van der Waals interactions with the residues lining the innermost segment of V-shaped active site tunnel (Figure 3A). For instance, any modification of the hydrogen-bonding groups on the dihydrobenzimidazole ring turned out to be detrimental for binding (Supporting Information Table S1).

Among the tested ZINC-69435460 derivatives, only 1a and 1e featured detectable AGPS inhibition (Table 1, Figure 1B). 1e was synthesized with the idea that a double bond on the linker moiety would increase rigidity. As gathered from enzymatic and thermal shift assays, the compound demonstrated good binding and inhibition although not as strong as the parent compound. Interestingly, the structural analysis revealed some alterations in the binding conformation with the dihydrobenzimidazole being flipped compared to the structure observed for ZINC-69435460, although the hydrogen-bonding interactions between inhibitor and the protein remain unaffected (Supporting Information Figure S1B). The more rigid double-bond containing linker of 1e possibly causes a suboptimal fit in the active site accounting for the observed reduction in binding affinity and inhibitory efficacy ($\Delta T_m 2^\circ C$ smaller than that of ZINC-69435460; Table 1).

1a differs from the parent compound simply for a methyl-to-hydrogen substitution on the linker portion of the inhibitor (Table 1). The molecule demonstrated to be as effective in AGPS inhibition as ZINC-69435460 (estimated $K_i \sim 500 \text{ nM}$). Furthermore, the crystal structure showed that the binding was identical to that of the parent compound, the removal of the methyl substituent having no effect on the conformation of both inhibitor and interacting protein residues (Figure 2B, Supporting Information Figure S1A). 1a is more attractive as a lead compound as it lacks one of the two chiral centers of ZINC-69435460, due to the elimination of a methyl substituent (Table 1). Therefore, given its strong binding affinity and inhibitory activity, we chose this molecule as a lead compound for further biological evaluation.

AGPS Inhibitor 1a Lowers Ether Lipid Levels in Cancer Cells. We previously demonstrated that genetic knockdown of AGPS with RNA interference in malignant breast and melanoma cancer cells dramatically lowered both structural and oncogenic signaling ether lipid levels. We thus next sought to determine whether the representative inhibitor 1a leads also lower ether lipid levels in human cancer cells. We treated C8161 melanoma, 231MFP breast, and SKOV3 ovarian cancer cells with 1a (500 $\mu M$) and subsequently performed targeted single-reaction monitoring (SRM)-based lipidomic profiling of >120 lipid species, encompassing fatty acids, neutral lipids, phospholipids, sphingolipids, eicosanoids, and ether lipids. We show that 1a primarily lowers the levels of ether lipids across all three cancer cell lines, largely without affecting the levels of lipids from other classes (Figure 4). More importantly, we show that the levels of the oncogenic signaling lipid LPae, which we previously showed to be a driver of AGPS-mediated effects upon cancer pathogenicity, were also lowered upon 1a treatment (Figure 4). We did not observe ether lipid lowering upon treatment of cancer cells with a lower concentration of 1a (50 $\mu M$), possibly due to issues with cell (and/or peroxisome) penetrance (data not shown). Nonetheless, our results indicate that 1a inhibits AGPS in situ and rather selectively lowers the levels of ether lipids across multiple different types of human cancer cells.

AGPS Inhibitor 1a Impairs Cancer Pathogenicity. We previously showed that AGPS genetic knockdown in cancer cells impaired cell survival and migration. Having established that 1a treatment evidently inhibits AGPS in situ and is capable of lowering ether lipid levels in cancer cells, we next tested whether 1a and a pharmacological blockade of AGPS also impairs cancer pathogenicity. Indeed, we show that 1a treatment impairs serum-free cell survival and migration in C8161 melanoma, 231MFP breast, and SKOV3 ovarian cancer cells (Figure 5). Importantly, these results overall recapitulate the pathogenic impairments observed previously with genetic knockdown of AGPS in cancer cells.

Conclusion. In this study, we put forth the first report of an AGPS inhibitor that directly binds to the enzyme active site, inhibits enzymatic activity, lowers ether lipid levels, and impairs pathogenicity in several human cancer cells. We also present a medium-throughput pipeline to screen for AGPS inhibitors, which can be used in subsequent studies, coupled with insights from our structural analysis of inhibitor-AGPS interactions, to optimize the potency, selectivity, and in vivo efficacy of future AGPS inhibitors.

Targeting dysregulated lipid metabolism has arisen as an attractive strategy toward combatting human cancer. Prominent examples include targeting de novo fatty acid synthesis through fatty acid synthase inhibition, targeting dysregulated lipolysis and fatty acid release by inhibiting monoacylglycerol lipase, manipulating tumor-suppressing lipids by inhibiting platelet activating factor acetylhydrolase 1B2 and 1B3 (PAFAH1B2 and PAFAH1B3), or targeting specific aspects of ether lipid metabolism by inhibiting the 2-acetylmonoaicylglycerol ether hydrolase KIAA1363. We recently characterized AGPS as a dysregulated enzyme in cancer that drives the heightened ether
Figure 4. Data showing that AGPS inhibitor 1a lowers ether lipid levels in cancer cells. Shown are lipidomic profiling data from C8161 melanoma (A), 231MFP breast (B), and SKOV3 ovarian (C) cancer cells treated with vehicle (DMSO) or 1a (500 μM) for 24 h. Shown in heat maps are relative levels compared to control for each lipid measured by targeted SRM-based LC-MS/MS lipidomics. Darker and lighter blue compared to control indicates elevated or reduced levels, respectively, upon 1a treatment compared to controls. Bar graphs show lipid species that were significantly (p < 0.05) changed >2-fold upon 1a treatment compared to vehicle-treated controls. Data shown are from the n = 5/group. Data in bar graphs are shown as average ± SEM. Lipid abbreviations are as follows: LPA, lysophosphatidic acid; AC, acyl carnitine; MAG, monoalkylglycerol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; LPI, lysophosphatidylinositol; PA, phosphatidic acid; PI, phosphatidyl inositol; NAE, N-acylethanolamine; TAG, triacylglycerol; DAG, diacylglycerol; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol. Lower case “e” and “p” letters indicate ether and plasmalogen phospholipids, respectively.
lipid metabolism that has been historically correlated with highly proliferative human tumors. We showed that genetic knockdown of AGPS by RNA interference impaired cellular survival, motility, and invasiveness through lowering key oncogenic signaling lipids and altering the overall landscape of fatty acid metabolism to impair other protumorogenic lipid signaling molecules that subserve cancer aggressiveness. We also showed that AGPS knockdown had dramatic effects upon in vivo tumor xenograft growth in mice, indicating that AGPS inhibitors may have therapeutic benefit in cancer treatment.

We report here 1a as a lead AGPS inhibitor which shows in vitro inhibition of AGPS activity and apparent in situ AGPS inhibition as demonstrated by the lowering of ether lipid levels and impaired cancer pathogenicity that recapitulate the effects observed with genetic knockdown of this enzyme. The lead inhibitors reported and characterized here can serve as scaffolds for future structure−activity relationship studies to optimize potency, selectivity, and pharmacokinetic parameters so that AGPS inhibitors can be tested for their safety and efficacy in vivo in preclinical and clinical settings. While loss-of-function mutations in AGPS in humans have been shown to cause neurodevelopmental and neurobehavioral deficits through impairing peroxisomal function, whether pharmacological inhibition of AGPS in adulthood would cause toxicity remains unknown. Thus, the development of in vivo efficacious AGPS inhibitors will be crucial in assessing the future promise of AGPS inhibitors for cancer therapy. They will also be very valuable tools for further investigations of the biology of ether phospholipids.

■ MATERIALS AND METHODS

Protein Production and Crystallization. Expression, purification, and crystallization of Cavia porcellus AGPS were performed according to the methods previously described. Enzyme crystals obtained by siting-drop vapor diffusion at 20 °C mixing protein 8 mg mL⁻¹ and 20−24 wt %/vol PEG 1500 in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) at pH 7.5 were soaked with the cryo-protectant solution (30 wt %/vol PEG 1500 in 100 mM Hepes, pH 7.5) containing 1 mM inhibitor o/n at 20 °C and then washed in cryo-protectant solution without inhibitor before freezing (Table 2).

ThermoFAD Assays. ThermoFAD experiments were performed with 5 μM AGPS, 180 μM inhibitors, 50 mM K₂HPO₄ buffer at pH 7.5, 50 mM NaCl, 5% glycerol, and 20 μL final volume. The temperature gradient was set to 25−70 °C with fluorescence detection every 0.5 °C at 485 ± 30 nm excitation and 625 ± 30 nm emission for 5 s (BioRad MiniOpticon Real-Time PCR System). To estimate the binding of the identified compounds, the same ThermoFAD protocol was followed, using serial dilutions of the compounds from 500 μM to 10 nM. To estimate the affinity constant, ΔH⁻⁻ of AGPS was
calculated according to eq 6 in Matulis et al.14 and Δ\textsubscript{235}C\textsubscript{4} was estimated according to AGPS size and structure.21 The calculation of the estimated K\textscript{a} was performed according to eq 14 in Matulis et al.14 considering a range of uncertainty due to the reasonable assumption of Δ\textsubscript{A}H\textsubscript{ep} being 40 kJ/mol (relatively unimportant for the overall K\textsubscript{a} calculation).

**Enzymatic Assays.** Activity assays were performed under the same conditions previously described using radioactive [1-14C]-hexadecanol.\textsuperscript{9,22} The final concentrations were as follows: 500 nM AGPS, 50 mM Tris/HC1 at pH 8.2, 50 mM NaF, 0.1% (v/w) Triton X-100, 100 μM palmitoyl-DHAP\textsuperscript{23} 96 μM [1-14C]-hexadecanol (Sigma; specific radioactivity adjusted to 13 000 dpm/nmol) in a total volume of 100 μL at 36 °C. The palmitoyl-DHAP stock solution was sonicated before usage. Aliquots (10 μL) were withdrawn at different times and spotted on DEAE cellulose disks. After an extensive wash with ethanol, the disks were transferred to scintillation vials and counted by means of a Tri-Carb 2100TR (Packard) scintillation counter to measure the amount of the radioactive [1-14C]-hexadecanol-DHAP product. When appropriate, an inhibitor or the vehicle (DMSO) was added to the reaction mixture to a final concentration of 180–200 μM after preincubation with the protein for 30 min on ice.

**Targeted Lipidomic Analyses.** Metabolite measurements were conducted using modified previous procedures.\textsuperscript{9} Cancer cells were grown in serum-free media for 24 h to minimize the contribution of serum-derived metabolites to the cellular profiles. Cells were treated with 1a (500 μM) for 24 h. Cancer cells (1 × 10\textsuperscript{6} cells/6 cm dish or 2 × 10\textsuperscript{6} cells/6 cm dish) were washed twice with phosphate buffer saline (PBS), harvested by scraping, and isolated by centrifugation at 1400 g at 4 °C, and cell pellets were flash frozen and stored at −80 °C until metabolome extractions. Lipid metabolites were extracted in 4 mL of a 2:1:1 mixture of chloroform:methanol:phosphate buffer (pH 7.4, 50 mM) with inclusion of internal standards C12:0 dodecylglycerol (10 nmol) and pentadecanoic acid (10 nmol). Organic and aqueous layers were separated by centrifugation at 1000 g for 5 min, and the organic layer was collected. The aqueous layer was acidified (for metabolites such as LPA) by adding 0.1% formic acid, followed by the addition of 2 mL of chloroform. The mixture was vortexed, and the organic layers were combined, dried down under N\textsubscript{2}, and dissolved in 2 mL of chloroform:methanol:phosphate buffer saline (pH 7.4, 50 mM) before seeding cells into survival and migration assays. For 231 MFP cells, rather than the addition of WST-1 cell proliferation reagent, cell viability was measured at 450 nm using a spectrophotometer. For 231 MFP cells, migration was measured at 5, 6, and 8 h, respectively.

**Additional experimental details, compound characterization, and assays (PDF)**

**Accession Codes**

Atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org, with accession codes 5adz (complex with 1a), Sae1 (complex with ZINC-69435460), Sae2 (complex with 1e), and Sae3 (complex with antimycin A).

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00466.

Additional experimental details, compound characterization, and assays (PDF)


