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

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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*-effective pharmacological tools for inhibiting AGPS, which may provide chemical scaffolds for future AGPS inhibitor development for cancer therapy.

Introduction

Cancer cells possess fundamentally altered lipid metabolism that underlies cancer pathogenicity, including heightened *de novo* lipogenesis and lipolysis as well as aberrant

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The atomic coordinates and structures have been deposited in the Protein Data Bank, www.pdb.org.

AUTHOR CONTRIBUTIONS

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SUPPORTING INFORMATION

Additional experimental, compound characterization, and assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

incorporation of exogenous lipids. This altered lipid metabolism is required both 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 [1].

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 [2–5]. 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 is not yet clear, their particular physicochemical properties contribute to their biological importance in cellular structure, membrane fusion and vesicle formation, free radicals 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 acyl-transferase (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) [6–8].

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 lysophosphatidic acid ether (LPAe) and prostaglandins. These studies indicated that AGPS may serve as an attractive therapeutic target for combatting 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 lower ether lipids and impair cancer pathogenicity in multiple 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 using fatty alcohol and fatty acyl fatty acyl DHAP as substrates. The flavin of AGPS enables the acyl/alkyl exchange by covalently reacting with DHAP through an unusual non-redox catalytic mechanism [7–9].

Protein thermal stabilization assay is a well-established medium-throughput method to screen for strongly binding ligands. A variant of the classic ThermoFluor assay, ThermoFAD, measures the unfolding temperature of the protein by monitoring the increase in cofactor flavin adenine dinucleotide (FAD) fluorescence upon release from the protein [10]. In this way, artifacts caused by usage of fluorescent dyes are bypassed. We chose AGPS from *C. porcellus*, as a suitable system for inhibitor screening because of its stability and suitability for crystallographic studies [8]. We screened an initial set of 1360 small molecules from the Prestwick Chemical Library[®] that encompasses 1280 approved drugs and a subset of the Zinc database [11], at 180 μ M against purified AGPS (5 μ M protein). We identified lead compounds that affected the thermal stability of AGPS, increasing the melting temperature of the protein by 4° C (Table 1). They included (3R)-3-(2-fluorophenyl)-N-[(1R)-1-(2-oxo-1,3-dihydrobenzimidazol-5-yl)ethyl]butanamide (ZINC-69435460), and the anti-fungal agent Antimycin A (from Prestwick Chemical Library) [12].

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]hexadecanol as substrates and detecting the formation of [1-¹⁴C]hexadecanyl-DHAP as function of time in presence of an excess of inhibitor (180 μ M) (Figure 1B). We were unable to calculate K_i values using this approach due to the insolubility of the lipid substrates and the low sensitivity of radioactive detection, but our data show that the three lead compounds inhibit AGPS enzymatic activity. We also attempted to measure the binding of the inhibitors to AGPS using surface plasmon resonance and isothermal calorimetry, but were unsuccessful due to insolubility of the compounds. Nonetheless, we estimated the affinity constants of the identified compounds as 200–700 nM for both Antimycin A and ZINC-69435460 through thermal shift assay according to procedures established by *Matulis et al* [13].

Three-Dimensional Structure of AGPS in Complex with 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 2.1–2.2 Å resolution, enabling a detailed view of the inhibitor binding (Figure 2, 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 partly overlapping, druggable sites within the protein.

ZINC-69435460 (Table 1) makes multiple specific interactions with residues in the AGPS active site. 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 S1A, 2B). On the other hand, the more hydrophobic amidic 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”) [8–9]. ZINC-69435460 binding causes localized rearrangements in the side chains of residues in the active site to enable inhibitor binding (Figure S1A).

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). Antimycin A consists of three portions: the dilactone ring in the middle, the 3-formylamino salicylic acid group, that is linked to the dilactone ring through an amide bond, and the hydrophobic alkyl and a 1-methyl butanoate tails on the other side of the dilactone ring. Commercial Antimycin A is a mixture of four compounds, differing in the length of the alkyl chain: Antimycin A₁ (R = C₆H₁₃), A₂ (R = C₅H₁₁), A₃ (R = C₄H₉) and A₄ (R = C₃H₇) (Table 1). From the crystal structure in complex with AGPS, it is evident that Antimycin A₁ is binding to the enzyme as its bulky six-carbon side chain nicely fits the electron density. The dilactone ring is sitting in hydrophobic pocket in the central segment of the active-site tunnel with the alkyl chain pointing towards (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” (Figure 2A, 2C, 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 known inhibitor of cytochrome C reductase, it is not an attractive lead compound for further investigations [14]. Along this line, we focused our efforts on the synthesis and evaluation of several structurally related analogues 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 (Table S1). These results highlighted the specificity of inhibitor recognition by AGPS as all three inhibitor moieties, fluorophenyl, benzamide 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 2B, 3A). For instance, any modification of the hydrogen-bonding groups on the dihydrobenzimidazole ring turned out to be detrimental for binding (Table S1). Among the tested ZINC-69435460 derivatives and analogues, 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 to retain 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 (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 (T_m 2° smaller than that of ZINC-69435460; Table 1, Figure 1B). **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 ~500 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, S1A). **1a** is more attractive as lead compound since it lacks a chiral center due to the elimination of a methyl-substituent. Therefore, given its strong binding affinity and inhibitory activity, we chose this molecule as 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 [15]. We thus next sought to determine whether the representative inhibitor **1a** leads also lowers ether lipid levels in human cancer cells. We treated C8161 melanoma, 231MFP breast, and SKOV3 ovarian cancer cells with **1a** (500 μ 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 4A, 4B, 4C). More importantly, we show that the levels of the oncogenic signaling lipid LP Ae, 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 μ 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 inhibited AGPS *in situ* and was capable of lowering ether lipid levels in cancer cells, we next tested whether **1a** and 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 5A–5F). Our 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 towards 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-acetylmonoalkylglycerol ether hydrolase KIAA1363 [16–17]. We recently characterized AGPS as a dysregulated enzyme in cancer that drives the heightened ether 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 protumorigenic 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. While the inhibitors reported here are unlikely to be *in vivo*-active, we hope that 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 [18], 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

Protein expression, purification and crystallization were performed according to the methods previously described [8]. AGPS crystals obtained by sitting-drop vapor diffusion at 20° C mixing protein 8 mg/ml and 20–24% wt/vol PEG 1500 in Na/Hepes pH 7.5–100 mM were soaked with the cryo-protectant solution (30% wt/vol PEG 1500 in Na/Hepes pH 7.5–100

mM) containing 1 mM inhibitor o/n at 20°C and then washed in cryo-protectant solution without inhibitor before freezing.

ThermoFAD assays

ThermoFAD experiments were performed with cpAGPS 5 μ M, inhibitors 180 μ M, KPO₄ buffer 50 mM pH 7.5, 50 mM NaCl, 5% glycerol, 20 μ l final. Temperature gradient 25–70°C, fluorescence detection every 0.5°C at 485 $\pm$ 30 nm excitation and 625 $\pm$ 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 μ M to 10 nM, tested with 5 μ M AGPS in KPO₄ buffer 50 mM pH 7.5, 50 mM NaCl, 5% glycerol, 20 μ l final. To estimate the affinity constant, ΔH_T of AGPS was calculated according to equation 6 [13] using ThermoFAD output, and ΔC_p was estimated according to AGPS size and structure [19]. The calculation of the estimated K_d was performed according to equation 14 [13], intentionally considering a range of uncertainty due to the reasonable assumption of ΔH_{Tm} being 40 kJ/mol (relatively unimportant for the overall K_d calculation).

Enzymatic assays

Activity assays were performed in the same conditions previously described using radioactive [1-¹⁴C]hexadecanol [9, 20]. The following final concentrations were used: 500 nM AGPS, 50 mM Tris/HCl pH 8.2, 50 mM NaF, 0.1% (w/v) Triton X-100, 100 μ M palmitoyl-DHAP [21], 96 μ M [1-¹⁴C]hexadecanol (Sigma; specific radioactivity adjusted to 13000 dpm/nmol) in a total volume of 100 μ l at 30° C. The palmitoyl-DHAP stock solution was sonicated before usage. Aliquots (10 μ l) 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]hexadecanyl-DHAP product. When appropriate, an inhibitor or the vehicle (DMSO) was added to the reaction mixture to final concentration of 180–200 μ M after pre-incubation with the protein for 30 min on ice.

Targeted lipidomic analyses

Metabolite measurements were conducted using modified previous procedures [15]. Cancer cells were grown in serum-free media for 24 hr to minimize the contribution of serum-derived metabolites to the cellular profiles. Cells were treated with **1a** (500 μ M) for 24 h. Cancer cells (1 $\times$ 10⁶ cells/6 cm dish or 2 $\times$ 10⁶ cells/6cm dish) were washed twice with phosphate buffer saline (PBS), harvested by scraping, and isolated by centrifugation at 1400 $\times$ 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:Tris buffer 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 $\times$ 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 chloroform. The mixture was vortexed, and the organic layers were combined, dried down under N₂ and dissolved in 120 μ l chloroform, of which 10 μ l was

analyzed by both single-reaction monitoring (SRM)-based LC-MS/MS or untargeted LC-MS. LC separation was achieved with a Luna reverse-phase C5 column (50 mm × 4.6 mm with 5 µm diameter particles, Phenomenex). MS analysis was performed with an electrospray ionization (ESI) source on an Agilent 6430 QQQ LC-MS/MS. Representative metabolites were quantified by SRM of the transition from precursor to product ions at associated collision energies.

Cell survival and migration studies

Cell survival studies were performed as previously described [15, 22]. Cells were pre-incubated with **1a** (500 µM) before seeding cells into survival and migration assays. For survival assays, cells were washed twice in PBS, harvested by trypsinization, washed in serum-free media and cells were seeded into 96-well plates (30,000 cells per well in a volume of 200 µl containing serum-free media) for 48 h prior to addition of WST-1 (10 µl) for 1 h at 37°C in 5 % CO₂. Absorbance was then measured at 450 nm using a spectrophotometer. For 231MFP cells, rather than the addition of WST-1, cell viability was measured by first aspirating the media followed by the addition of 100 µl of fixation solution containing 5 µM Hoescht in formalin. The fixation solution was aspirated and the cells were washed with PBS. Absorbance was then measured using a fluorescent plate reader with an excitation of 350 nm and an emission of 461 nm. Migration assays were performed as previously described [15]. Migration assays were performed in Transwell chambers (Corning) coated with collagen. SKOV3, C8161, and 231MFP migration was measured at 5, 6, and 8 h, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. AGPS functional role and inhibition of AGPS activity by lead inhibitors

(A) AGPS catalyzes the formation of alkyl-DHAP from displacement of the acyl group by a fatty alcohol from the substrate acyl-DHAP. The enzyme is located in the peroxisomes. **(B)** Inhibition of *Cavia porcellus* AGPS activity was assessed by a radioactivity assay using 100 μ M palmitoyl-DHAP, 100 μ M [1-14C]hexadecanol, 180 μ M inhibitor and detecting the formation of [1-14C]hexadecanoyl-DHAP as function of time. The controls were performed using AGPS alone and the catalytically inactive AGPS mutant Thr578Phe. Measurements were performed at least in triplicate [8].

Figure 2. Structural studies on *Cavia porcellus* AGPS in complex with the inhibitors **1a and Antimycin A**

(A) Overall view of the dimeric enzyme bound to **1a** (blue spheres). The inhibitor is extended along the hydrophobic substrate tunnel, delimited by the “gating helix” (residues 126–137). AGPS is known to be associated to the luminal side of the peroxisomal membrane as outlined in the figure [9]. FAD is shown in yellow. The overall rms deviation between the unbound protein (PDB entry 4bby) and the inhibitor complex is 0.36 Å. (B) Weighted 2Fo-Fc final electron density map contoured at 1.2 σ level for **1a** in the same orientation of the left subunit shown in Figure 2A. Binding involves specific H-bonds (black dashed lines) with the catalytic residues Tyr580, His616, and His617. The position and the distance of the inhibitor suggest also π interactions with the cofactor isoalloxazine ring. A few side chains change their conformation with respect to the unbound enzyme (light orange). (C) Final weighted 2Fo-Fc map (1.2 σ contour level) for Antimycin A-enzyme complex. The electron density is fully consistent with Antimycin A₁ (6-carbon alkyl moiety) being bound to the protein. Inhibitor binding causes the rearrangement of several residues around the active site (in orange superposition with native AGPS residues).

Figure 3. Electrostatic potential surfaces of AGPS in complex with the inhibitors **1a (A) and Antimycin A (B)**

Negatively and positively charged regions are shown in red and blue, respectively. The orientation highlights the tripartite organization of the active site: the catalytic core close to the flavin, the positively charged entrance tunnel, and the more hydrophobic tunnel which is proposed to accommodate the alkyl moiety of the substrate [8]. The two inhibitors bind in only partly overlapping active-site regions. The aliphatic portion of Antimycin A is mainly extended along the lipophilic tunnel whereas the formamide-substituted aromatic ring interacts with the positively charged patch of the entrance tunnel. Conversely, **1a** binds deeper inside active site as compared to Antimycin A. The dihydrobenzimidazole moiety stacks in front of FAD whereas the benzene ring extends along the lipophilic tunnel, as for the Antimycin A alkyl chain. In both cases, the ligands are not solvent accessible and far from the surface.

Figure 4. 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 μ M) 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 $n = 5$ /group. Data in bar graphs are shown as average $\pm$ SEM.

Figure 5. AGPS inhibitor impairs cancer cell pathogenicity

Shown are effects of **1a** upon cell survival and migration in C8161 melanoma (**A, B**), 231MFP breast (**C, D**), and SKOV3 ovarian (**E, F**) cancer cells. Vehicle (DMSO) or **1a** were pre-incubated with cancer cells before seeding cells into serum-free survival or migration assays. Data shown as average $\pm$ SEM, $n=3-4$ /group. Significance is represented as * $p<0.05$ in relation to control groups.

Table 1

Structure and effect on AGPS thermal stability of the identified inhibitors.

		T_m
ZINC-69435460		+3.5°C
1a		+3.5°C
1e		+2.0°C
Antimycin A ^a		+4°C

^aCommercial Antimycin A from Sigma-Aldrich is a mixture of Antimycin A1 (R = C₆H₁₃), A2 (R = C₅H₁₁), A3 (R = C₄H₉) and A4 (R = C₃H₇)