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SPHK1 is a novel target of metformin in ovarian cancer

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Abstract

The role of phospholipid signaling in ovarian cancer (OvCa) is poorly understood. Sphingosine-1-phosphate (S1P) is a bioactive metabolite of sphingosine that has been associated with tumor progression through enhanced cell proliferation and motility. Similarly, sphingosine kinases (SPHK), which catalyze the formation of S1P and thus regulate the sphingolipid rheostat, have been reported to promote tumor growth in a variety of cancers. The findings reported here show that exogenous S1P or overexpression of SPHK1 increased proliferation, migration, invasion and stem-like phenotypes in OvCa cell lines. Likewise, overexpression of SPHK1 markedly enhanced tumor growth in a xenograft model of OvCa, which was associated with elevation of key markers of proliferation and stemness. The diabetes drug, metformin, has been shown to have anti-cancer effects. Here, we found that OvCa patients taking metformin had significantly reduced serum S1P levels, a finding that was recapitulated when OvCa cells were treated with metformin and analyzed by lipidomics. These findings suggested that in cancer the sphingolipid rheostat may be a novel metabolic target of metformin. In support of this, metformin blocked hypoxia-induced SPHK1, which was associated with inhibited nuclear translocation and transcriptional activity of hypoxia-inducible factors (HIF1 α and HIF2 α). Further, OvCa cells with high SPHK1 were found to be highly sensitive to the cytotoxic effects of metformin while OvCa cells with low SPHK1 were resistant. Together, the findings reported here show that hypoxia-induced SPHK1 expression and downstream S1P signaling promote OvCa progression and that tumors with high expression of SPHK1 or S1P levels might have increased sensitivity to the cytotoxic effects of metformin.

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Conflict of Interest:

The authors declare that there are no competing interests.

Keywords

S1P; SPHK1; metformin; ovarian cancer

Introduction

Biologically active lipids are key signaling molecules that mediate various aspects of cellular communication and play a role in several oncogenic processes, including cell proliferation, migration, survival, and pluripotency (1). Sphingolipids are a unique group of bioactive lipids thought to regulate tumor progression (2). For example, ceramides and sphingosine induce apoptosis and cell senescence, whereas sphingosine-1-phosphate (S1P) promotes cell growth, proliferation and migration (3-5). The balance between ceramide/sphingosine and S1P may influence cancer cell fate and is referred to as the sphingolipid rheostat (Figure 1A, reviewed in (6)).

Sphingosine kinases (SPHKs), including SPHK1, catalyze the formation of S1P from sphingosine and are pivotal regulators of the balance between ceramides, sphingosine and S1P. Increased expression of SPHK1 promotes tumor growth and metastasis in breast, colorectal and lung cancers (1, 3, 7, 8), and the role of the S1P pathway is becoming increasingly relevant in OvCa (9, 10). Two older studies reported higher S1P levels in the serum and ascites in OvCa patients compared to controls (11, 12) and a more recent study showed that SPHK1 is highly expressed in tumor stroma of high-grade serous ovarian cancer (13). Since increasing evidence suggests that SPHK1 is tumor promoting, attempts have been made to inhibit tumor growth by targeting this kinase (14). Unfortunately, progress with this approach has been limited by the toxicity of the agents (1). One approach to overcome drug toxicity is to utilize *drug repurposing* where efforts are focused on agents that already have demonstrated safety and are widely used for non-cancer indications (15).

Metformin is a lead candidate for drug repurposing in cancer. The diabetes drug started receiving considerable attention as a potential cancer therapeutic after retrospective studies reported that use of metformin by patients with diabetes was associated with improved survival in several types of cancer, including OvCa (16, 17). Pre-clinical data then began to accumulate demonstrating anti-cancer effects of metformin in several types of cancer (18). In OvCa, we demonstrated that metformin inhibits cancer growth *in vitro* and in multiple mouse models (19, 20). Although the preliminary findings in multiple cancer types are promising, metformin's mechanism of action in cancer has been difficult to define. Early studies focused on metformin's activation of AMPK (21) and alteration of serum insulin levels (22). In a recent study, we focused on defining global metabolic changes and demonstrated that metformin accumulates in human ovarian tumors at concentrations that inhibit cell intrinsic mitochondrial function including altering levels of tricarboxylic acid (TCA) cycle intermediates (23).

Pre-clinical data demonstrating anti-cancer effects of metformin has led to several prospective clinical trials testing metformin as a treatment for various cancers (24), including our ongoing phase II randomized trial of metformin as upfront treatment for OvCa (NCT02122185). However, if metformin is to advance as a cancer treatment, it is critically

important that the molecular mechanisms of action in cancer be defined and that biologic predictors of response be identified. Given the effects of metformin on tumor metabolism (23) and the drug's ability to alter lipid signaling (22, 25), in this study, we postulated that metformin's anti-cancer effects may be mediated, in part, through regulation of the S1P rheostat and that activity of this pathway might predict response to metformin in OvCa.

The findings show that elevated SPHK1 expression and S1P levels promote OvCa growth by increasing multiple oncogenic processes including proliferation and self-renewal *in vitro* as well as increased tumor growth *in vivo*. Moreover, we identified a novel mechanism by which metformin inhibits activity of hypoxia inducible factors (HIF1 α and HIF2 α) to repress SPHK1. Together, these findings support a critical role for S1P homeostasis in OvCa tumor progression, and show that tumors that possess an activated S1P metabolic profile might be uniquely susceptible to the anti-cancer effects of metformin.

Materials and Methods

Patient samples

Subjects provided informed consent and fasting serum and tumor samples were collected as part of an ongoing biorepository approved by the University of Chicago Institutional Review Board. Tissue microarrays were constructed from tumor implants collected at multiple abdominal sites during debulking surgery. The intensity, frequency and distribution of SPHK1 immunohistochemical staining was quantified by two subspecialty-trained gynecologic pathologists (RL and SM). Weak staining was scored for very focal and light intensity of SPHK1, whereas strong staining was scored for high frequency and dark intensity. Serum metabolite analysis was performed as described previously (26). For analysis of serum S1P levels, global metabolic profiling was performed during a prior study by our group (23), and re-analyzed for S1P content between control and metformin-treated groups. Briefly, Q-Exactive mass spectrometer was used for untargeted metabolomics with the SIEVE package (Thermo Fisher Scientific, Waltham, MA), and ions with larger changes in the treatment group (fold change > 2) were tentatively assigned by database searching using ChemSpider (<http://www.chemspider.com/>) using the detected mass to charge ratio (m/z) and accurate mass within 5ppm (26).

Reagents, plasmids and cell culture

OvCa cells were maintained in standard DMEM supplemented with 10% FB-Essence (VWR Life Science SeraDigm, Radnor, PA) with 1% penicillin/streptomycin (Corning, Corning, NY), 1% MEM non-essential amino acids (Corning) and 1% MEM vitamins (Corning). All cell lines were genotyped to confirm their authenticity (IDEXX Bioresearch short tandem repeat marker profiling every 3 months), and were used between 2-12 passages from first thaw. All experiments were carried out in low glucose DMEM (VWR Life Science) supplemented as above. The pLX304 SPHK1-V5 lentiviral expression vector was purchased from DNASU (Tempe, AZ). The constitutively active HIF1 α (P402A/P564A) and HIF2 α (P405A/P531A) plasmids were a gift from William Kaelin (27) obtained through Addgene (Cambridge, MA). 5HRE-GFP reporter was a gift from Martin Brown and Thomas Foster obtained through Addgene (28). SPHK1 siRNA was obtained from GE Dharmacon

(Lafayette, CO). Metformin, phenformin, CoCl_2 , and thiazolyl blue tetrazolium bromide were purchased from Sigma Aldrich (St. Louis, MO). S1P (Huzzah S1P) was purchased from Avanti Polar Lipids (Alabaster, AL). Hypoxia was induced through 1% O_2 /5% CO_2 using Hypoxia Incubator Chamber by STEMCELL Technologies (Vancouver, BC, Canada).

Cell line lipidomic analysis

Cellular lipids were extracted by a modified Bligh and Dyer procedure (29) with the use of 0.1N HCl for phase separation as described in (30). C17-S1P (40 pmol) was employed as internal standard, and was added during the initial step of lipid extraction. The extracted lipids were dissolved in methanol/chloroform (4:1, v/v), and aliquots were taken to determine the total phospholipid content (31). Analyses of sphingoid base-1-phosphates were performed by electrospray ionization tandem mass spectrometry (ESI-LC/MS/MS). Briefly, API4000 Q-trap hybrid triple quadrupole linear ion-trap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a turboionspray ionization source interfaced with an automated Agilent 1100 series liquid chromatograph and autosampler (Agilent Technologies, Wilmington, DE), as performed in (31).

Migration and invasion assays

Assays measuring cell migration and invasion were performed as done previously by our lab (32). Briefly, to test migration, a wound closure assay was utilized with cells were seeded in a 96-well plate 24 hours prior to the assay. The scratch was made by using Woundmaker^{TM} (Essen Bioscience, Ann Arbor, MI), and images were taken at 0 min and 16 hours. Migration was quantified using the ImageJ (NIH, Bethesda, MD) to measure relative distance over time. To assess invasion, OvCa cells were seeded onto 0.8 μm pore transwell insert (Corning, Corning, NY) with 300 μl serum-free DMEM, as we had done previously (33). In the lower chamber (24-well plate), 700 μl of serum free low glucose DMEM supplemented with 0.1% bovine-serum albumin (BSA) was treated with S1P (100nM) in the presence or absence of metformin (5mM) for 24 hours. Cells in the top of the chamber were removed using a Q-Tip, and invaded cells were stained using Wright Stain (Sigma) for 20 min, fixed with 4% paraformaldehyde (Sigma) for 10min, and then imaged using Zeiss Axiovert 200M (Zeiss, Oberkochen, Germany) and quantified using ImageJ (NIH).

Colony formation assay

OvCa cells were seeded at 5×10^2 or 1×10^3 cells, respectively, per 6-well dish. Cells were allowed to grow overnight, and then media was replaced and cells were treated for an additional 9 days prior to analysis. Cells were washed with 1X PBS, fixed with 4% paraformaldehyde for 10min, and then stained with crystal violet (0.5%) for 30min at room temperature. After removing crystal violet, plates were imaged and number of colonies were manually counted using ImageJ (NIH).

Cell viability and proliferation assays

Cells were plated in 96-well plates in quintuplicate overnight and subsequently treated as indicated. Cell viability was determined via MTT assay with 0.5 mg/ml thiazolyl blue tetrazolium bromide (Sigma), as previously described (34). Following $\log(x)$ transformation

and normalization per dataset, IC₅₀ values were determined using nonlinear regression (using log (inhibitor) vs. response – variable slope [four parameters]).

Determination of HIF transcriptional activity

Cells were plated in 6-well dishes overnight and transfected with 5HRE-GFP the following day. After 24h recovery, cells were treated with CoCl₂ with or without metformin for 4h. Cells were imaged using Zeiss Axiovert 200M (Zeiss) and then number of cells positive was quantified using ImageJ software (NIH).

Western immunoblotting

Cell lysates were prepared in RIPA buffer and immunoblotting was performed as previously described (19). Nuclear fractionation was performed using the REAP cellular fractionation method, as described in (35). Antibodies used were as follows: SPHK1 (Bethyl Laboratories, Montgomery, TX), SGPL1 and HIF1 α (Abcam, Cambridge, MA), HIF2 α (Novus Biologicals, Littleton, CO), phospho-AKT (S473), AKT, SOX2, phospho-AMPK (T172), AMPK, c-MYC, OCT-1, PKM1/2, β -Tubulin, Histone H3, cleaved caspase 3, Snail, V5-tag and β -Actin (Cell Signaling Technology, Beverly, MA).

Quantitative reverse transcription–polymerase chain reaction (PCR)

RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and transcribed into cDNA using cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MS). Quantitative real-time reverse transcriptase–polymerase chain reaction (quantitative RT-PCR, qRT-PCR) was performed as described (36) using Applied Biosystems 7500 Real-Time PCR System. SPHK1, SPHK2, SGPL1, ASAH1, ASAH2 and GAPDH Taqman probes were obtained from Applied Biosystems. Relative levels of messenger RNA (mRNA) expression was calculated by the 2^{-CT} method (37), using GAPDH as the housekeeping gene for normalization.

Mouse xenograft studies

All animal experiments were approved by the University of Chicago Institutional Animal Care and Use Committee. SPHK1 or vector control stably transfected OVCAR5 cells (2.5×10^4) were injected subcutaneously into the left flank of 7-week-old female athymic mice. The tumors were measured with calipers as *Tumor volume* = $1/2(\text{length} \times \text{width}^2)$. 18 days after injection of the cancer cells, the mice were sacrificed and tumors excised. Tumor samples were stained by IHC with antibodies against Ki67 (Thermo Fisher Scientific) and SOX2 (Cell Signaling Technologies). Slides were imaged using Zeiss Axiovert 200M (Zeiss) and number of cells with positive staining per high power field were recorded.

Statistics

Specific analyses performed for each experiment are described in the figure legends. In all analyses, data were evaluated using a one-way ANOVA with post-hoc Tukey, or two-tailed *t*-test, as appropriate; *p* < 0.05 was considered statistically significant (GraphPad Prism 7, San Diego, CA).

Results

Metformin regulates the sphingolipid rheostat and attenuates S1P-induced OvCa invasion

S1P levels are determined by activity of a rheostat whereby ceramide is converted to sphingosine, and phosphorylation of sphingosine generates S1P which can then be degraded [review in (2, 6)] (Figure 1A). Clinically, if S1P activity contributes to OvCa growth, identifying a safe and readily available agent that modulates this pathway could benefit patients. The biguanide metformin has been shown to affect intracellular and circulating levels of lipids in diabetes (22, 25, 38), metabolic syndrome (39), and cardiovascular disease (40). Therefore, we asked if metformin modulates the S1P rheostat in OvCa. Using serum from patients with stage III/IV OvCa, serum metabolites were measured using metabolomics (26). The analysis demonstrated that patients with OvCa who were using metformin for the treatment of type II diabetes mellitus had significantly lower serum S1P levels than patients not using metformin (Figure 1B). To further characterize the effects of metformin on the sphingolipid rheostat, an OvCa cell line was treated with metformin or control and lipidomic profiling was performed. Mirroring the findings in the patient serum, metformin treated cells had markedly reduced S1P and sphingosine levels while ceramide levels were increased (Figure 1C). Further lipidomic analysis of the components of the S1P pathway indicated that dihydroceramide (DH-ceramide) and dihydrosphingosine (DH-sphingosine) were unaltered by metformin treatment, while dihydro-S1P (DH-S1P) was markedly decreased (Figure 1D). Combined, these findings support the hypothesis that metformin shifts the rheostat towards reduced S1P production. To determine whether S1P does indeed promote OvCa, cancer cell migration and invasion, critical events in OvCa progression (41), were tested. Wound closure assays demonstrated that exogenous S1P promoted migration of OvCa cancer cells and that this effect was attenuated by pretreatment with metformin (Figure 1E). Similarly, S1P promoted invasion of the HeyA8 OvCa cell line and metformin treatment blunted this effect (Figure 1F). Together, this data suggest that S1P production and S1P-driven signaling are novel targets of metformin.

SPHK1 promotes ovarian cancer and is targeted by biguanides

Given the observation that metformin shifts the rheostat from S1P toward ceramide, we evaluated the effect of metformin on the regulatory steps of the S1P synthesis pathway (shown in Figure 1A). The metabolic profile of decreased S1P and DH-S1P levels suggested that metformin might inhibit SPHK1, the key regulatory enzyme that catalyzes the conversion of DH-sphingosine to DH-S1P. To test if SPHK1 is a target of metformin, OvCa cell lines were treated with metformin or another biguanide, phenformin, and SPHK1 expression was evaluated. The results demonstrate that both drugs significantly decreased SPHK1 mRNA levels in a dose-dependent manner in two different cell lines (Figure 2A). Protein expression of SPHK1 was also reduced by both drugs (Figure 2B). The effects of biguanides on other key regulators of the S1P rheostat, including ASAH1, SPHK2, and SGPL1, were inconsistent and only observed at very high doses (Supplemental Figure 1). This evaluation of the regulators of S1P rheostat suggested that metformin targets SPHK1. If SPHK1 is important in metformin's anti-cancer effect, there should be strong evidence that SPHK1 promotes OvCa growth. To understand if this was the case, we sought to test if SPHK1 is tumor promoting in OvCa by directly evaluating the effect of overexpression of

SPHK1 on several hallmarks of cancer growth by using OvCa cell lines that possess otherwise nearly undetectable endogenous levels of SPHK1 (Supplemental Figure 2). The results show that cells with ectopic SPHK1 expression had a higher rate of migration (Figure 2C) and proliferated more rapidly than control transfected cells (Figure 2D). Consistent with recent reports of S1P's role in AKT-dependent cell proliferation (8, 42), we also found markedly increased activation of AKT (phospho-S473) in SPHK1 overexpressing cells (Figure 2E). Next, using a colony formation assay, we tested whether SPHK1 promotes clonogenic growth. SPHK1 overexpressing cells formed significantly more colonies (Figure 2F), and SOX2, a key factor involved in stem cell-like phenotypes including self-renewal (43, 44), was markedly upregulated at both the mRNA and protein levels (Figure 2G and 2H, respectively). The ability of SPHK1 expression to promote proliferation, migration, clonogenicity and SOX2 expression was recapitulated in an additional cell line representative of high-grade serous OvCa (Supplemental Figure 3). Finally, in a xenograft mouse model of OvCa, overexpression of SPHK1 resulted in increased tumor burden compared to control mice (Figure 2I). Mirroring the findings in cell lines, immunohistochemical analysis of mouse tumors identified increased expression of Ki67 (Figure 2J) and SOX2 (Figure 2K) in the SPHK1 overexpressing tumors compared to controls. Altogether, these data suggest that SPHK1 promotes tumorigenicity in OvCa through induction of an aggressive phenotype characterized by increased motility, proliferation and self-renewal.

Metformin inhibits hypoxia-induced expression of SPHK1

In endothelial cells and glioma (45, 46), SPHK1 expression is induced by hypoxia, and SPHK1 has been shown to possess two hypoxia-response elements (HRE) in its promoter which are targeted by HIFs (46); therefore, we asked if hypoxia-inducible factors (e.g., HIF1 α , HIF2 α) upregulate SPHK1 in OvCa. To answer this, three OvCa cell lines were transfected with the constitutively stable forms of either HIF1 α (P402A/P564A) or HIF2 α (P405A/P531A) (32) and SPHK1 expression was evaluated. In all three cell lines tested, induction of either HIF1 α or HIF2 α significantly increased SPHK1 protein expression (Figure 3A). Exposure to metformin prevented the induction of SPHK1 protein and mRNA expression by ectopic expression of HIF 1 α (left) or HIF 2 α (right) (Figure 3B and Supplemental Figure 4). Likewise, exposure to metformin prevented the induction of SPHK1 protein by hypoxia (Figure 3C). Interestingly, HIF expression itself was not robustly repressed by metformin across both cell lines tested; therefore, we next asked if metformin might instead alter the activity of HIFs through reducing its nuclear localization. Cell fractionation demonstrated that, following HIF stabilization with CoCl₂ (47), metformin significantly reduced HIF1 α in both the nucleus and the cytosol as well as nuclear HIF2 α (Figure 3D and Supplemental Figure 5). These findings were confirmed using cells with constitutive expression of HIF1 α or HIF2 α . Here, it was noted that metformin reduced HIF1 or HIF2 solely in the nuclear fraction (Figure 3E). Finally, to directly test the impact of metformin on HIF transcriptional activity, OvCa cells were co-transfected with the hypoxia-response element (HRE) plasmid reporter, 5HRE (28), concurrently with either HIF1 α or HIF2 α stable mutants and then subsequently treated with metformin. Figure 3F shows that in normoxia, when HIF is stabilized using constitutively expressing mutants, metformin

significantly inhibited HIF transcriptional activity. Combined these findings indicate that metformin suppresses hypoxia-induced SPHK1 expression.

SPHK1 expression is associated with sensitivity to metformin

While there is enthusiasm for metformin's potential as a cancer therapeutic, it is unlikely to be effective for all patients. In OvCa cell lines, we have found the cytotoxic effects of metformin to be variable, with some cell lines being very sensitive to metformin (HeyA8, TYKnu) and others to a lesser extent (CAOV3, OVCAR5, Kuramochi and SNU119) (Figure 4A). Clinically, it is important to understanding *a priori* which patients will have the greatest anti-cancer effects from metformin. Interestingly, when evaluating metformin- sensitive and resistant cell lines, we noted that, compared to metformin resistant cells, metformin-sensitive cells expressed high levels of SPHK1 and low levels of the enzyme responsible for S1P degradation, S1P lyase (SGPL1) (Figure 4B/C). This finding indicated that a SPHK1^{high}/SGPL1^{low} profile may predispose OvCa cells to the cytotoxic effects of metformin. Supporting this concept, in three different cell lines the IC₅₀ for metformin was reduced by >50% in SPHK1 overexpressing cells compared to controls (Figure 4D). Likewise, the induction of apoptosis by metformin was greater in SPHK1 overexpressing than in control transfected cells, as indicated by increased cleavage of caspase 3 (Figure 4E). Given the marked sensitization of OvCa tumor cells to metformin by ectopic expression of SPHK1, we next asked if decreased levels of SPHK1 were linked to resistance to the drug, and whether this was associated with hypoxia-dependent regulation of the kinase. To answer this question, SPHK1 was silenced in the metformin-sensitive cell line TYKnu, which express relatively high levels of SPHK1 compared to other OvCa cell lines. Knockdown of SPHK1 using siRNA markedly increased the IC₅₀ of metformin compared to control cells as measured by MTT assay, indicating that reduced SPHK1 increases resistance to the drug (Figure 4F-G and Supplemental Figure 6). Intriguingly, hypoxia dramatically sensitized OvCa cells to metformin which, importantly, was potentially abrogated in cells with reduced SPHK1 expression (Figure 4F and Supplemental Figure 6), suggesting that SPHK1 expression downstream of hypoxic signaling is central to metformin's impact on cell viability in this setting. In addition, knockdown of SPHK1 in these cells prevented the induction of caspase 3 cleavage by metformin (Figure 4H). Interestingly, regulation of metformin sensitivity by SPHK1 expression appeared to be independent of other putative markers of metformin's efficacy, as neither overexpression nor knockdown of SPHK1 altered expression of metformin's membrane transporter OCT1, its canonical target AMPK, or glycolytic enzymes that may mediate its cytotoxic effects such as c-MYC or PKM1/2 (20) (Supplemental Figure 7). Taken together, these data indicate that in OvCa cells, SPHK1 expression independently modulates sensitivity to the cytotoxic effects of metformin.

Metformin overcomes cancer-promoting effects of SPHK1

To understand whether these preclinical findings are relevant to patient's with OvCa, we analyzed the expression of SPHK1 in patient pathology specimens (n=389) from common anatomical sites of tumor growth. The results show that SPHK1 is strongly expressed in the cytoplasm of cells in >40% of primary ovarian tumors, omental metastases and peritoneal metastases (Figure 5A), indicating that SPHK1/S1P signaling may be an important pathway throughout OvCa tumor progression. Given that the prior experiments indicated that SPHK1

activity may be associated with metformin response, we asked if metformin would attenuate the cancer promoting effects of SPHK1. The results show that colony formation promoted by overexpression of SPHK1 was abrogated by metformin (Figure 5B). Conversely, silencing SPHK1 markedly reduced the ability of OvCa cells to form colonies and treatment with metformin did not further inhibit clonogenicity in these cells (Figure 5C). Consistent with a reduction in colony formation by metformin, expression of the self-renewal factor SOX2 and its downstream target Snail induced by SPHK1 was prevented by the drug (Figure 5D). In a final experiment, we tested the necessity of targeting SPHK1 to metformin's ability to prevent invasion. In addition, based on the previous observations, we asked if metformin's effect on OvCa invasion was dependent on the upregulation of SPHK1 by hypoxia. The results show that OvCa cell invasion was significantly increased under hypoxia and that the hypoxia-induced increase in cancer cell invasion was prevented by concurrent treatment with metformin (Figure 5E). Importantly, silencing SPHK1 inhibited invasion of cancer cells in normoxia and significant reduced invasion induced by hypoxia (Figure 5E and Supplemental Figure 8). Notably, silencing SPHK1 prevented the ability of metformin to reduce hypoxia-driven invasion in these cells (Figure 5E). Combined, these findings support the concept that the tumor promoting effects of SPHK1 in OvCa can be targeted using metformin and directly relate to the efficacy of the drug in preventing tumor growth (Figure 5F).

Discussion

The biguanide metformin has been shown to affect intracellular and circulating levels of lipids in diabetes (22, 25, 38) and significant effort is underway to understand if the diabetes medication has potential as cancer treatment (48). Here, we show that the S1P rheostat is a novel target of metformin in OvCa. As depicted in Figure 1, serum levels of S1P are lower in patients with OvCa that were taking metformin and, *in vitro*, metformin shifts the sphingolipid rheostat away from oncogenic S1P. Increasing evidence supports an important role of bioactive lipids like S1P and the upstream kinase, SPHK1, in cancer (3) and the experiments reported here demonstrate that metformin suppresses migration and chemotaxis induced by ectopic SPHK1 and downstream S1P signaling.

Further investigation of the effects of metformin on the S1P rheostat revealed that inhibition of SPHK1 is the central mechanism by which metformin represses S1P. Functional assays indicated that ectopic SPHK1 expression alone was sufficient to recapitulate the pro-tumorigenic effects of S1P, suggesting SPHK1's pivotal role in S1P-mediated oncogenesis. While earlier studies have shown S1P and SPHK1 to increase proliferation and migration in OvCa *in vitro* (11, 49), here we demonstrate that SPHK1 directly induces activation of AKT and enhances SOX2 expression, thereby promoting OvCa tumorigenicity *in vivo*. Together, these data support the S1P pathway and SPHK1 in particular, as potential targets for OvCa treatment. An IHC analysis of over 300 OvCa patient samples confirmed expression of SPHK1 in all the metastatic sites affected by OvCa; however, to our knowledge no significant attempts have been made to target SPHK1 for OvCa treatment. There are phase I clinical trials targeting SPHK1 in other solid tumors (14, 50, 51) and, in a mouse model, pharmacologic inhibition of SPHK1 with fingolimod decreased mouse ovarian tumor weight (10). Unfortunately, traditional inhibitors of SPHK1 may prove to have limited clinical

utility as the inhibitors induce hemolysis and hepatotoxicity in preclinical models (1) and use of fingolimod in humans is limited by immunosuppression (52).

To increase safety, decrease cost, and expedite clinical use of new cancer therapeutics a *drug repurposing* paradigm is attractive. In this approach anti-cancer properties are identified in agents that are already widely used for non-cancer indications (18). A cancer therapeutic directed against S1P would, ideally, shift the S1P rheostat towards reduced S1P production (1). This concept is supported by pre-clinical studies in multiple cancer types that demonstrate increased tumor growth when S1P is elevated either due to increased synthesis as result of SPHK1/2 upregulation (7, 8) or due to decreased S1P degradation as result of SGPL1/2 inhibition (53). In the current study, we demonstrate that metformin reduces S1P levels by downregulating SPHK1. Given the limitations of other compounds that target SPHK1, the data presented here demonstrate the potential to expedite the exploitation of the S1P pathway through repurposing metformin as SPHK1 inhibitor in OvCa.

To understand the mechanism by which metformin inhibits SPHK1, we focused on HIFs as it has been reported that metformin inhibits hypoxia-induced HIF1 α expression through repression of complex I-dependent ROS production (54) and prior reports suggested that SPHK1 expression and activity may be induced by hypoxia (45, 46, 55). The results show that, in OvCa, HIF stabilization potently induce SPHK1 mRNA and protein expression and that metformin inhibits hypoxia-induced SPHK1 upregulation. It is notable, that metformin did not reliably repress HIF expression in the cell lines tested, which, while inconsistent with some studies (47), metformin has been reported by others to be ineffective at reducing HIF expression at the dose and duration used here (56). However, our data does support that, in the current system, metformin abrogated the nuclear accumulation and concomitant transcriptional activity of HIFs in both normoxic and hypoxic conditions *in vitro*, lending to a novel mechanism by which the drug alters HIF signaling. While metformin was capable of repressing HIF-induced SPHK1 expression, it is still unclear whether HIFs specifically are necessary targets of the drug's action on SPHK1, as indirect targets or co-factors, such as ARNT1, CBP and p300 (57), could be involved in its transcriptional activity. Further, our data indicates that metformin may in some cases inhibit translocation or import of HIFs into the nucleus; however, the exact mechanism by which the drug elicits this effect remain undetermined. Also, given the importance of SOX2 as a key regulator in pluripotency and tumor development (58), the finding that SPHK1 promotes self-renewal through the upregulation of SOX2 lends to a new understanding of the contribution of sphingolipid signaling to tumor progression. The ability of metformin to abrogate SOX2 expression and the associated clonogenicity of cells with ectopic SPHK1 expression identifies a new mechanism by which the diabetic drug inhibits vital oncogenic processes in OvCa.

New therapeutic approaches for OvCa are desperately needed as improvement in outcomes for patients have been constrained by limited understanding of the drivers of ovarian carcinogenesis and the introduction of very few new treatments in 20 years (59). The results reported here demonstrate that targeting SPHK1 expression and downstream S1P signaling may be a novel approach to OvCa treatment. Demonstrating that SPHK1 is directly enhanced by hypoxia identifies a novel, actionable, mechanism by which SPHK1 and concomitant sphingolipid signaling may be targeted in OvCa. Importantly, our data supports

that metformin can be repurposed to inhibit SPHK1, thereby repressing S1P oncogenic signaling in OvCa.

Although there is significant enthusiasm for the potential of metformin as a cancer treatment, it is unlikely that that drug is going to be effective for all patients and, clinically, it is critically important to be able to identify in advance the patients most likely to benefit from this approach. The findings reported here show that SPHK1 expression levels within the tumor cell directly influence the IC₅₀ of metformin's cytotoxic effects as well as the induction of apoptosis by the drug, with elevated SPHK1 increasing sensitivity and reduced SPHK1 levels conferring resistance to metformin. Importantly, the influence of SPHK1 on metformin's effects was found to be independent of previously described putative markers that indicate sensitivity to the drug. Together, these results suggest that high SPHK1 expression in the tumor, or perhaps even serum S1P levels, may predict increased response to metformin and support a decision for its use therapeutically. This finding could be relevant to the >55 clinical trials testing metformin as treatment for breast, endometrial, prostate, pancreas, and lung cancer (24) and future large clinical trials of metformin could consider incorporating high expression of SPHK1 as a predictive biomarker.

While this report strongly indicates SPHK1 expression level predict metformin sensitivity or resistance, studies identifying serum S1P levels as a potential, and importantly a more accessible, biomarker are needed. Indeed, one limitation of the current study analyzing our clinical samples is that no cancer-free subjects were assessed, and while patients were matched on age and cancer stage, there was no inclusion of diabetics on other drugs, which may affect these results. Furthermore, while SPHK1 and S1P had similar effects on tumor cell behavior *in vitro*, it is unclear whether SPHK1 in OvCa tumors directly relates to circulating S1P levels in patients. While suppression of SPHK1 using inhibitors has been shown to reduce serum S1P in xenograft models *in vivo* (60), it is clear that other sources of the metabolite result from SPHK1-dependent secretion into plasma, such as from erythrocytes (61), and thus secretion from tumor cells may not entirely account for circulating S1P levels in cancer patients. Future studies are warranted to evaluate the relationship between SPHK1 and S1P within the tumor and their impact on systemic S1P levels in OvCa, as well as to further characterize whether the circulating metabolite alone may directly provide an indication for metformin's clinical efficacy. Lastly, it is worth noting that the metformin dose used in the present study is significantly lower than doses used in a large number of prior studies, but still is higher than what is observed physiologically in humans (20). These constraints of pre-clinical investigation make prospective clinical testing of metformin in patients imperative. Looking ahead, recognizing the importance of bioactive lipid pathways in carcinogenesis and targeting those pathways with safe, readily available repurposed drugs might prove to be an unexpected path forward in the journey to improve outcomes for women battling this insidious disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Implication Statement:

Metformin targets sphingolipid metabolism through inhibiting SPHK1, thereby impeding ovarian cancer cell migration, proliferation and self-renewal.

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Figure 1. Metformin regulates the sphingolipid rheostat and represses S1P-driven migration in OvCa.

(A) Schematic of the key regulatory steps regulating the sphingolipid rheostat and the resultant bioactive lipids. (B) S1P levels in serum of age matched patients with stage III/IV OvCa using metformin for diabetes (n=9) compared to control counterparts (n=10). (C) Lipidomic profiling measuring ceramide, sphingosine, and S1P concentration in CAOV3 OvCa cells after metformin treatment (1mM, 72h) (n=3). (D) Lipidomic profiling showing the effects of metformin on DH-S1P, DH-ceramide and DH-sphingosine in CAOV3 OvCa cells (n=3). (E) Wound healing assay in TYKnu and CAOV3 cells with exogenous treatment with S1P (10 and 100nM) following metformin pretreatment (1mM, 24 hours) (n=6). (F)

Transwell invasion assay in HeyA8 OvCa cells using S1P (100nM in serum-free media) as a chemoattractant with concurrent metformin treatment (1mM, 15h) (n=3). FBS (10%) as a chemoattractant was used as a positive control. Data represent mean value $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ of one-way ANOVA with post-hoc Tukey test. *N.S.*; not significant.

Figure 2. SPHK1 is a novel target of metformin.

(A) SPHK1 mRNA expression was measured by qRT-PCR in TYKnu (left) and CAOV3 (right) OvCa cells treated with metformin or phenformin at the indicated doses for 24h. Results expressed as relative quantity over GAPDH housekeeping gene (n=3) (B) SPHK1 protein expression was assessed by immunoblot in TYKnu (left) and CAOV3 (right) cells treated with metformin or phenformin at the indicated doses for 72h, (n=3). (C) Wound healing assay in OVCAR5 cells stably overexpressing SPHK1 (SPHK1-OE) (n=6). (D) MTT assay measuring proliferation in SPHK1 overexpressing and control transfected OVCAR5 cells (n=6). (E) Immunoblot of AKT activation (S473 phosphorylation) in SPHK1 overexpressing and control transfected OVCAR5 cells (n=3). (F) Colony formation assay

showing number of colonies formed after 10 days in SPHK1 overexpressing and control transfected OVCAR5 cells (n=3). **(G)** SOX2 mRNA expression was measured by qRT-PCR in OVCAR5 cells overexpressing SPHK1 or control vector. Results expressed as relative quantity over GAPDH housekeeping gene (n=3). **(H)** SOX2 protein expression was assessed by immunoblot in OVCAR5 cells overexpressing SPHK1 or control vector. **(I)** Xenograft mouse model with OVCAR5 cells overexpressing of SPHK1 was compared to vector control OVCAR5 cells (Control n=9, SPHK1-OE n=10). X-axis is expressed as fold change in mean tumor weight (g) over control. IHC analysis of tumors from SPHK1 overexpressing group and control group measuring expression of Ki67 **(J)** and SOX2 **(K)**. Data represent mean value $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ of one-way ANOVA with post-hoc Tukey test. *N.S.*; not significant.

Figure 3. Metformin inhibits hypoxia-induced expression of SPHK1 in OvCa.

(A) SPHK1 protein expression was assessed by immunoblot in OVCAR5, Kuramochi and TYKnu cells overexpressing constitutively stable HIF1α with P402A/P564A (left) or HIF2α with P405A/P531A (right) mutations (n=3). (B) SPHK1 protein expression was analyzed by immunoblot after metformin treatment (1mM, 72h) in OVCAR5 and Kuramochi cells overexpressing constitutively stable HIF1α with activating substitution P402A/P564A (left) or HIF2α with P405A/P531A (right) mutations (n=3). (C) HIF1α, HIF2α and SPHK1 protein expression were assessed by immunoblot in response to hypoxia (1% O₂, 5% CO₂) with metformin concurrent treatment (1mM, 48h) (n=3). (D) HIF1α and HIF2α protein expression and localization in OVCAR5 cells was measured by immunoblot following

cellular fractionation. HIF expression was stabilized using CoCl_2 (100 μM) and concurrently treated with metformin (1mM, 24h). Histone H3 and $\beta\text{Tubulin}$ were used to normalize nuclear and cytosolic localization, respectively (n=3). (E) HIF1 α and HIF2 α protein expression and localization in OVCAR5 cells was measured by immunoblot following cellular fractionation following transfection with HIF1 α or HIF2 α constitutively stable mutants and subsequent treatment with metformin (1mM, 24h). Histone H3 and $\beta\text{Tubulin}$ were used to confirm nuclear and cytosolic fractions. (F) HIF transcriptional activity was measured in HeyA8 cells transiently co-transfected with the 5HRE reporter system in conjunction with either constitutively active HIF1 α or HIF2 α plasmids. Cells were then treated $\pm$ metformin (1mM, 24h) (n=3). Data represent mean value $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$ of one-way ANOVA with post-hoc Tukey test. *N.S.*; not significant.

Figure 4. SPHK1 expression is associated with metformin sensitivity in OvCa cells.

(A) MTT viability assay of six OvCa cell lines analyzing sensitivity to metformin using a dose range up to 40mM in 5mM glucose DMEM (n=6). SPHK1 and SGPL1 mRNA (B) and protein (C) expression was assessed in multiple OvCa cell lines (n=3). (D) MTT viability assay IC₅₀ values for metformin (mM) in TYKnu, OVCAR5 and Kuramochi cells overexpressing SPHK1 or control vector (n=6). (E) Expression of cleaved caspase 3 was assessed through immunoblot in OVCAR5 and Kuramochi cells overexpressing SPHK1 or control vector with concurrent treatment with metformin (1mM, 48h) (n=3). (F) MTT viability assay IC₅₀ values for metformin (mM) in TYKnu cells with transient knockdown of SPHK1 using siRNA, in either normoxia or hypoxia (1% O₂, 5% CO₂) (n=6). (G)

Knockdown of SPHK1 mRNA expression was confirmed by qRT-PCR following knockdown using siRNA in HeyA8 cells (n=3). **(H)** Western blot of SPHK1 and cleaved caspase 3 in TYKnu cells following knockdown of SPHK1 using siRNA, with or without metformin treatment (1mM, 48h) (n=3). Data represent mean value $\pm$ S.D.

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Figure 5. Metformin inhibits SPHK1-dependent tumorigenicity.

(A) IHC staining of tumor microarrays of common anatomical sites in OvCa for expression of SPHK1 (n=389). Representative images (20x magnification). Scale bar represents 100μm.

(B) Colony formation assay showing number of colonies formed in 10 days in SPHK1 overexpressing and control transfected OVCAR5 (left) or Kuramochi (right) cells in the presence of metformin (1mM) (n=3). (C) Colony formation assay over 10 days in TYKnu cells with SPHK1 knockdown using siRNA, with or without metformin treatment (1mM) (n=3). (D) SOX2 and Snail protein expression were assessed by immunoblot in OVCAR5 and Kuramochi cells overexpressing SPHK1 or control vector with metformin treatment (1mM, 72h) (n=3). (E) Transwell invasion assay in HeyA8 cells with transient knockdown

of SPHK1 using siRNA. Cells were treated with hypoxia (1% O₂) in the presence or absence of metformin (1mM) during the invasion assay. 10% FBS was used as a chemoattractant below the transwell inserts, and cells were allowed to invade for 16h following seeding. **(F)** Schematic depicting inhibition of SPHK1 by metformin, resulting in a shift away from tumorigenic S1P signaling. Data represent mean value $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ of Student's t Test, or one-way ANOVA with post-hoc Tukey test.