Abstract. ABC294640, a selective inhibitor of sphingosine kinase-2, inhibits the formation of sphingosine 1-phosphate (S1P), a signaling lipid implicated in promoting tumor survival. We investigated the anticancer activity of ABC294640 in two ovarian cancer cell lines, BG-1 and Caov-3. ABC294640 dose-dependently inhibited clonogenic survival and cell viability of both ovarian cancer lines in vitro. Using enzyme-linked immunosorbant assays and western blot detection in chemoresistant Caov-3 cells, treatment with ABC294640 alone also potentiated bcl-2-associated X-protein and caspase-9 transcription levels, although it did not significantly increase apoptotic cell death. Interestingly, ABC294640 administered to Caov-3 ovarian cancer cells in conjunction with paclitaxel induced apoptotic cell death through activation of caspase-9. Induction of apoptosis may mediate the anticancer effect of ABC294640 in ovarian cancer, although its precise antitumor mechanism is unclear. Ultimately, through its inhibition of S1P formation and subsequent effects on critical survival signaling cascades, ABC294640 may prove to be a useful adjunct to help re-sensitize tumors to standard therapy.

In the United States, ovarian cancer represents approximately 3% of all cancers in women, with an estimated 22,240 new cases and 14,030 deaths in 2013 (1). The five-year ovarian cancer survival rate between 2002-2008 was 43%, with women under 65 years demonstrating a 56% survival rate, and women over 65 years a 27% survival rate. Initial diagnosis of ovarian cancer is usually made after the cancer has already metastasized, which drastically reduces five-year survival to just 28% (1). Treatment options include surgery and chemotherapy. Common chemotherapy treatments are the taxanes (paclitaxel or docetaxel) and platinum agents (cisplatin and carboplatin). Many women die due to treatment failure, therefore it is important to develop new agents that target different aspects of tumor progression and resistance for use along with standard regimens. Sphingolipid compounds involved in cell signaling are targets for manipulation.

The rheostat. Sphingolipids are ubiquitous structural components of cell membranes. Recently, their metabolites have gained prominence as signaling molecules involved in regulation of cell functioning including cell death and differentiation, proliferation, and aspects of inflammation. Most of these important aspects are mediated by the sphingolipid metabolites ceramide, sphingosine, and sphingosine-1-phosphate (S1P). The precise balance and equilibrium (a.k.a. the rheostat) between these metabolites has been extensively studied and recent literature has defined their roles in disease states, inflammatory conditions, and apoptosis (2, 3). S1P is generated from ceramide by the action of ceramidase and one of two isoforms of sphingosine kinase, SphK1 or SphK2. Ceramidase deacylates ceramide to form sphingosine, which is then phosphorylated to S1P by sphingosine kinase(s) (4). Some of the actions of S1P include cell proliferation, inhibition of apoptosis, migration, induction of stress fiber formation, and up-regulation of adhesion molecules (5-7). Both Huwiler et al. (6) and Hait et al. (2) have shown that the balance between ceramide and S1P levels is critical in determining a cell’s life death decision, which reinforces targeting the rheostat for rational anticancer regimens. Furthermore, the many contributions of sphingolipids to growth factor signaling, cell-cycle regulation, apoptosis regulation, angiogenesis and metastasis, and other targets of prolongation of cell life support the study of their role in cancer development and resistance mechanisms. They appear to be expedient targets for anticancer drug therapy (2, 4).
SphK2 and its inhibitor, ABC294640. Although its sister enzyme, SphK1, has received much attention as a target for anticancer therapies, because of the contributions S1P makes to growth, progression, and differentiation (8, 9), the role of SphK2 in cancer cells is still emerging. Studies indicate differential functions and effects on apoptosis between SphK1 and SphK2. SphK2 itself has unique characteristics: it has a nuclear localization sequence which accounts for its presence in the nucleus, cytosol, or endoplasmic reticulum depending on specific needs of the cell (9). SphK2 also has a BH3 domain, and overexpression of SphK2 can induce apoptosis (10), although this is attenuated when its catalytic domain is mutated (11). Recent evidence supports SphK2 as being a useful target in cancer therapeutics. Selective targeting of SphK2 with siRNA knockdown results in stronger ablation of extracellular signal-regulated kinase-mediated proliferation, invasion, and migration when compared with selective SphK1 knockdown in kidney and breast cancer (12). Lung cancer cell SphK2 activity that has been enhanced by hypoxia can mediate chemoresistance to the apoptosis-inducing agent, etoposide, and this effect is relieved by SphK2 knockdown (13). SphK2 knockdown in colon cancer resulted in increased sodium butyrate-induced apoptosis, while overexpression of Sphk2 promoted survival and sodium butyrate resistance (14). Recent efforts have been made in developing and evaluating the effect of small-molecule inhibitors of SphK2 in cancer and other inflammatory states as effective therapeutic options.

ABC294640 is a potent, orally bioavailable inhibitor of SphK2, which is capable of dose-dependently competing with sphingosine for the enzyme. ABC294640 has demonstrated dose-inhibition of growth of a variety of solid tumors including breast, colon, lung, ovary, and prostate (15). ABC294640 has also shown dose-dependent growth reduction in xenografted mammary adenocarcinoma and hepatocellular carcinoma in vivo (15, 16). Antoon and colleagues from this laboratory indicate that it is both a powerful inducer of apoptosis as well as a chemical resensitizer to standard chemotherapy in cell and animal models of chemoresistant breast cancer (17-19). This effect is thought to be mediated by adjusting the rheostat toward favoring apoptotic signaling by increasing the ceramide:S1P ratio (17-19). This finding, coupled with those from previous studies, led us to hypothesize a similar induction of apoptosis in chemoresistant ovarian cancer. Currently, there are no studies of which we are aware that examine the effect of SphK2 inhibition in ovarian cancer in the depth that the present study does. Our aim was to determine the efficacy of this agent and the mechanism by which ABC294640 may inhibit ovarian cancer growth and resensitize tumors to traditional therapies. Use of chemoresistant Caov-3 ovarian and chemosensitive BG-1 ovarian cells allowed us to address this specific aim.

Materials and Methods

Reagents. ABC294640 [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)-amide] was provided by Apogee Biotechnology Corporation (Hummelstown, PA, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Hudson, NH, USA) and paclitaxel from Sigma-Aldrich (St Louis, MO, USA).

Cell culture. BG-1 cells were generously provided by Syreeta Tilghman, and Caov-3 cells were generously provided by Melyssa Bratton, both at Tulane University. All cancer cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; pH 7.4) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Salt Lake City, UT, USA), 1% non-essential amino acids, 1% minimum-essential medium amino acids, 1% sodium pyruvate, antibiotic/anti-mitotic, and insulin at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Colony assay. The BG-1 and Caov-3 cells were plated in 6-well plates at a density of 10^3 cells per well in 10% DMEM. Cells were allowed to adhere overnight then treated with DMSO or ABC294640 and monitored for 10-14 days for colony growth. Cells were then processed and fixed with 3% glutaraldehyde (15 min), plates were washed and stained with 0.4% solution of crystal violet in 20% methanol (30 min), and cells were then washed with phosphate buffered saline, dried, and counted. Any colony with >30 cells was counted as positive. Results were normalized to those of the DMSO vehicle control and half maximal inhibitory concentration (IC50) and statistical significance values were calculated using dose–response curves with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Viability assay. The BG-1 and Caov-3 cells were plated at a density of 7,500 cells per well in a 96-well plate in 10% DMEM and allowed to adhere overnight. Cells were then treated with ABC294640 (1 μM to 100 μM) or DMSO for 24 h. Following treatment, 20 μl of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml) reagent was added to each well and plates incubated for 4 h. MTT/media were removed and cells were lysed with 100 μl DMSO. The absorbance was read on an Elx808 MicroTek plate reader (Bio-tek, Winooski, VT, USA) at 550 nm, with a reference wavelength of 630 nm.

Proteomics analysis. Proteome Profiler Antibody Array was purchased from R&D Systems (Minneapolis, MN, USA) and used according to the manufacturer’s instructions. Briefly, Caov-3 cells were plated at 70-80% confluence and allowed to adhere overnight. Cells were then treated with either DMSO or ABC294640 for 6 and 24 h. Cell lysates were prepared using the included reagents and instructions. The array procedure was performed as outlined in the manual. Lysates were incubated with arrays embedded with capture antibodies for apoptotic proteins overnight in the included buffers, and were washed, and probed with an antibody detection cocktail. The only deviation from the included protocol was the use of infrared-conjugated streptavidin to permit scanning on a LiCor Odyssey imager (LiCor Biosciences, Lincoln, NE, USA) and software. Protein levels were quantified using pixel densitometric analysis. Positive signaling was confirmed with western blotting.
Western Blotting. Studies were performed as previously described (18). Briefly, Caov-3 cells were plated at 70-80% confluence in 25 mm² flasks and allowed to adhere overnight. The following day, cells were treated with either DMSO or ABC294640 for 6 h. Cells were then lysed with lysis buffer (mammalian protein extraction reagent; Pierce, Rockford, IL, USA) HALT protease inhibitors (Pierce), and phosphatase inhibitor cocktail I and II (Sigma-Aldrich), and transferred to 1.5 ml centrifuge tubes and placed in ice for 30 min. The lysate was then centrifuged at 4°C at 12,000 xg for 10 min. Supernatants were combined with loading buffer and 5% 2-mercaptoethanol in 4x LDS loading buffer (Invitrogen), boiled for 5 min, loaded onto a 4-12% bis-tris polyacrilamide gel (Invitrogen), followed by polyacrylamide gel electrophoresis at 150 V for 1.25 h. Proteins were transferred to nitrocellulose membrane using an iBlott transfer machine (Invitrogen), blocked in 2.5% milk (Bio-Rad Labs, Hercules, CA, USA) in tris-buffered saline-Tween20 (TBS-T; USB, Cleveland OH, USA) at room temperature (RT) for 1 h. Primary antibodies for bcl2-associated X-protein (BAX) and actin were obtained from Cell Signaling Technology (Beverly, MA, USA) and diluted in 5% bovine serum albumin (Sigma-Aldrich) at the recommended 1:1000 dilution. Membranes were probed with primary antibodies overnight at 4°C with gentle agitation. Blots were washed three times with 1x TBS-T wash buffer. Infrared-conjugated secondary antibodies (LiCor Biosciences) were diluted 1:10,000, and blots were incubated at RT for 1 h, washed and scanned on a LiCor Odyssey infrared scanner using the corresponding software. Western blots and protein quantification were performed following three independent experiments, with representative blots shown. Protein levels were quantified using densitometric analyses.

Caspase-9 Glo assay. Caov-3 cells were plated at 10,000 cells per well in a 96-well plate in DMEM and allowed to adhere overnight. Cells were then treated with DMSO, ABC294640, paclitaxel, or both ABC294640 and paclitaxel for 24 h. Caspase-Glo-9 buffer, substrate, and MG-132 inhibitor reagent mix (Promega Corp) were added to cells at a 1:1 ratio with media for 35 min. Following incubation, mixtures were transferred to white-walled, clear bottom, well plates and the luminescence was read. Absorbance was read on an Elx808 MicroTek plate reader (Bio-tek Instruments) at 405 nm. The signal was normalized for the fraction of surviving cells using MTT analysis and treatment response in relation to vehicle control.

ELISA assay for apoptosis. Caov-3 cells were plated at 10,000 cells per well in DMEM and allowed to adhere overnight. Cells were then treated with ABC294640, paclitaxel, or vehicle for 24 h. A Cell Death Detection ELISA PLUS Kit from Roche Applied Science (Indianapolis, IN, USA) was used to process cells. Briefly, cells were lysed and transferred to an anti-nucleosome-coated microplate, incubated with colorimetric detection reagent, and the absorbance was read on a MicroTek plate reader at 405 nm.

Statistical analysis. IC₅₀ values were obtained by entering transformed raw data into a dose–response curve evaluation with GraphPad Prism software using the equation: \( Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10\log EC₅₀} \cdot X \) assuming a standard slope, where response goes from 10-90% maximal as X increases over two log units. Statistical analysis of drug vs. vehicle combinations for differences in cell death and caspase-9 signaling were compared using two-way Dunnet’s ANOVA post-hoc analysis comparing treatment with control. Student’s unpaired t-test was used for analysis of BAX transcription levels for vehicle vs. treatment. All statistical analyses were performed using GraphPad Prism software 4.0 evaluating at least three independent experiments.

Results

Pharmacological inhibition of SphK2 by ABC294640 reduces ovarian cancer cell viability and clonogenic survival. As stated previously, there are no studies which examine the selective inhibition of SphK2 in ovarian cancer cell lines. Here we show that the inhibition of SphK2, using ABC294640, resulted in decreased ovarian cancer cell viability and survival. Utilizing MTT assays, the IC₅₀ values for ABC294640 in BG-1 and Caov-3 cells were 16.3 and 30.5 μM, respectively (Figure 1). We also examined the efficacy of this drug in long-term colony formation and survival in ovarian cancer cells lines. As seen in previous studies, ABC294640 had IC₅₀ values in the low micromolar range: 2.6 μM for BG-1 cells and 9.4 μM for Caov-3 cells (Figure 1). Previous studies from our laboratory demonstrated similar effects in chemoresistant breast cancer cell lines (17-19), and efficacy in prostate and pancreatic cancer models has also been confirmed (unpublished data). Our experiments provide further evidence that targeting SphK2 activity can reduce survival, with potent cytotoxic effects in a multitude of cancer types.

Pharmacological inhibition of SphK2 with ABC294640 induces apoptotic signaling in ovarian cancer cells and enhances cell death with traditional chemotherapeutics. There is conflicting evidence regarding the role of SphK2 in apoptosis. It is known that inhibition of SphK2 can mediate both intrinsic apoptosis as well as autophagy (18, 20). We previously investigated the ability of ABC294640 to induce apoptosis in chemoresistant breast cancer and we hypothesized that similar treatment with ABC294640 with consequent inhibition of SphK2 for 24 h would induce similarly significant apoptosis in chemoresistant Caov-3 ovarian cancer cells. The treatment led to an increased but insignificant induction of apoptosis of Caov-3 cells (Figure 2), and yielded a marked reduction in cell viability (data not shown). Interestingly, when combined with paclitaxel, ABC294640 significantly (p<0.0001) increased apoptosis compared with the vehicle (Figure 2). Although ABC294640 did not impart strong apoptotic signaling alone, it did induce increased apoptosis when combined with other apoptotic agents, and while these results are somewhat in conflict with anticipated anticancer effects on ovarian cancer cells, there is indication of other apoptotic signaling taking place. Caspase-9 activation, released downstream from cytochrome c, proceeds to propagate the apoptotic cascade via the intrinsic apoptosis pathway. Treatment with ABC294640 resulted in nearly a two-fold induction of caspase-9 activity (p<0.05) (Figure 2),
aligned with a significant reduction in cell viability (not shown). In addition, combination of ABC294640 with paclitaxel significantly ($p<0.0001$) increased caspase-9 signaling compared with vehicle control (Figure 2). Our
results suggest that a combination treatment of taxane/SphK2 inhibitors may help resensitize chemoresistant tumors to traditional therapeutics by increasing their apoptotic effect.

**ABC294640 treatment results in increased BAX levels.** Given the slight induction of apoptotic nucleosomes with solitary treatment of ABC294640, but confronted with an increase in caspase-9 signaling, we sought to determine if there were other markers of apoptosis active in response to treatment with ABC294640, as seen in other cancer cell lines. Utilizing a proteomic array specific to 35 proteins involved in apoptosis and cell-cycle regulation, we observed differences in several protein levels and confirmed these with western blotting. Indeed, the largest difference shown was in the level of BAX, which was found to be significantly ($p<0.05$) elevated in response to ABC294640 at 6 h (Figure 3), although this response was diminished by 24 h (not shown). We speculate that the increase in pro-apoptotic signaling as a result of SphK2 inhibition may be partially responsible for the cytotoxic effect and diminished survival in Caov-3 cells.

**Discussion**

Recent research supports an important role of SphK/S1P in chemoresistance. Our laboratory has determined that selective targeting of the rheostat in classic cancer cell model systems confers tumor ablation which is synergistic with traditional chemotherapeutics (17–19). This evidence suggests that targeting SphK2 may resensitize resistant tumors to current therapies. Our data provide further evidence of the efficacy of SphK2 inhibition in the treatment of multiple tumor types, including ovarian cancer. We have shown that ABC294640, a selective inhibitor of SphK2, dose-dependently inhibited ovarian cancer cell survival, an outcome supported by similar results for breast cancer cells from our laboratory (17, 18). ABC294640 is also shown to potentiate mediators of apoptosis in chemoresistant cell lines by an undetermined mechanism, which warrants further discussion.

There is some indication that the antitumor effect of ABC294640 is mediated by cellular targets known to be under the regulation, although independent of, p53. Importantly, the Caov-3 cell line contains non-wild-type p53, therefore we could not confirm a direct effect of SphK2 inhibition on p53 signaling. We were able to evaluate downstream targets of p53 which are known to mediate apoptosis, such as BAX. We evaluated BAX signaling for several reasons. Increased BAX concentration via transfection in ovarian cancer cells is known to increase apoptosis in response to chemotherapy, even when cells lack functional p53. Therefore an increased BAX level confers apoptosis and relieves the need for upstream apoptosis regulation by functional p53 (21). Conversely, Caov-3 cells exposed to gamma radiation have increased BAX expression despite lacking functional p53. In other words, treatments can bypass non-functional p53 and affect BAX expression (22). Thus, there is an indication that the BAX level can mediate and act as a surrogate for apoptotic signaling, even in cells lacking functional p53. Lastly, and perhaps most importantly, potentiating BAX has useful clinical implications; one study revealed that increased BAX levels in tumors of patients newly diagnosed with ovarian cancer resulted in a complete response to paclitaxel and platinum-containing compounds (23). This effect was due to increased apoptotic cell death. Therefore treatments that can increase the level of BAX may be useful to clinicians for primary treatments, but also in efforts to re-sensitize tumors to conventional therapy. Together with the caspase-9 results, the observed increased BAX signaling provides further evidence that apoptosis is taking place and reflects the antitumor activity of
Although Caov-3 cells lack functional p53, the literature suggests they are still capable of apoptosis, but the effect that non-wild-type p53 may exert on our apoptosis assay, if any, is not yet certain. The ability of ABC294640 to affect a multitude of signaling cascades [ERK, nuclear factor kappa B (NFκB) and protein kinase B (AKT)] and apoptosis (17, 18) and the presence of mediators of cell death in the p53 family (BAX), indicate that further investigation to elucidate its antitumor mechanisms is warranted.

Although there is some indication that apoptotic signaling does occur on treatment with ABC294640, the possibility of a separate mechanism by which this drug imparts its cytotoxic effect in ovarian cancer needs to be considered. Beljanski et al. recently discovered that in kidney, breast, and prostate cancers, ABC294640 mediates its anticancer effect primarily through autophagic, not apoptotic mechanisms. The authors state that the exact autophagic mechanism is unknown, but may include key metabolic and apoptotic proteins that ultimately push the cell into non-apoptotic (and autophagic) death (20). Our findings may reflect some part of this alternate mechanism. Although their study did not examine the effect of ABC294640 on ovarian cancer cells, it is plausible that autophagic and not apoptotic cell death was the primary action mechanism of this drug in ovarian cancer. Alternatively, both processes may be occurring. Autophagy as the primary mechanism may help support our observation of the discordance between the drug’s cytotoxic effect and its failure to induce robust DNA fragmentation into mono- and oligo-nucleosomes. It has also been suggested that BAX and other B-cell lymphoma 2 (Bcl-2)-related proteins may have a role in regulating autophagy (24). Therefore, the increase in BAX and caspase-9 signaling may not be suitable surrogates for apoptosis in the present study. Another possibility is that potentiation of BAX and caspase-9 in response to treatment is quantitatively dwarfed when compared with the autophagic signaling that may be taking place. More studies are needed to determine if autophagy plays a substantive role in the antitumor effect of ABC294640 on ovarian carcinoma.

We have, to our knowledge, for the first time, evaluated the antitumor activity and mechanism of a selective SphK2, ABC294640, in chemoresistant ovarian cancer. We have shown increased cell death signaling when ABC294640 was used in combination with conventional therapy. Based on this evidence, we conclude that the selective targeting of SphK2 with ABC294640 may prove to be a valuable tool in cancer therapeutics.

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References


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