Targeting Sphingosine Kinase Induces Apoptosis and Tumor Regression for KSHV-Associated Primary Effusion Lymphoma

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Abstract

Sphingosine kinase (SPHK) is overexpressed by a variety of cancers, and its phosphorylation of sphingosine results in accumulation of sphingosine-1-phosphate (SIP) and activation of antiapoptotic signal transduction. Existing data indicate a role for SIP in viral pathogenesis, but roles for SPHK and SIP in virus-associated cancer progression have not been defined. Rare pathologic variants of diffuse large B-cell lymphoma arise preferentially in the setting of HIV infection, including primary effusion lymphoma (PEL), a highly mortal tumor etiologically linked to the Kaposi’s sarcoma-associated herpesvirus (KSHV). We have found that ABC294640, a novel clinical-grade small molecule selectively targeting SPHK (SPHK2 >> SPHK1), induces dose-dependent caspase cleavage and apoptosis for KSHV+ patient-derived PEL cells, in part through inhibition of constitutive signal transduction associated with PEL cell proliferation and survival. These results were validated with induction of PEL cell apoptosis using SPHK2-specific siRNA, as well as confirmation of drug-induced SPHK inhibition in PEL cells with dose-dependent accumulation of proapoptotic ceramides and reduction of intracellular SIP. Furthermore, we demonstrate that systemic administration of ABC294640 induces tumor regression in an established human PEL xenograft model. Complimentary ex vivo analyses revealed suppression of signal transduction and increased KSHV lytic gene expression within drug-treated tumors, with the latter validated in vitro through demonstration of dose-dependent viral lytic gene expression within PEL cells exposed to ABC294640. Collectively, these results implicate interrelated mechanisms and SPHK2 inhibition in the induction of PEL cell death by ABC294640 and rationalize evaluation of ABC294640 in clinical trials for the treatment of KSHV-associated lymphoma. Mol Cancer Ther; 13(1); 154–64. ©2013 AACR.

Introduction

Rare and aggressive pathologic variants of diffuse large B-cell lymphoma (DLBCL) arise more frequently in patients infected with HIV, and the majority of these tumors, including primary effusion lymphoma (PEL), are etiologically linked to the human oncogenic γ-herpesviruses: Kaposi’s sarcoma-associated herpesvirus (KSHV) and Epstein–Barr virus (EBV; ref. 1). PEL tumors are causally associated with KSHV, typically infected with both KSHV and EBV (KSHV+EBV+) or KSHV alone (KSHV+EBV−), arise preferentially within pleural or peritoneal cavities, and progress rapidly despite chemotherapy with a median survival of around 6 months (2–4). Combination cytotoxic chemotherapy represents the current standard of care for PEL (3, 5, 6), but lack of efficacy, off-target effects (including bone marrow suppression), and chemotherapeutic resistance (generated through virus-associated mechanisms) continue to limit the utility of this approach (3, 5–9). A combination of highly active antiretroviral therapy and rituximab, an anti-CD20 monoclonal antibody, has led to short-term responses, but PEL cells generally do not express CD20 (10, 11). The proteasome inhibitor bortezomib and the combination of arsenic trioxide and interferon both reduce NF-κB activation and may work synergistically with cytotoxic chemotherapy to reduce PEL viability (12, 13). Unfortunately, proteasome inhibition and arsenic incur significant toxicities limiting
their clinical application. Antiviral agents inhibit \( \gamma \)-herpesvirus replication (14, 15), but these drugs do not affect latent gene expression in PEL cell lines and have limited effects on PEL cell growth in vitro (15). Targeting the mammalian target of rapamycin (mTOR) suppresses constitutive signal transduction associated with PEL survival and has proven successful in preclinical models (16), but rapamycin may paradoxically induce activation of alternative signaling pathways, and whether drug concentrations necessary for an antitumoral effect are achievable in patients remains unclear (17). Finally, efficacy data for autologous stem cell transplantation for PEL are also limited, and the problems inherent with additive immune suppression in HIV-infected patients currently preclude routine use of this approach (18). In summary, safer and more effective therapeutic strategies for PEL are urgently needed.

Sphingolipid biosynthesis involves hydrolytic conversion of ceramide to sphingosine, which is subsequently phosphorylated by one of two sphingosine kinase isoforms (SPHK1 or SPHK2) to generate bioactive sphingosine-1-phosphate (SIP; ref. 19). The relative cellular concentrations of ceramide and SIP ultimately determine tumor cell fate, with accumulation of ceramides favoring apoptosis, and accumulation of SIP favoring proliferation (19, 20). SPHK is activated by tumor-promoting cytokines and growth factors, leading to rapid increases in the intracellular levels of SIP and depletion of ceramide species (21). SPHK activity and SIP induce activation of signal transduction, including mitogen-activated protein kinase (MAPK) and NF-\( \kappa \)B pathways (22, 23) relevant to KSHV pathogenesis (24, 25), and a small number of studies support a role for sphingolipid biosynthetic pathways in regulation of viral pathogenesis (26, 27). However, functional consequences of targeting SPHK and reducing SIP for virus-infected tumor cells have not been explored.

A novel small molecule, 3-(4-chlorophenyl)-adaman-tane-1-carboxylic acid (pyridin-4-ylmethyl)amide (ABC294640), inhibits SPHK activity and is highly selective for the SPHK2 isoform at concentrations less than 100 \( \mu \)mol/L (28). ABC294640 displays in vitro and in vivo activity against a variety of nonviral tumors in preclinical studies, including significant reductions in SIP expression within intratumoral and noncellular fractions (28, 29). In addition, the drug’s selectivity for SPHK, evidenced by lack of inhibition of other kinases (30), underscores its observed safety in preclinical studies and, thus far, in a clinical trial enrolling patients with solid tumors (Clinicaltrials.gov identifier, NCT01488513). Therefore, we sought to characterize the impact of ABC294640 inhibition of SPHK for KSHV-infected PEL cell viability in vitro, as well as associated tumor progression in vivo. In addition, we sought to identify relationships between SPHK inhibition and mechanisms associated with PEL cell death, including induction of apoptosis, accumulation of proapoptotic ceramides, and perturbations in KSHV gene expression.

Materials and Methods

Cell culture and reagents

Body cavity–based lymphoma cells (BCBL-1, KSHV\(^+\)/EBV\(^{-}\)) and a Burkitt lymphoma cell line (BL-41, KSHV\(^{-}\)/EBV\(^{+}\)) were kindly provided by Dr. Dean Kedes (University of Virginia School of Medicine, Charlottesville, VA) and maintained in RPMI 1640 medium (Gibco) with supplements as described previously (31). BC-1 (KSHV\(^+\)/EBV\(^{-}\)), BC-3 (KSHV\(^+\)/EBV\(^{+}\)), and BCP-1 (KSHV\(^+\)/EBV\(^{+}\)) cells were purchased from American Type Culture Collection (ATCC) and maintained in complete RPMI 1640 medium (ATCC) supplemented with 20% FBS. KSHV infection was verified for all cell lines using immunofluorescence assays for detection of the KSHV latency-associated nuclear antigen (LANA). All cells were incubated at 37°C in 5% CO\(_2\). All experiments were carried out using cells harvested at low (<20) passages. The 3-(4-chlorophenyl)-adaman-tane-1-carboxylic acid (pyridin-4-ylmethyl)amide (ABC294640) was synthesized for all experiments using good laboratory practices as appropriate for clinical applications and previously described (30).

Cell viability assays

Metabolic activity of PEL cells was assessed using standard MTT assays as described previously (31). Apoptosis was quantified by flow cytometry using the fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) Apoptosis Detection Kit I (BD Pharmingen) as previously described (10) and according to the manufacturer’s instructions. Data were collected using a FACS-Calibur 4-color flow cytometer (BD Bioscience).

Transfection assays

BCBL-1 were transfected using pcDNA3.1-FLAG-ERK, pcDNA3.1-FLAG-NF-xB p65, or control vectors as described previously (32, 33). Transfection efficiency was assessed through cotransfection of a lacZ reporter construct and subsequent determination of \( \beta \)-galactosidase activity using a commercial \( \beta \)-galactosidase enzyme assay system according to the manufacturer’s instructions (Promega). For RNA interference (RNAi) assays, SphK2 ON-TARGETplus SMARTpool siRNA (Dharmacon), or negative control siRNA, were delivered using the DharmaFECT transfection reagent according to the manufacturer’s instructions. To confirm initial transfection efficiency for siRNA experiments, PEL cells were transfected with GFP-tagged siRNA, and GFP expression was determined by flow cytometry 24 hours later. Three independent transfections were performed for each experiment, and all samples were analyzed in triplicate for each transfection.

Immunoblotting

Cells were lysed in buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% NP40, 1 mmol/L EDTA, 5 mmol/L NaF, and 5 mmol/L Na\(_2\)VO\(_4\). Total cell lysates (30 \( \mu \)g) were resolved by 10% SDS-PAGE, transferred to
none
95°C under vacuum for 40 minutes and allowed to cool for 30 minutes at room temperature, then rinsed with PBS and incubated in MeOH/3% H2O2 for 20 minutes to quench endogenous peroxidase. Slides were then washed with PBS and blocked with 5% normal goat serum in 0.1% PBS/BSA for 2 hours at room temperature, then incubated overnight with a rat monoclonal anti-LANA antibody (ABI) using a 1:500 dilution in 0.1% PBS/BSA. The following day, slides were incubated with a goat anti-rat IgG secondary antibody at room temperature for 1 hour, followed by avidin–biotin peroxidase complexes for 1 hour at room temperature. Finally, slides were developed using a diaminobenzidine substrate, counterstained with hematoxylin, dehydrated through an ascending alcohol gradient, cleared in xylene, and coverslipped with Permount. Images were collected at ×200 and ×600 magnification using a Olympus BX61 microscope equipped with a high resolution DP72 camera and CellSense image capture software.

**Isolation of circulating human B cells**

Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood from two healthy donors following Ficoll gradient separation. PBMC were washed and resuspended in 500 μL total volume, including 440 μL buffer composed of 2% FBS and 1 mmol/L EDTA in 1× PBS (EasySep Buffer; STEMCELL Technologies), 30 μL Fc-receptor blocker (eBiosciences), and 30 μL of a PE-conjugated anti-CD19 monoclonal antibody (BD-Pharmagen), for incubation at room temperature for 20 minutes. Of note, 100 μL EasySep PE selection cocktail (STEMCELL Technologies) was added for an additional 15 minutes, and 2.5 mL of additional buffer was then added before magnetic column separation of CD19+ cells. Following column separation, supernatants were discarded and cells resuspended in fresh 2.5 mL buffer for each of two additional column separation steps. Thereafter, cells were resuspended in complete RPMI 1640 medium supplemented with 20% FBS for overnight culture with ABC294640, or in 1× PBS for flow cytometry to determine the purity of selection. For both donors, 92% to 95% pure populations of CD19+ cells were recovered (data not shown).

**Statistical analysis**

Significance for differences between experimental and control groups were determined using the two-tailed Student t test (Excel 8.0), and P values less than 0.01 were considered significant.

**Results**

**Pharmacologic targeting of SphK with ABC294640 results in dose-dependent induction of apoptosis for PEL cells**

To first determine whether pharmacologic targeting of SphK in vitro impacts metabolic activity for PEL cells, we incubated either KSHV+/EBVneg BC-1 cells or KSHVneg/EBVneg BL-41 cells with ABC294640 at concentrations previously shown to impact SphK2 function, but not SphK1 (28). We found that the drug induces significant dose-dependent suppression of metabolic activity of BCBL-1 based on MTT assays, with little or no impact on BL-41 cells (Fig. 1A). Next, using flow cytometry, we found that ABC294640 induces apoptosis in dose-dependent fashion for BCBL-1 cells, but has little impact on BL-41 cells over the same range of concentrations (Fig. 1B and C). We also found that ABC294640 induced dose-dependent apoptosis for other KSHV+ PEL cell lines, including KSHV+/EBV+ BC-1 cells, KSHV+/EBVneg BC-3 cells, and KSHV+/EBVneg BCP-1 cells (Fig. 1D–F). Providing additional evidence for drug selectivity, we observed a lack of discernible drug-induced toxicity for primary human CD19+ B cells isolated from peripheral blood of healthy donors (Supplementary Fig. S1) at concentrations commensurate with those achievable in human plasma following administration of the predicted therapeutic dose of ABC294640 (20 μmol/L or less based on the ongoing clinical trial; NCT01488513). Of note, we observed significant increases in toxicity for B-cell tumors at 20 μmol/L in vitro (Fig. 1).

**ABC294640 induces apoptosis for PEL cell lines through suppression of KSHV-associated signal transduction**

As mentioned previously, SIP induces activation of NF-kB and MAPKs, including extracellular signal-regulated kinase (ERK), and these pathways are also activated by KSHV-encoded latent proteins and increase viability for infected cells (22, 23, 32, 35). Therefore, we sought to determine whether ABC294640 impairs constitutive signal transduction in PEL cells, and whether reversal of drug-induced suppression of these pathways restores PEL viability. We observed dose-dependent suppression of ERK, Akt, and NF-kB p65 phosphorylation, as well as cleavage of caspases-3 and 9, within BCBL-1 cells exposed to ABC294640, but not within drug-resistant BL-41 cells (Fig. 3A). To verify whether ABC294640-induced apoptosis is associated with drug-mediated suppression of signal transduction, we transiently transfected BCBL-1 cells with constructs encoding either ERK or p65 before their incubation with ABC294640. We found that overexpression of either ERK or p65 (and resultant increases in phospho-ERK/p65) partially suppressed apoptosis induced by ABC294640 (Fig. 3B and C). To validate these observations, we used RNAi targeting SphK2 given the
preferential inhibitory activity of the drug for this isof orm at the concentrations used in our studies. Achieving 60% to 70% reduction in basal SPHK2 transcript expression for BCBL-1 and BCP-1 cells using this approach (Fig. 4A and B), we found that PEL cells exhibited reduced activation of ERK, Akt, and p65 phosphorylation (Fig. 4C), as well as a 5-to 6-fold increase in apoptosis and cell death (Fig. 4D and E).

**ABC294640 suppresses PEL progression and induces regression of established PEL tumors in vivo**

As noted earlier, ABC294640 displays in vivo activity in preclinical tumor models (28, 29), but its activity against virus-associated tumors, and hematologic tumors generally, has not been explored. Therefore, we sought to determine the activity of ABC294640 against PEL tumors in vivo using an established xenograft model in which PEL cells are introduced into the peritoneal cavity of immune-compromised mice. This results in intracavitary tumor expansion with development of ascites and increases in abdominal girth associated with “free-floating” PEL cells, as well as formation of solid PEL tumors within the peritoneal cavity and distal organs following hematogenous dissemination (36, 37). We injected BCBL-1 cells into NOD/SCID mice for these experiments, given a high degree of resistance for BCBL-1 cells to conventional cytotoxic agents relative to other PEL cell lines (7), which is due in part to increased surface expression of drug efflux pumps (9). We observed clear PEL expansion within 3 to 4 weeks using our protocol, including time-dependent weight gain and increased abdominal girth, as well as ascites accumulation and splenic enlargement due to tumor infiltration at the time of necropsy (Fig. 5). These observations were validated through identification of human CD138+ PEL cells recovered from within ascites fluid (Supplementary Fig. S2A), as well as observation of intranuclear expression of KSHV-encoded LANA within tumor cells from ascites fluid (Supplementary Fig. S2B) and within splenic tissue (Supplementary Fig. S2C).
For initial experiments, we administered ABC294640, either intraperitoneally or orally, within 24 hours of PEL cell injections. ABC294640 significantly reduced tumor expansion with either route of administration (Fig. 5). To obtain sufficient tumor tissue for relevant ex vivo analyses, we performed additional experiments in which ABC294640 therapy was initiated following establishment of PEL tumors (beginning 21 days after PEL cell injections). Using this approach, drug-treated mice exhibited significant regression of PEL tumor burden relative to untreated animals (Fig. 6A–C).

Sufficient and homogenous populations of ascites-derived PEL cells were retrievable from drug-treated and untreated mice in these latter experiments for subsequent analyses (Supplementary Fig. S2). To assess SPHK inhibition in the model, lipid mass spectrometry...
was performed using ascites-derived PEL cell lysates from individual animals. Higher concentrations of ceramides and dh-ceramides were observed within live tumor cells from drug-treated animals, although these differences were not significant (Supplementary Fig. S3A and S3B). However, cells from drug-treated animals exhibited significantly reduced concentrations of intracellular S1P (Supplementary Fig. S3C). Lipid mass spectrometry performed on cell-free fluid requires large sample volumes, limiting the utility of this approach for comparison of cell-free intracavitary sphingolipid concentrations between individual animals. Nevertheless, we performed these assays after pooling cell-free ascites fluid, and we observed increases in ceramides and dh-ceramides, as well as reductions in S1P, within pooled samples from ABC294640-treated mice relative to their untreated counterparts (Supplementary Fig. S3D–S3F). Plasma-based assays were also performed, but low concentrations of bioactive sphingolipids in this compartment precluded meaningful comparisons (data not shown).

To determine whether ABC294640 induces apoptosis and suppresses KSHV-associated signal transduction for PEL cells in vivo, we performed immunoblot analyses using ascites-derived PEL cell lysates from representative animals. Increases in annexin expression, along with increases in caspase-3 and -9 cleavage and reductions in ERK, Akt, and p65 phosphorylation, were noted for cells from drug-treated mice (Fig. 6D and E), commensurate with our in vitro data. We also quantified expression of representative KSHV transcripts from ascites-derived PEL cell lysates from individual animals. We found that...
PEL cells from drug-treated animals exhibited significantly increased expression of all lytic genes profiled, including ORF50, but no change in expression of latent ORF73 transcripts (which encode LANA; Fig. 6F). To validate these findings, we repeated in vitro experiments incubating BCBL-1 cells with ABC294640 and noted dose-dependent increases in expression of lytic transcripts with drug exposure, but once again no change in expression of ORF73 (Fig. 6G).

Discussion

To our knowledge, this is the first report directly implicating bioactive sphingolipids in KSHV pathogenesis. Our data are also consistent with previous reports correlating antitumor effects for ABC294640 with intratumoral accumulation of proapoptotic ceramide species, reduced S1P levels, and suppression of MAPK and NF-κB activation (28, 29, 38, 39). One recent study reported induction of autophagy within renal, prostate, and breast cancer cell lines following exposure to ABC294640, and use of an inhibitor of autophagy enabled apoptosis while reducing tumor cell death with ABC294640 exposure (40). These data suggest that for some tumors, ABC294640 may synergize with other proapoptotic agents to cause cell death through complimentary induction of autophagy. However, induction of autophagy may actually facilitate use of alternative energy sources and promote cancer-cell proliferation within established tumors (41). We found that ABC294640 induces dose-dependent apoptosis for a variety of patient-derived PEL cell lines in vitro and in vivo, and parallel experiments using ascites tumor lysates from drug-treated animals failed to reveal protein signatures consistent with activation of autophagy (data not shown).

The relative importance of apoptosis and autophagy pathways for PEL cell death during ABC294640 treatment requires further clarification to inform future preclinical studies and clinical trials using combination therapies targeting these pathways.

On the basis of a wealth of published data and our own observations, suppression of constitutive signal transduction, accumulation of proapoptotic ceramides, reductions in antiapoptotic signaling associated with S1P, and/or induction of KSHV lytic gene expression may all logically contribute to PEL cell apoptosis induced by ABC294640. Restoration of ERK and p65 activation during drug
treatment only partially restored PEL viability, and we observed coincident dose-dependent activation of viral lytic gene expression with the drug. Induction of KSHV lytic gene expression, governed by the lytic "switch" gene ORF50, typically results in PEL cell death (42–44), and this concept has been explored as a therapeutic strategy given that standard cytotoxic agents, as well as bortezomib and valproic acid [the latter a histone deacetylase (HDAC) inhibitor used for a variety of clinical applications], induce lytic gene expression and reduce PEL cell viability (36, 37, 42, 45). KSHV lytic gene expression is inhibited through HDAC binding to ORF50, (46), and viral genes expressed predominantly during lytic replication suppress HDAC activity (47). A role for SPHK in epigenetic regulation was revealed through its direct association with core histone H3 and generation of intranuclear S1P that binds active sites on HDACs to inhibit their enzymatic activity (48). These data suggest a potential role for SPHK and S1P in regulating the KSHV lytic switch. Because KSHV microRNAs also regulate the lytic switch, induce antiapoptotic signal transduction, promote viral latency, and suppress caspase-3–mediated apoptosis for KSHV-infected cells (49–53), future mechanistic studies might logically focus on whether SPHK and S1P regulate KSHV microRNA expression and function. Whether the observed lack of toxicity for KSHVneg/EBVneg BL-41 cells and human peripheral B cells isolated from healthy donors in our studies is related to the lack of established viral infection within these cells (and, by extension, lack of induction of death-associated lytic viral gene expression) requires confirmation. Additional studies are also needed to address potential mechanisms for drug resistance, including drug transporter expression or compensatory signaling associated with cellular oncogenes.

ABC294640 exhibits little or no inhibitory activity for SPHK1 at concentrations up to 100 μmol/L (28), a

Figure 6. ABC294640 (ABC) induces regression of established PEL tumors in vivo. A to C, NOD/SCID mice were injected intraperitoneally with 10^7 BCBL-1 cells. Beginning 21 days later, 100 mg/kg ABC or vehicle (n = 10 per group) were administered intraperitoneally once daily, 5 days per week, for an additional 21 days for each of three independent experiments. Weights were recorded weekly, and images of representative animals and their respective spleens, as well as ascites fluid volumes, were collected at the conclusion of experiments on day 42. D, apoptosis was quantified by flow cytometry for live PEL cells recovered from ascites fractions as described in Materials and Methods and Supplementary Fig. S2. E, immunoblots were performed to identify phosphorylated signaling intermediates and caspase cleavage within live PEL cells recovered from ascites fractions from each of 2 mice representing vehicle- and drug-treated groups. F, RNA was recovered from live PEL cells from ascites fractions from each of 4 mice representing vehicle- or drug-treated groups, and this was done for two independent experiments. Quantitative real-time PCR (qRTPCR) was used to quantify viral transcripts representing either latent (ORF73) or lytic genes, including the lytic "switch" gene ORF50. Data were normalized to samples representing vehicle-treated mice, and using β-actin as a loading control. G, BCBL-1 cells were incubated with the indicated concentrations of ABC or vehicle for 16 hours and representative viral transcripts quantified using qRT-PCR as earlier. Error bars, SEM for two (F) or three (B and G) independent experiments; *, P < 0.01.
concentration that exceeds those used in our in vitro studies. Moreover, we find that RNAi targeting of SPHK2 alone is sufficient to suppress ERK, Akt, and NF-κB activation and induce apoptosis for PEL cells. Induction of apoptosis with SPHK2 knockdown was incomplete, although this could reflect limitations of our assays given low-level persistence of SPHK2 transcripts and protein with our siRNA protocol. Therefore, although we feel it is unlikely that ABC294640 reduces S1P production in PEL cells through inhibition of Spk1 at clinically achievable concentrations, we cannot categorically exclude drug inhibition of SPHK1 as a mechanism for the observations in the present studies.

SIP binds to one of five G-protein–coupled SIP receptors (SIPR1–5) that activate diverse downstream signaling pathways (54). Our identification of extracellular SIP within ascites fluid from tumor-bearing mice suggests that PEL cells may support their own growth through secretion of SIP and augmentation of signal transduction through autocrine or paracrine mechanisms. Therefore, additional experiments are under way to characterize SIPR expression by PEL cells and to determine their relative importance for constitutive PEL signaling, as well as the role for exogenous SIP in survival for KSHV-infected cells. Our data also indicate that SIP may be useful as either a diagnostic tool or prognostic biomarker in ascites fluid, and future clinical trials including these assays should provide additional information.

Collectively, our data imply that SPHK (specifically SPHK2) and S1P play a role in PEL pathogenesis through regulation of well-characterized signal transduction pathways and possibly KSHV gene expression. Most importantly, our studies highlight SPHK as a potential therapeutic target for PEL and justify performance of early-phase clinical trials evaluating ABC294640 in HIV+ patients with PEL or other aggressive B-cell lymphomas who have thus far been excluded from clinical trials with this agent.

Disclosure of Potential Conflicts of Interest

C.D. Smith is the President and Chief Executive Officer and has ownership interests (including patents) in Apogee Biotechnology Corporation. No potential conflicts of interest were disclosed by the other authors.

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