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## 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 (S1P) and activation of anti-

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#### Disclosure of Potential Conflicts of Interest

CDS is the President and Chief Executive Officer of Apogee Biotechnology Incorporated. Apogee produced clinical-grade ABC294640 for this study, but no additional funds were received from the company. None of the other authors have formal relationships with the company or other conflicts of interest.

#### Authors' Contributions

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apoptotic signal transduction. Existing data indicate a role for S1P in viral pathogenesis, but roles for SphK and S1P 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<sup>+</sup> 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 pro-apoptotic ceramides and reduction of intracellular S1P. 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.

## Keywords

KSHV; sphingosine kinase; S1P; lymphoma; HIV

## Introduction

Rare and aggressive pathologic variants of diffuse large B-cell lymphoma (DLBCL) arise more frequently in patients infected with the human immunodeficiency virus (HIV), and the majority of these tumors, including primary effusion lymphoma (PEL), are etiologically linked to the human oncogenic  $\gamma$ -herpesviruses: Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) (1). PEL tumors are causally associated with KSHV, typically infected with both KSHV and EBV (KSHV<sup>+</sup>EBV<sup>+</sup>) or KSHV alone (KSHV<sup>+</sup>EBV<sup>neg</sup>), 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 (HAART) 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- $\kappa$ B 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 pre-clinical models (16), but rapamycin may paradoxically induce activation of alternative signaling pathways, and whether drug concentrations necessary for an anti-tumoral effect are achievable in patients remains unclear (17). Finally, 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 (S1P) (19). The relative cellular concentrations of ceramide and S1P ultimately determine tumor cell fate, with accumulation of ceramides favoring apoptosis, and accumulation of S1P favoring proliferation (19, 20). SphK is activated by tumor-promoting cytokines and growth factors, leading to rapid increases in the intracellular levels of S1P and depletion of ceramide species (21). SphK activity and S1P 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 S1P for virus-infected tumor cells have not been explored.

A novel small molecule, 3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide (ABC294640) inhibits SphK activity and is highly selective for the SphK2 isoform at concentrations less than 100 $\mu$ M (28). ABC294640 displays *in vitro* and *in vivo* activity against a variety of non-viral tumors in preclinical studies, including significant reductions in S1P expression within intratumoral and non-cellular 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*, and to identify relationships between SphK inhibition and mechanisms associated with PEL cell death, including induction of apoptosis, accumulation of pro-apoptotic ceramides, and perturbations in KSHV gene expression.

## Materials and Methods

### Cell culture and reagents

Body cavity-based lymphoma cells (BCBL-1, KSHV<sup>+</sup>/EBV<sup>neg</sup>) and a Burkitt's lymphoma cell line (BL-41, KSHV<sup>neg</sup>/EBV<sup>neg</sup>) were kindly provided by Dr. Dean Kedes (University of Virginia) and maintained in RPMI 1640 medium (Gibco) with supplements as described previously (31). BC-1 (KSHV<sup>+</sup>/EBV<sup>+</sup>), BC-3 (KSHV<sup>+</sup>/EBV<sup>neg</sup>), and BCP-1 (KSHV<sup>+</sup>/EBV<sup>neg</sup>) cells were purchased from ATCC and maintained in complete RPMI 1640 medium (ATCC) supplemented with 20% FBS. KSHV infection was verified for all cells lines using immunofluorescence assays for detection of the KSHV latency-associated nuclear antigen (LANA, see below). All cells were incubated at 37°C in 5% CO<sub>2</sub>. All experiments were carried out using cells harvested at low (<20) passages. KSHV infection The 3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl) amide (ABC294640) was synthesized for all experiments using good laboratory practices as appropriate for clinical applications as 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 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- $\kappa$ B p65, or control vectors as described previously (32, 33). Transfection efficiency was assessed through co-transfection 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, Madison, WI). For RNA interference assays, SphK2 ON-TARGET plus SMART pool siRNA (Dharmacon, Chicago, IL), 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 green fluorescent protein (GFP)-tagged siRNA, and GFP expression determined by flow cytometry 24 h 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 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM EDTA, 5 mM NaF and 5 mM Na<sub>3</sub>VO<sub>4</sub>. Total cell lysates (30  $\mu$ g) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and incubated with 100–200  $\mu$ g/mL of the following antibodies: phospho-Akt (Ser473), phospho-p44/42 ERK (Thr202/Tyr204), phospho-NF- $\kappa$ B p65 (Ser536) and respective total kinase proteins, pro-/cleaved caspase-3, and pro-/cleaved caspase-9 (Cell Signaling Technologies). For loading controls, lysates were also incubated with antibodies detecting  $\beta$ -Actin (Sigma). Immunoreactive bands were developed using an enhanced chemiluminescence reaction (Perkin-Elmer).

## qRT-PCR

Total RNA was isolated using the RNeasy Mini kit (QIAGEN), and cDNA was synthesized from equivalent total RNA using a SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen) according to the manufacturer's instructions. Primers used for amplification of target genes are displayed in Supplemental Table 1. Amplification was carried out using an iCycler IQ Real-Time PCR Detection System, and cycle threshold (Ct) values were tabulated in duplicate for each gene of interest in each experiment. "No template" (water) controls were used to ensure minimal background contamination. Using mean Ct values tabulated for each gene, and paired Ct values for  $\beta$ -actin as a loading control, fold changes for experimental groups relative to assigned controls were calculated using automated iQ5 2.0 software (Bio-rad).

## Quantification of sphingolipids

Quantification of ceramide and dihydro-ceramide species was performed using a Thermo Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer operating in Multiple Reaction Monitoring positive ionization mode (Thermo Fisher Scientific). Quantification was based on calibration curves generated by spiking an artificial matrix with known amounts of target standards and an equal amount of the internal standard. The target analyte:internal standard peak area ratios from each sample were compared with the calibration curves using linear regression. Final results were expressed as the ratio of sphingolipid normalized to total phospholipid phosphate level using the Bligh and Dyer lipid extract method (34). Concentrations of S1P in ascites fluid supernatants and PEL cell lysates were determined using the S1P Assay Kit (Echelon) according to the manufacturers' instructions.

## PEL xenograft model

BCBL-1 cells maintained at early passage number in cell culture were washed twice in sterile-filtered PBS prior to performance of trypan blue and flow cytometry assays for

verification of their viability. Aliquots of  $10^7$  viable cells were diluted in 200 $\mu$ l sterile PBS, and 6–8 week-old male non-obese diabetic/severe-combined immunodeficient (NOD/SCID) mice (Jackson Laboratory, Taconic Inc.) received intraperitoneal (i.p.) injections with a single cell aliquot. For drug delivery, ABC294640 was diluted in sterile-filtered 0.375% Tween-80 (Sigma) in PBS to achieve 100 $\mu$ l total volume. The drug, or vehicle alone, was administered using an insulin syringe for i.p. injections, or a 20-gauge 1.0-inch animal feeding needle (Popper and Sons, item #7921) for oral gavage. Drug was administered either 1 day or 21 days after BCBL-1 injections. Three experiments, with 10 mice per group for each experiment, were performed for each route of administration. For confirmation of PEL expansion within the murine model, ascites fluid was collected 3–4 weeks after injection. Cells from ascites fluid were resuspended in PEL media (described above) for 2 h in fibronectin-coated plates, and cells remaining in suspension were separated from adherent cells and analyzed by flow cytometry. For the latter, cells were resuspended in 3% BSA in 1X PBS, incubated on ice for 10 minutes, then incubated with a 1:50 dilution of primary antibodies recognizing human CD138 for an additional 30 minutes. Following two subsequent wash steps, cells were incubated for 30 minutes with goat anti-rabbit IgG conjugated to Alexa-647 and diluted 1:200. Control cells were incubated with secondary antibodies only. 10,000 cells were resuspended in 1X PBS for flow cytometry analysis. As a second method of validation,  $10^5$  non-adherent cells freshly isolated from ascites fluid cultures as above were placed on glass slides (using a pap pen) for 2 h at 37°C, then incubated in 1:1 methanol-acetone at 20°C for fixation and permeabilization. Following incubation with a blocking reagent (10% normal goat serum, 3% bovine serum albumin, and 1% glycine) for an additional 30 minutes, cells were incubated for 1 h at 25°C using a 1:1000 dilution of rat anti-LANA monoclonal antibodies (ABI), followed by 1:100 dilution of goat anti-rat secondary antibodies conjugated to Texas Red (Invitrogen). For nuclear localization, cells were subsequently counterstained with 0.5  $\mu$ g/mL 4', 6-diamidino-2-phenylindole (DAPI, Sigma) in 180 mM Tris-HCl (pH 7.5), washed once in 180 mM Tris-HCl for 15 min, and prepared for visualization using a Leica TCPS SP5 AOBs confocal microscope. Weights were recorded weekly as a surrogate measure of tumor progression, and ascites fluid volumes were tabulated for individual mice at the completion of each experiment. All protocols were approved by the Louisiana State University Health Science Center Animal Care and Use Committee in accordance with national guidelines.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were microtome-sectioned to a thickness of 4  $\mu$ m, placed on electromagnetically charged slides (Fisher Scientific), and stained with hematoxylin & eosin (H&E) for routine histologic analysis. Immunohistochemistry was performed using the Avidin-Biotin-Peroxidase complex system, according to the manufacturer's instructions (Vectastain Elite ABC Peroxidase Kit; Vector Laboratories). In our modified protocol, sections were deparaffinized in xylene and re-hydrated through a descending alcohol gradient. For non-enzymatic antigen retrieval, slides were heated in 0.01 M sodium citrate buffer (pH 6.0) to 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% H<sub>2</sub>O<sub>2</sub> 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 h 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 h, followed by avidin-biotin peroxidase complexes for 1 h 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 200x and 600x 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 500uL total volume, including 440uL buffer composed of 2% FBS and 1mM EDTA in 1X PBS (EasySep buffer, STEMCELL Technologies), 30uL Fc-receptor blocker (eBiosciences), and 30uL of a PE-conjugated anti-CD19 monoclonal antibody (BD-Pharmagen), for incubation at RT for 20 minutes. 100uL EasySep PE selection cocktail (STEMCELL Technologies) was added for an additional 15 minutes, and 2.5ml of additional buffer was then added prior to magnetic column separation of CD19<sup>+</sup> cells. Following column separation, supernatants were discarded and cells resuspended in fresh 2.5ml 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 1X PBS for flow cytometry to determine the purity of selection. For both donors, 92–95% pure populations of CD19<sup>+</sup> cells were recovered (data not shown).

### Statistical analysis

Significance for differences between experimental and control groups were determined using the two-tailed Student's t-test (Excel 8.0), and p values <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<sup>+</sup>/EBV<sup>neg</sup> BCBL-1 cells or KSHV<sup>neg</sup>/EBV<sup>neg</sup> 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 (Figs. 1B and C). We also found that ABC294640 induced dose-dependent apoptosis for other KSHV<sup>+</sup> PEL cell-lines, including KSHV<sup>+</sup>/EBV<sup>+</sup> BC-1 cells, KSHV<sup>+</sup>/EBV<sup>neg</sup> BC-3 cells, and KSHV<sup>+</sup>/EBV<sup>neg</sup> BCP-1 cells (Figs. 1D–F). Providing additional evidence for drug selectivity, we observed a lack of discernible drug-induced toxicity for primary human CD19<sup>+</sup> B cells isolated from peripheral blood of healthy donors (Fig. S1) at concentrations commensurate with those achievable in human plasma following administration of the predicted therapeutic dose of ABC294640 (20uM or less based on the ongoing clinical trial: NCT01488513). Of note, we observed significant increases in toxicity for B-cell tumors at 20uM *in vitro* (Fig. 1).

To confirm inhibition of SphK in cells for which ABC294640 induces appreciable toxicity, we used mass spectrometry analyses to quantify intracellular levels of bioactive ceramides and dihydro(dh)-ceramides, as well as a modified ELISA assay to quantify S1P. We observed dose-dependent accumulation of total ceramide and dh-ceramide levels, and reductions in S1P, within BCBL-1 cells in the presence of ABC294640 (Fig. 2), thereby confirming dose-dependent inhibition of SphK function in these cells.

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

As mentioned previously, SIP induces activation of NF- $\kappa$ B 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- $\kappa$ B 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 prior to 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 (Figs. 3B and C). To validate these observations, we used RNA interference targeting SphK2 given the preferential inhibitory activity of the drug for this isoform at the concentrations used in our studies. Achieving 60–70% reduction in basal SphK2 transcript expression for BCBL-1 and BCP-1 cells using this approach (Figs. 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 (Figs. 4D and E).

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

As noted above, 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* utilizing an established xenograft model wherein 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 3–4 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<sup>+</sup> PEL cells recovered from within ascites fluid (Fig. S2A), as well as observation of intranuclear expression of KSHV-encoded latency-associated nuclear antigen (LANA) within tumor cells from ascites fluid (Fig. S2B) and within splenic tissue (Figs. S2C).

For initial experiments, we administered ABC294640, either i.p. or orally, within 24 hours of PEL cell injections. ABC294640 significantly reduced tumor expansion with either route of administration (Fig. 5). In order to obtain sufficient tumor tissue for relevant *ex vivo* analyses, we performed additional experiments wherein 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 (Figs. 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 (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 (Fig. S3A and B). However, cells from drug-treated animals exhibited significantly reduced concentrations of intracellular S1P (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 (Figs. S3D–F). 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 (Figs. 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 anti-tumor effects for ABC294640 with intratumoral accumulation of pro-apoptotic ceramide species, reduced S1P levels, and suppression of MAPK and NF- $\kappa$ B 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 pro-apoptotic 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 pre-clinical studies and clinical trials employing combination therapies targeting these pathways.

Based on a wealth of published data and our own observations, suppression of constitutive signal transduction, accumulation of pro-apoptotic ceramides, reductions in anti-apoptotic 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 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 histone deacetylase (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 which 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. Since KSHV microRNAs also regulate the lytic switch, induce anti-apoptotic 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 KSHV<sup>neg</sup>/EBV<sup>neg</sup> 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 100uM (28), a 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, while we feel it is unlikely that ABC294640 reduces S1P production in PEL cells through inhibition of SphK1 at clinically achievable concentrations, we cannot categorically exclude drug inhibition of SphK1 as a mechanism for the observations in the present studies.

S1P binds to one of five G protein-coupled S1P receptors (S1PR1–5) that activate diverse downstream signaling pathways (54). Our identification of extracellular S1P within ascites fluid from tumor-bearing mice suggests that PEL cells may support their own growth through secretion of S1P and augmentation of signal transduction through autocrine or paracrine mechanisms. Therefore, additional experiments are underway to characterize S1PR expression by PEL cells and to determine their relative importance for constitutive PEL signaling, as well as the role for exogenous S1P in survival for KSHV-infected cells. Our data also indicate that S1P 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<sup>+</sup> patients with PEL or other aggressive B-cell lymphomas who have thus far been excluded from clinical trials with this agent.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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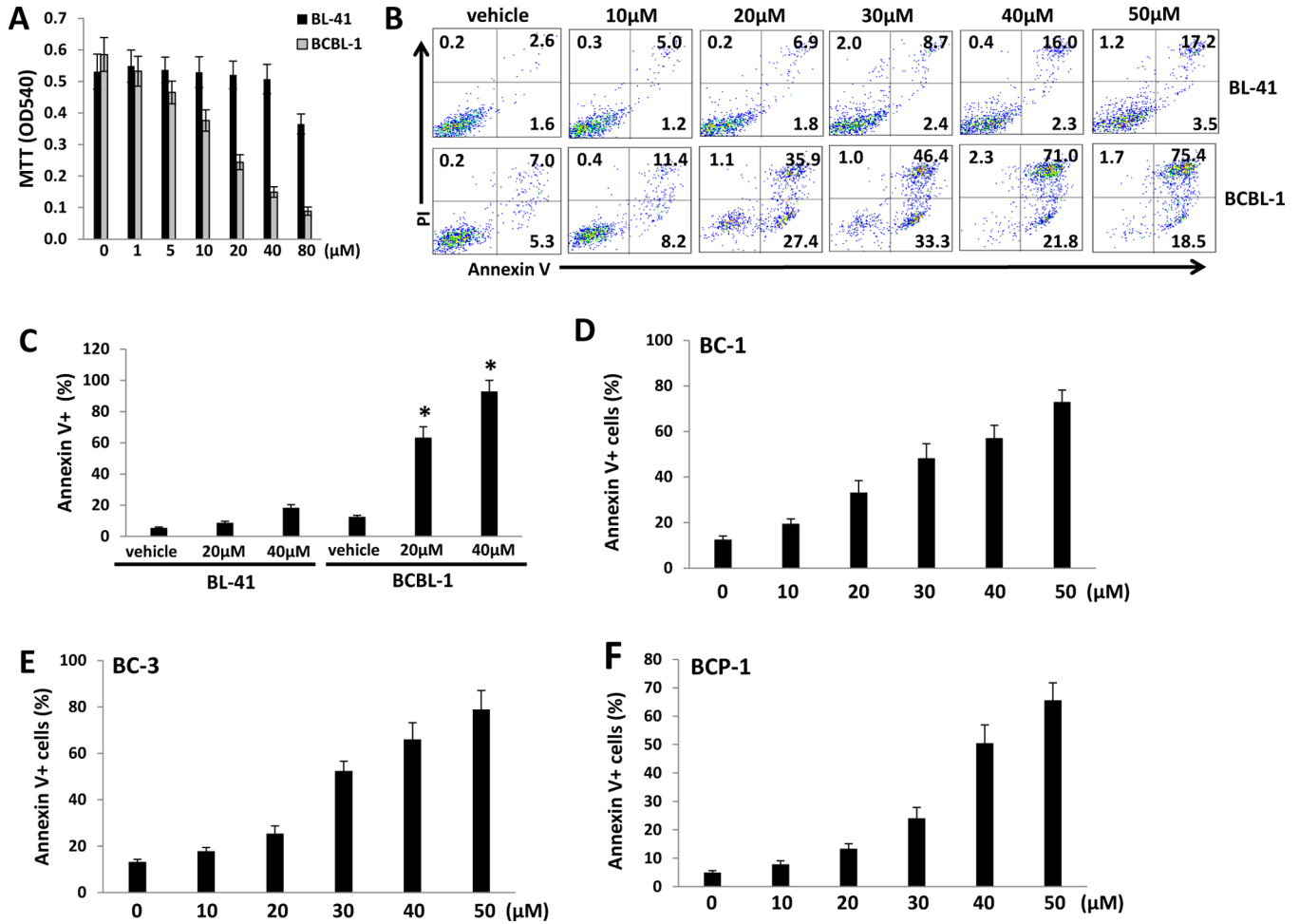
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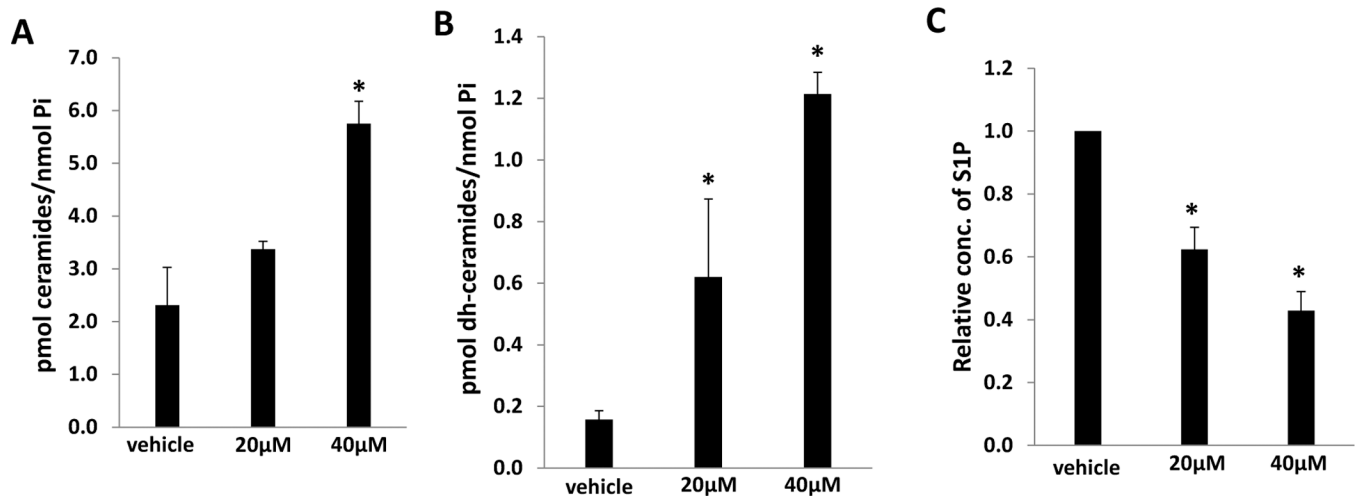
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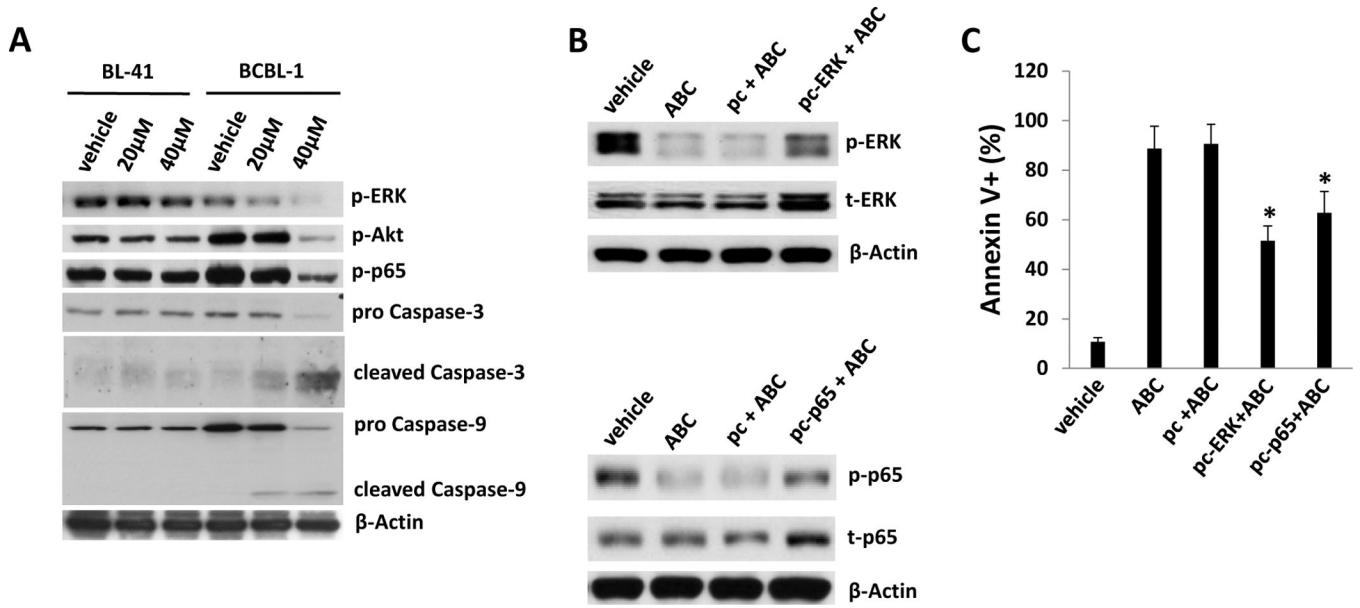


**Figure 1. Pharmacologic targeting of SphK with ABC294640 results in dose-dependent reductions in viability and apoptosis for PEL cells**  
 (A) KSHV<sup>neg</sup>/EBV<sup>neg</sup> BL-41 and KSHV<sup>+</sup>/EBV<sup>neg</sup> BCBL-1 cells were incubated with the indicated concentrations of ABC294640 (ABC) or vehicle for 16 h. Cell viability was quantified using MTT assays. (B,C) BL-41 and BCBL-1 cells were treated as (A) and apoptosis quantified using flow cytometry assays as described in Methods. (D–F) KSHV<sup>+</sup>/EBV<sup>+</sup> BC-1 cells (D), KSHV<sup>+</sup>/EBV<sup>neg</sup> BC-3 cells (E), and KSHV<sup>+</sup>/EBV<sup>neg</sup> BCP-1 cells (F), were incubated with the indicated concentrations of ABC294640 (ABC) or vehicle for 16 h and apoptosis quantified as above. Error bars represent the S.E.M. for three independent experiments.



**Figure 2. ABC294640 increases accumulation of ceramide species and reduces S1P levels within PEL cells**

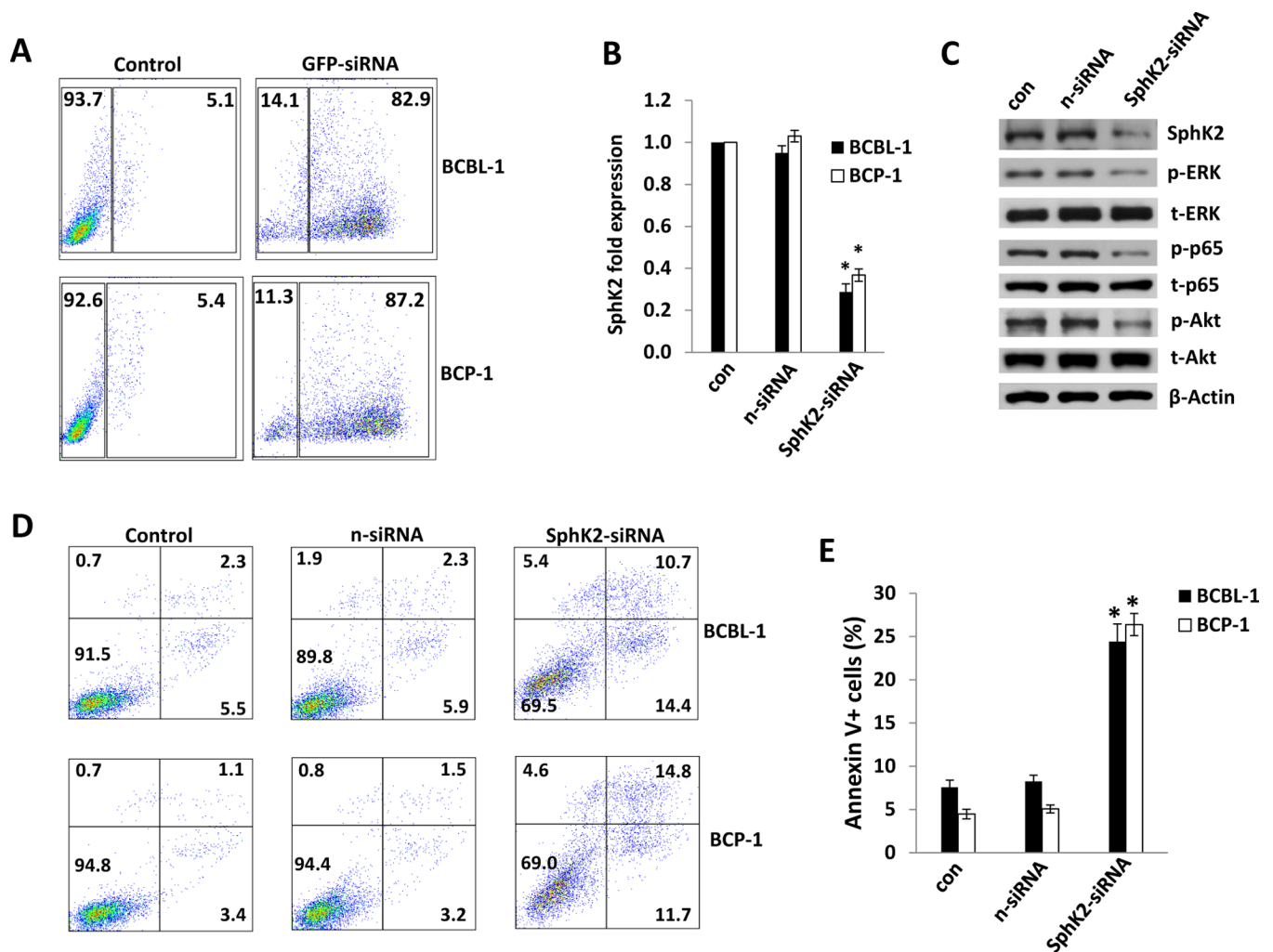
(A,B) BCBL-1 cells were incubated with the indicated concentrations of ABC294640 (ABC) or vehicle for 16 h, then ceramide and dihydro-ceramide species quantified as described in Methods. (C) Cells were treated as (A) and the concentration of intracellular S1P quantified by ELISA. Error bars represent the S.E.M. for three independent experiments. \* =  $p < 0.01$ .



**Figure 3. ABC294640 induces apoptosis for PEL cells through suppression of intracellular signal transduction**

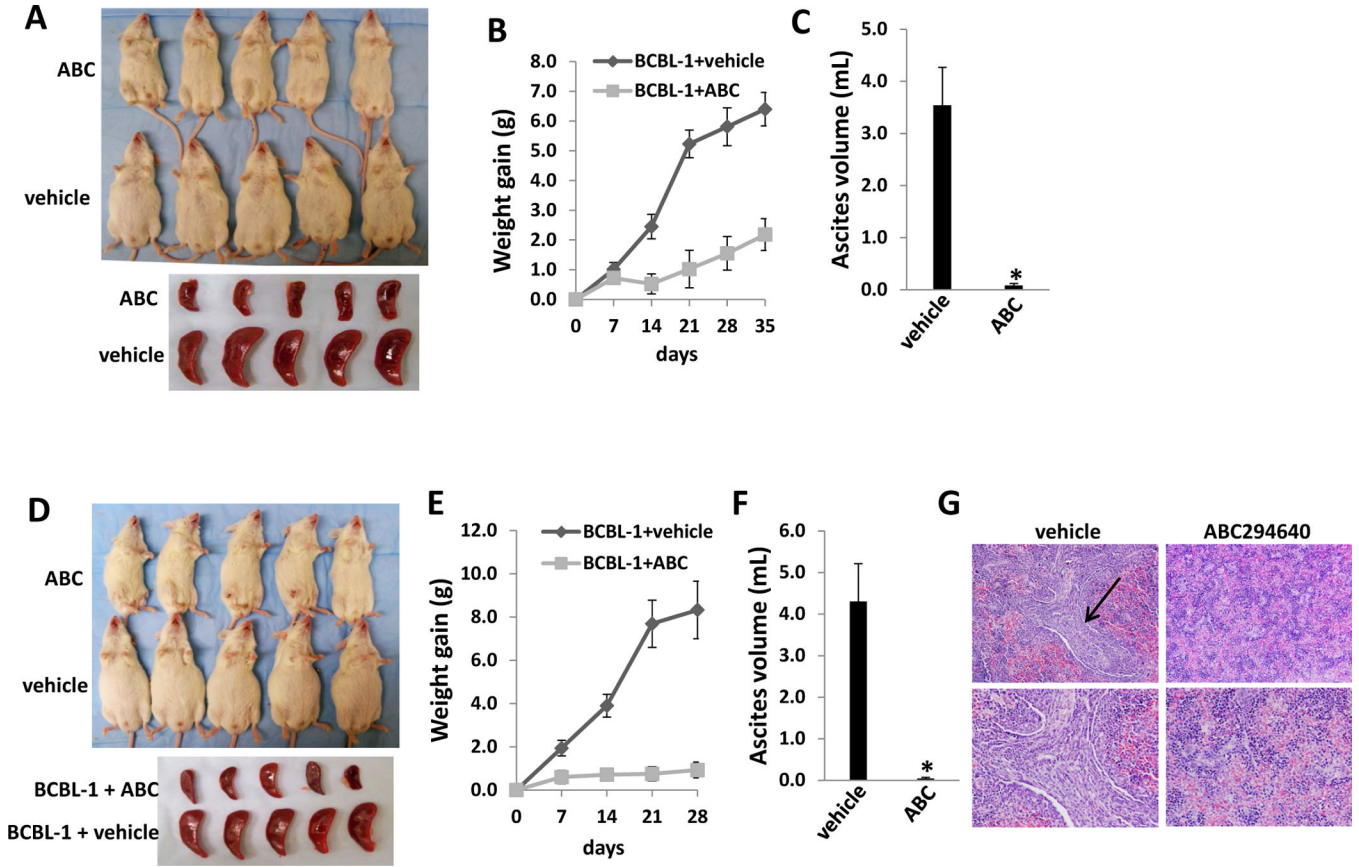
(A) BL-41 and BCBL-1 cells were incubated with the indicated concentrations of ABC294640 (ABC) or vehicle for 16 h, and immunoblots were performed to identify phosphorylated signaling intermediates and caspase cleavage, with β-Actin used as an internal control. (B,C) BCBL-1 cells were transfected with control vector (pc), or vectors encoding ERK (pc-ERK) or NF-κB p65 (pc-p65) for 24 h, then incubated with either vehicle or 40µM ABC294640 (ABC) for an additional 16 h. Protein expression was detected using immunoblots (B) and apoptosis quantified using flow cytometry (C). Error bars represent the S.E.M. for three independent experiments. \* = p<0.01.



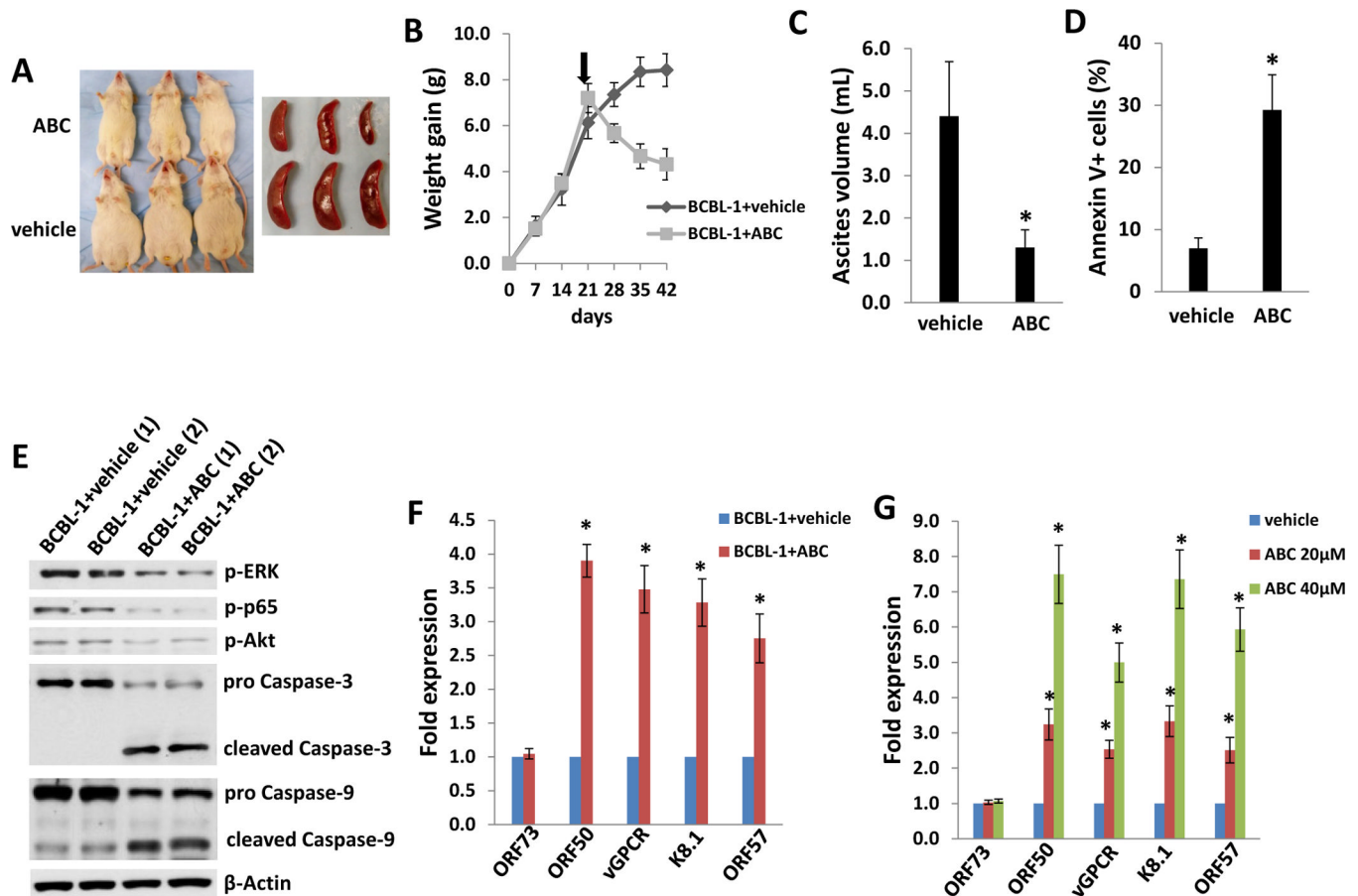


**Figure 4. Targeting SphK2 by RNA interference induces PEL cell apoptosis**

(A) BCBL-1 and BCP-1 cells were transfected with siRNA conjugated to green fluorescent protein (GFP) for 24 h and transfection efficiency determined by flow cytometry. Data shown represent one of three independent experiments. (B) BCBL-1 and BCP-1 cells were transfected with control (n-siRNA) or SphK2-specific siRNA (SphK2-siRNA) for 48 h then SphK2 transcripts quantified using qRT-PCR. Data are normalized to non-transfected cells, and using  $\beta$ -actin as a loading control. (C) BCBL-1 cells were treated as (B) and protein expression determined using immunoblots including  $\beta$ -actin as loading control. Data shown represent one of three independent experiments. (D,E) BCBL-1 and BCP-1 cells were treated as (B), and apoptosis was quantified using flow cytometry as described in Methods. Error bars represent the S.E.M. for three independent experiments, \* =  $p < 0.01$ .



**Figure 5. ABC294640 suppresses PEL progression *in vivo*** (NOD/SCID mice were injected intraperitoneally (i.p.) with  $10^7$  BCBL-1 cells. Beginning 24 h later, 50 mg/kg ABC294640 (ABC) or vehicle (n=10 per group) were administered intraperitoneally (i.p.) (A–C) or by oral gavage (D–F), once daily, 5 days per week, for each of 3 independent experiments. Weights were recorded weekly. Images of representative animals and their respective spleens, as well as ascites fluid volumes, were collected at the conclusion of experiments on day 35 (A–C) or day 28 (D–F). Error bars represent the S.E.M. for three independent experiments, \* =  $p < 0.01$ . (G) Spleens from vehicle- or ABC- (50 mg/kg) treated mice were prepared for routine hematoxylin and eosin (H&E) staining as described in Methods for identification of tumors infiltrating along vascular channels (arrow). Data shown represent individual animals from one of three independent experiments (200x, upper panel; 600x, lower panel).



**Figure 6. ABC294640 induces regression of established PEL tumors *in vivo***  
 (A–C) NOD/SCID mice were injected i.p. with  $10^7$  BCBL-1 cells. Beginning 21 days later, 100 mg/kg ABC294640 (ABC) or vehicle (n=10 per group) were administered i.p. once daily, five days per week, for an additional 21 days for each of 3 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 Methods and 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 two 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 2 independent experiments. qRT-PCR 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  $\beta$ -actin as a loading control. (G) BCBL-1 cells were incubated with the indicated concentrations of ABC294640 (ABC) or vehicle for 16 h and representative viral transcripts quantified using qRT-PCR as above. Error bars represent the S.E.M. for 2 (F) or 3 (B and G) independent experiments, \* =  $p < 0.01$ .