Original Research

Sphingosine kinase isoforms as a therapeutic target in endocrine therapy resistant luminal and basal-A breast cancer

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Abstract

Sphingosine kinase signaling has become of increasing interest as a cancer target in recent years. Two sphingosine kinase inhibitors, sphingosine kinase inhibitor (SKI)-II and ABC294640, are promising as potential breast cancer therapies. However, evidence for their therapeutic properties in specific breast cancer subtypes is currently lacking. In this study, we characterize these drugs in luminal, endocrine-resistant (MDA-MB-361) and basal-A, triple-negative (MDA-MB-468) breast cancer cells and compare them with previously published data in other breast cancer cell models. Both SKI-II and ABC294640 demonstrated greater efficacy in basal-A compared with luminal breast cancer. ABC294640, in particular, induced apoptosis and blocked proliferation both *in vitro* and *in vivo* in this triple-negative breast cancer system. Furthermore, Sphk expression promotes survival and endocrine therapy resistance in previously sensitive breast cancer cells. Taken together, these results characterize sphingosine kinase inhibitors across breast cancer cell systems and demonstrate their therapeutic potential as anti-cancer agents.

Keywords: sphingolipids, chemoresistance, sphingosine kinase, breast cancer, ceramide, experimental therapeutics, sphingosine-1-phosphate

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Introduction

The sphingolipid-signaling pathway has been of increasing interest in recent years as a cancer therapeutic target.[1] The sphingolipid, ceramide, is known to be an important element of both endogenous and chemotherapy-induced apoptosis. Furthermore, its metabolite, sphingosine-1-phosphate (S1P), is known to promote cancer survival, proliferation and metastasis.[2,3] The sphingosine kinase (Sphk) converts ceramide into S1P and is known to be overexpressed in a number of cancer tissues, including the breast, kidney, thyroid and prostate.[1] Of particular interest, breast cancer has become a model tissue for aberrant sphingolipid signaling in cancer.[4,5] Sphingosine kinase has been shown to increase proliferation and survival in estrogen receptor (ER)-positive breast cancer as well as poor prognosis in ER-negative breast cancer.[3,6]

Breast cancer is a complex disease consisting of multiple genotypes resulting in various phenotypes in the clinic. There is much debate in the literature concerning the relevance and translational potential of breast cancer cells; however, their significance and contribution to our understanding of the biology of cancer is crucial. There are numerous breast cancer cell lines representing dozens of phenotypes, and determining the proper cell to best represent the clinical environment can be difficult.[7] These cell lines originate from various biopsy sources (Table 1) and exhibit varying genetic profiles (Table 2). Considering the restrictions on using genetic mouse models of cancer because of the 'OncoMouse patents' held by private companies, many human breast cancer cell lines are also used *in vivo* in the form of xenografts in immunocompromised mice.[8] The ability of a drug to target a broad range of breast cancer subtype is an important feature of any experimental therapeutic.

Although clinical breast cancer is a heterogeneous disease, a new genetic classification of breast cancer was established to characterize tumors. Four broad subtypes of clinical breast cancer have been defined based on genetic studies of tumor samples: luminal (generally ER-positive), basal-like (ER-negative), HER2-postive and, most recently, claudin.[7,9–12] These cancers have distinct genetic profiles

Table 1 Tumor type and clinical source of human breast cancer cell lines[7]

Cell line	Tissue	Tumor type	Age (years)	Ethnicity	Source
MCF10A	Breast	Normal	36	Caucasian	Primary breast
MCF-7	Breast	Invasive ductal	69	Caucasian	Pleural effusion
MCF-7TN-R	Breast	Invasive ductal	36	Caucasian	Pleural effusion
MDA-MB-231	Breast	Adenocarcinoma	51	Caucasian	Pleural effusion
MDA-MB-361	Breast	Adenocarcinoma	40	Caucasian	Primary breast
MDA-MB-468	Breast	Adenocarcinoma	51	African-American	Pleural effusion

Table 2 Genetic and phenotypic characterization of human breast cancer cell lines[7]

Cell line	Gene cluster	ER status	PR status	Metastatic	Drug status
MCF10A	Basal B	Negative	Negative	No	Sensitive
MCF-7	Luminal	Positive	Positive	No	Sensitive
MCF-7TN-R	Basal-like	Negative	Negative	Yes	Chemoresistant
MDA-MB-231	Basal B	Negative	Negative	Yes	Endocrine-resistant
MDA-MB-361	Luminal	Positive	Negative	Yes	Endocrine-resistant
MDA-MB-468	Basal A	Negative	Negative	Yes	Endocrine-resistant

ER, estrogen receptor; PR, progesterone receptor

as well as clinical outcomes. Luminal cancers are the most common, making up approximately 69% of breast tumors, whereas basal-like tumors account for 12–15%.[10] Basal-like cancers are fairly heterogeneous but can be further broken down into basal-A and basal-B groups. These tumor subtypes possess varying clinical characteristics, with basal cancers being more aggressive and correlating with increased mortality compared with the luminal and HER2 subtypes. Basal-A and basal-B have distinct protein and gene expression profiles.[7,13] In general, basal-B cells are less differentiated, exhibit greater epithelial-to-mesenchymal transition changes, and are more invasive compared with basal-A breast cancer.[7] The basal-A gene expression profile is more similar to luminal cells compared with basal-B. Furthermore, the response to both endocrine and chemotherapies varies depending on subtype.[9,14] For example, HER2 cancers are responsive to Herceptin, making the overall mortality rate of this subtype low compared with others. Luminal cancers usually respond to first-line endocrine therapies such as tamoxifen. Yet, there are currently no targeted therapies for basal or endocrine therapy-resistant luminal cancers.

There are multiple non-cancerous human mammary cell lines commercially available. One commonly used cell model is the Michigan Cancer Foundation 10A (MCF10A) cell line. MCF10A cells were originally isolated from fibrocystic breast tissue obtained from a reduction mammoplasty of a 36-year-old woman with no evidence or family history of breast cancer. Subsequently, MCF10A cells have been characterized as immortalized, semi-transformed breast epithelial cells and generally represent a normal breast phenotype for comparison with breast cancer cell lines.[7,15–18] Alternatively, several ER-positive cell lines are frequently studied in context of human breast cancer. The mainstay of endocrine responsive, endocrine therapy-sensitive luminal breast cancer is the MCF-7 cell line. The MCF-7 cell line was isolated from the pleural effusion of a 69-year-old, postmenopausal, Caucasian woman with metastatic breast cancer in 1973. These cells are ER-, progesterone receptor (PR)-positive, and HER2/Neu-negative and have become the model for ER-positive breast adenocarcinoma in the laboratory.[19] MCF-7 cells represent a drug-sensitive, minimally invasive, ductal phenotype of breast carcinoma found in the clinic. Owing to the wide dissemination of this cell line and its prolonged use in the laboratory, the sensitivity of MCF-7 cells to various agents is variable. In general, these cells are estrogen-responsive and sensitive to endocrine therapy in the form of selective estrogen receptor modulators (SERMs) and selective estrogen receptor downregulator (SERDs) as well as the apoptotic effects of tumor necrosis factor (TNF) and chemotherapeutic agents.[5,20] Alternately, the M.D. Anderson-Metastatic Breast-361 (MDA-MB-361) cell line was derived from a metastatic brain site of a adenocarcinoma tumor of a 40-year-old Caucasian woman. These cells are basal-A subtype, ER-positive, PR-negative and are metastatic *in vivo.* The MDA-MB-361 cell line poses as an alternative to MCF-7 as an ER-positive, breast cancer cell line and represents an estrogen-dependent, endocrine therapy-resistant, invasive breast cancer.[7,18,21]

There are a multitude of cell lines used to represent hormone-independent, endocrine therapy-resistant breast cancer, often referred to as 'triple-negative' because of their lack of ER/PR/HER2 expression. The most studied of these cell lines is the MDA-MB-231 cell line, in which the basal-B adenocarcinoma cells are derived from the pleural effusion of a 51-year-old Caucasian woman with metastatic breast cancer. Morphologically, these cells exhibit epithelial-to-mesenchymal transition changes, such as loss of E-cadherin and increased expression of N-cadherin. These cells are highly invasive and metastatic both *in vitro* and *in vivo*.[7,22] Similarly, MDA-MB-468 cells are triple-negative and metastatic, although genetically they belong to the basal-A subtype. The MDA-MB-468 cells were isolated in 1977 from a primary breast tumor tissue of a 51-year-old African- American woman after treatment with adriamycin and cytoxan. These cells overexpress endothelial growth factor (EGF), have decreased expression of PTEN and p53, and exhibit a constitutively active ERK/MAPK-signaling pathway. These cells are highly aggressive and, similar to

MDA-MB-231 cells, form large metastatic tumors *in vivo* that are SERM-, SERD- and herceptin-resistant.[7,23,24] MDA-MB-231 and MDA-MB-468 cell lines represent varying genetic profiles of hormone-independent, endocrine therapy-resistant breast cancer in the laboratory.

The basal-like MCF-7TN-R cell line is a MCF-7 derived, ER/PR/HER2-negative, chemoresistant breast cancer cell line. These cells were derived by growing MCF-7 cells in increasing concentrations of TNFα until resistance was established.[25–27] Altered death receptor signaling is a hallmark of these cells because they exhibit increased TNFR1 and p50 expression and high basal nuclear factor-kappa B transcriptional activity relative to MCF-7 cells.[28] These cells exhibit morphological epithelial-to-mesenchymal transition changes and are highly aggressive both *in vitro* and *in vivo*. The MCF-7TN-R cells display increased resistance to several chemotherapeutic agents compared with MCF-7 cells, including doxorubicin, etoposide and paclitaxel.[5,26] These cells represent a model of transition to chemoresistance and aberrant death receptor signaling in breast carcinoma.[4,5,27,29–31]

A number of sphingosine kinase inhibitors (SKIs) have been developed in recent years, with the two most prevalent being SKI-II and ABC294640. SKI-II is the first commercially available inhibitor and is known to target both isoforms of Sphk.[32,33] ABC294640 is a Sphk2 selective inhibitor and was developed using SKI-II as the parent compound.[34] Both SKI-II and ABC294640 are orally bioavailable and exhibit limited toxicity in animal model experiments. Currently, both of these inhibitors have been characterized in a number of breast model systems, ranging from normal breast tissue to multidrug resistant metastatic cancer cell systems.[32–38] Both inhibitors selectively target particular types of breast cancer cells without affecting normal breast cells.[35–37] Unfortunately, research with these inhibitors is noticeably lacking in several breast cancer subtypes. These include luminal, endocrine-resistant basal-A, triple-negative breast cancers. Both of these cancers are metastatic clinical tumors which exhibit endocrine therapy resistance. We further examine the use of sphingosine kinase inhibitors as a potential treatment in breast cancer cell systems that represent these cancers, MDA-MB-361 and MDA-MB-468. In addition, to facilitate our understanding of the overall potential of these inhibitors as breast cancer therapeutics, we compare the use of SKIs in these cell systems to previously published data in other breast cancer cell systems (MCF10A, MCF-7, MDA-MB-231 and MCF-7TN-R).

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). SKI-II (4-(4-(4-chloro-phenyl)-thiazol-2-ylamino)-phenol) was purchased from Sigma-Aldrich (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Waltham, MA, USA).

Cell culture

The MCF-7 human breast adenocarcinoma cell line from the American Type Culture Collection (ATCC; Manassas, VA, USA) was generously provided by Louise Nutter (University of Minnesota, Minneapolis, MN, USA).[20] MCF-7TN-R cells were generated by exposing MCF-7 cells to increasing concentrations of TNFα until resistance was established.[27] MDA-MB-468 cells were obtained from ATCC. ER-positive MDA-MB-361 cells were generously provided by Dr Hongwu Chen (University of California Davis, Sacramento, CA, USA). 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 (FBS; Hyclone, Salt Lake City, UT, USA), 1% non-essential amino acids, minimal essential medium amino acids, sodium pyruvate, antibiotic/anti-mitotic and insulin under mycoplasma-free conditions at 37°C in humidified 5% CO_2 and 95% air. For studies examining the role of estrogen, cells were washed with phosphate-buffered saline (PBS) three times and grown in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5%) for 72 h before plating for each particular experiment.

Generation of Sphk1-overexpressing cell line

The protocol for the generation of stable cell lines was modified from previously published transfection methods.[26,39] MCF-7-GFP cells were plated in 10 cm^2 polystyrene dishes and allowed to adhere at 37°C. After 24 h, plates were transfected with *Homo sapiens* sphingosine kinase 1 (SPHK1), transcript variant 1 or empty vector control (Origene Technologies, Inc., Rockville, MD, USA). Transfection was accomplished using Lipofectamine (3:1 ratio) as per the manufacturer's instructions. Three days after transfection, cells were treated with neomycin (200 μg/mL; Invitrogen Corp.). Cells were fed with media every 3 days and monitored for fluorescence. When fluorescent colonies formed, multiple clonal colonies were selected, passaged and growth monitored. Clones were then visually assessed for stable expression and Sphk1 expressions were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR).

Clonogenic survival assay

Colony assays were performed as described in previously published methods.[27,40] Cells were plated in six-well plates at a density of 1000 cells per well in full DMEM media. After 24 h, cells were treated with ABC294640 (0.1–10 μmol/L) and then monitored for colony growth. After 10 days, the cells were fixed with 3% glutaraldehyde. Following fixation for 15 min, the plates were washed and stained with a 0.4% solution of crystal violet in 20% methanol for 30 min, washed with PBS and dried. Colonies of $\geq$30 cells were counted as positive. Results were normalized to DMSO vehicle-treated control cells. Statistical analysis of IC_{50} values were calculated from concentration–response curves using GraphPad

Prism 5.0 (Graphpad Software, San Diego, CA, USA), using the equation:

$$Y = \text{Bottom} + (\text{Top} - \text{bottom})/1 + 10\text{LogEC}_{50} - X$$

Cell viability assay

Viability assays were performed as described previously.[25,27] In brief, cells were plated at a density of 7.5×10^5 cells per well in a 96-well plate in phenol-free DMEM supplemented with 5% FBS and allowed to attach overnight. Cells were then treated with SKI (ranging from 10 nmol/L to 100 μmol/L) for 24 h. Following treatment, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT: 5 mg/mL) reagent was incubated in each well for 4 h. Cells were lysed with 20% sodium dodecyl sulfate in 50% dimethylformamide. The pH and absorbances were read on an ELx808 Microtek plate reader (Bio-Tek Instruments, Winooski, VT, USA) at 550 nm, with a reference wavelength of 630 nm.

Cell death detection enzyme-linked immunosorbent assay

The induction of apoptosis was determined as previously described using the nucleosome enzyme-linked immunosorbent assay (ELISA) kit (Roche, Boulder, CO, USA).[37] Cells were plated at 10,000 cells per well in 96-well plates and treated for 24 h with SKI. The induction of apoptosis was determined by the amount of nucleosomes in the cytoplasm according to the manufacturer's protocol. Absorbances were read on an ELx808 Microtek plate reader (Bio-Tek) at 405 nm.

Cell proliferation immunofluorescence assay

Proliferation assays were performed as previously described.[35,37] Cells were plated at a density of 10,000 cells per well in a 96-well plate in 10% DMEM media and allowed to attach overnight. The following day, cells were treated with DMSO or SKI for 24 h. At the endpoint, cells were fixed using 100 μL of 3.7% formaldehyde in PBS for 10 min. Formaldehyde was removed and cells were permeabilized using cold methanol for 5 min at room temperature and washed twice with PBS. One hundred microliters of 3% FBS in PBS blocking buffer were then added. After 30 min, blocking buffer was removed and cells were incubated for 1 h with Ki-67 (BD Pharmingen, San Diego, CA, USA) antibody. Cells were washed with PBS and stained with 4′,6-diamidino-2-phenylindole nuclear stain for 5 min before imaging. For staining quantification, the numbers of positively stained cells were expressed as a percentage of the total number of cells per field of view/image. The vehicle control was set to 1 for comparison with SKI-II treatment.

Animals

All procedures involving animals were conducted in compliance with State and Federal laws, standards of the US Department of Health and Human Services, and guidelines established by the Tulane University Animal Care and Use Committee. The facilities and laboratory animal program of Tulane University are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All the animal surgeries and other procedures were performed under anesthesia using a mix of isofluorane and oxygen delivered by mask.

Xenograft models were performed similar to previously reported studies.[41] In brief, Nu/nu or SCID/BEIGE immunocompromised female ovariectomized mice (29–32 days old) were obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were allowed a period of adaptation in a sterile and pathogen-free environment *ad libitum*. Placebo or estradiol pellets (0.72 mg, 60-day release; Innovative Research of America, Sarasota, FL, USA) were implanted subcutaneously in the lateral area of the neck in the middle point between the ear and shoulder using a precision trochar (10 gauge). MDA-MB-468 cells in the exponential growth phase were harvested using PBS/ethylenediaminetetraacetic acid solution and washed. Viable cells (5×10^6) in a 50 μL sterile PBS suspension were mixed with 100 μL Matrigel (BD Biosciences, Bedford, MA, USA). Cells were injected in the mammary fat pad through a 5 mm incision in the hypogastric region, and the incision was closed using staples. Tumors were allowed to form over 10 days and mice were randomized to two treatment groups, vehicle control and ABC294640, with five mice per group. The ABC294640 mixture was suspended in a solution of DMSO and PBS and was given intraperitoneally at 50 mg/kg for 15 days starting after the tumors were measureable. Control mice were injected with vehicle daily for 15 days. Tumor size was measured every two days using a digital caliper. The volume of the tumor was calculated using the following formula: $4/3\pi \ LS^2$ (L = larger radius; S = shorter radius). At necropsy on day 24, animals were euthanized by cervical dislocation after exposure to a CO_2 chamber. Tumors were removed and either frozen in liquid nitrogen or fixed in 10% formalin for further analysis.

Immunohistochemistry

Immunohistochemistry was performed as described in previously published methods.[36,37] Tumor explants were collected at necropsy, and fixed in 10% buffered formalin phosphate. Formalin-fixed paraffin-embedded (FFPE) 4-lM-thick tumor sections were analyzed by immunohistochemistry using primary monoclonal antibodies against human Ki-67 and BCL-2 (DAKO North America, Inc., Carpinteria, CA, USA). The mouse antibodies on mouse tissue polymer detection kit (Biocare Medical, LLC, Concord, CA, USA) were used to perform immunohistochemistry. In brief, FFPE sections were deparaffinized and hydrated in a graded series of ethanol solutions followed by 3% H_2O_2 for 5 min to inactivate endogenous peroxides, then rinsed. Slides were subjected to 10-min incubation in avidin followed by 10-min incubation in biotin. For antigen retrieval, sections were exposed to Rodent decloaker (Biocare Medical) at 95°C

for 25 min, rinsed and allowed to cool to room temperature for 20 min. Slides were incubated with Rodent block for 30 min and then with primary antibodies or serum alone (negative control) for 75 min. Mouse-on-mouse horseradish peroxidase-polymer secondary antibody was added to the sections and incubated for 15 min. After rinsing, diaminobenzidine solution (Biocare Medical) was applied and incubated for 1 min, and sections were counterstained with hematoxylin (Biocare Medical) followed by Tacha Blueing reagent (Biocare Medical) for 30 s each. Slides were then allowed to air-dry and then coverslipped using Acrymount (Fisher Scientific Inc., Waltham, MA, USA). Sections were viewed and photographed using a Leica DM IRB Inverted Research microscope and SPOT RT color camera (Leica Microsystems Inc., Buffalo Grove, IL, USA). Five images at 40× were taken of each tumor. Care was taken to avoid areas of necrosis. For staining quantification, the numbers of positively stained cells were expressed as a percentage of the total number of cells per field of view/image.

Statistical analysis

Statistical analyses were performed as previously described.[36,37] In brief, IC_{50} values were calculated from concentration–response curves using GraphPad Prism 5.0 (GraphPad Software), using the equation:

$$Y = \text{Bottom} + (\text{Top} - \text{bottom})/1 + 10\text{LogEC}_{50} - X$$

assuming a standard slope, where the response goes from 10 to 90% of maximal as X increases over two log units. Differences in IC_{50} were compared using Student's unpaired *t*-test with $P < 0.05$ as the limit of statistical significance. Experiments comparing multiple concentrations to the control were tested with one-way analysis of variance with Bonferroni post-test to compare individual concentrations. All statistical analyses were done using GraphPad Prism 5.0 (GraphPad Software).

Quantitative realtime RT-PCR

Realtime RT-PCR was performed similar to previously reported studies.[42,43] In brief, total cellular RNA was extracted using the RNeasy® mini column (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The concentration of RNA was determined using an ultraviolet spectrophotometer. RT was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The level of Sphk1 and Sphk2 transcripts were determined using the iQ5 realtime quantitative PCR detection system (BioRad Inc., Hercules, CA, USA). Primers for PCR were designed to span intron/exon junctions to minimize amplification of residual genomic DNA. The primer sequences for genes studied were as follow: (sense and antisense, respectively): *Actin:* (F) 5′- TGAGCGCGGCTACAG CTT-3′, (R) 5′ – CCTTAATGTCACACACGATT – 3′, *SPHK1:* (F) 5′- GCCGATACTTCTCACTCTC - 3′, (R) 5′ – ATGAACCTGCTGTCTCTG - 3′; *SPHK2:* (F) 5′- GCCACCT ACGAAGAGAAC - 3′, (R) 5′ – TGACCAATAGAAGCA ACCG - 3′. All primers were obtained from Invitrogen. The PCR reaction was carried out in the following manner: step 1 – 95°C 3 min; step 2 – for 40 cycles 95°C 20 s, 60°C 1 min; step 3 – 70°C 10 s, hold at 4°C. Each reaction tube contained: 12.5 μL 2× SYBR Green supermix + 6.5 μL nuclease-free water + 1 μL 0.1 $\mu g/\mu L$ primer (pair) + 5 μL cDNA (0.2 $\mu g/\mu L$). Genes were amplified in triplicate. Data were analyzed by comparing relative target gene expression to actin control. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. RNA isolation, cDNA synthesis and quantitative PCR were performed as previously described and outlined above.

Results

Overexpression of Sphk increases long-term survival and confers endocrine resistance in invasive luminal carcinoma cells

Sphingosine kinase is known to mediate the promotion and proliferation of breast cancer.[4] Furthermore, studies have shown that Sphk1 overexpression confers resistance to TNF and doxorubicin.[44] Unfortunately, there have been few studies concerning the effects of sphingosine kinase on endocrine therapy resistance.[3] Therefore, to determine the effect of Sphk1 on endocrine therapy, we utilized the well-studied luminal MCF-7 system. We stably overexpressed Sphk1 in MCF-7 cells expressing a GFP tag (Figure 1a). These MCF-7-Sphk1 cells have a 7.60 ± 0.36-fold ($P < 0.05$) increase in Sphk1 mRNA expression (Figure 1b) and a 2.02 ± 0.34 ($P < 0.05$)-fold increase in downstream sphingosine-1-phosphate levels (Figure 1c). Using these cells, we determined the effect of Sphk on clonogenic survival and response to endocrine therapy treatment in comparison to parental MCF-7-VEC cells. Interestingly, overexpression of Sphk1 increased overall cancer cell clonogenic survival (Figure 1c) and conferred resistance to tamoxifen compared with parental MCF-7-VEC cells (Figure 1d). Thus, increased Sphk may play a role in endocrine therapy resistance in luminal subtype cancers. These results provide proof of principle that Sphk is a potential therapeutic target for endocrine resistant, ER-positive cancers, such as the MDA-MB-361 cell system.

Characterization of SKIs in ER-positive, endocrine-resistant breast cancer

Our findings suggest a potential link between Sphk and endocrine resistance. Therefore, we utilized the unstudied MDA-MB-361 cell system to determine the biological effects of SKIs in ER-positive, endocrine-resistant breast cancer. As discussed above, MDA-MB-361 cells are luminal and resistant to endocrine therapies such as tamoxifen.[7,39] Previous breast cancer studies determined that SKIs can decrease breast cancer viability in various cell systems.[26,45,46] We initially screened both SKI-II and ABC294640 on ER-positive, metastatic breast cancer viability. Unlike previous findings, neither SKI nor ABC294640

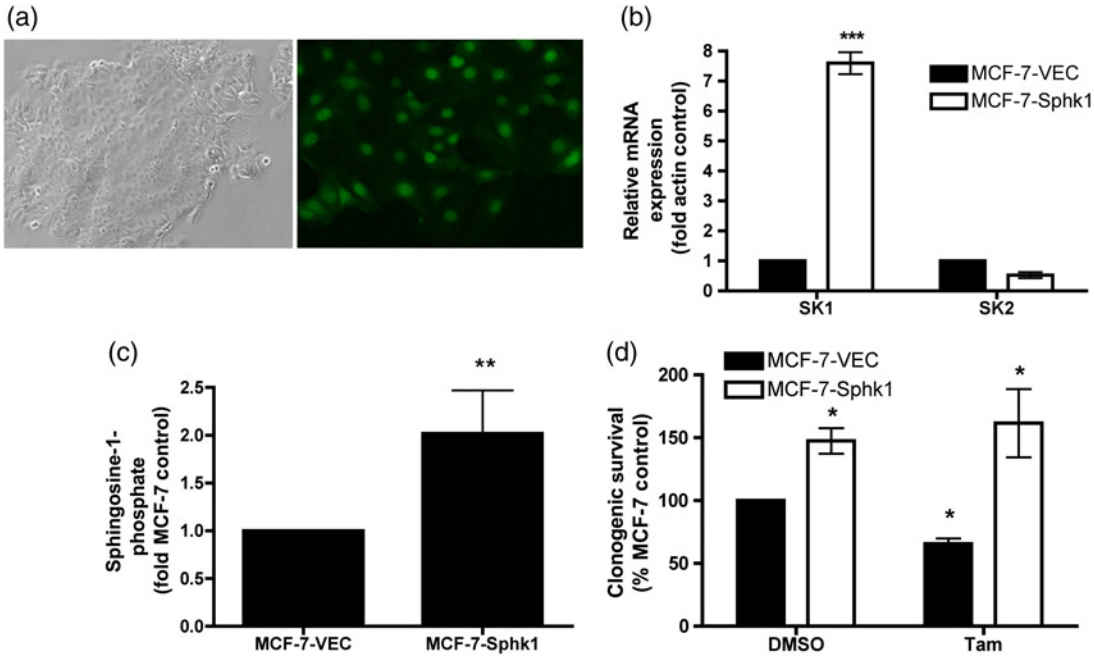

Figure 1 Generation and characterization of Sphk1-overexpressing MCF-7 cell line. MCF-7-GFP cells were stably transfected with Sphk1 or vector control. (a) Images of MCF-7-Sphk1 cells taken at 100×. (b) Quantitative RT-PCR expression of Sphk1 and Sphk2 in MCF-7-VEC and MCF-7-Sphk1 cells. Results shown as mean fold change of three independent experiments ± SEM. (c) Endogenous S1P levels in MCF-7-VEC and MCF-7-Sphk1 cells. S1P levels normalized to MCF-7-VEC control. Mean values of ± SEM of three different experiments in duplicate are reported. (d) MCF-7 and MCF-7-Sphk1 cells were plated at 500 cells per 60 mm^2. The following day, cells were treat with ICI 182,780 or tamoxifen for 10–14 days. Colonies greater than 50 cells were scored as positive for colony formation. Data are presented as percent of MCF-7 vehicle-treated samples. Mean values of ± SEM of three different experiments in duplicate are reported. (A color version of this figure is available in the online journal)

Table 3 Viability IC_{50} values across breast cancer cells systems[35–38]

Cell system	SKI-II	ABC294640	*P* value
MCF10A	>1000	>1000	<0.01
MCF-7	13.0 ± 1.21	21.85 ± 3.24	<0.001
MCF-7TN-R	4.43 ± 1.25	8.83 ± 1.13	<0.001
MDA-MB-231	11.77 ± 217	22.39 ± 5.11	<0.001
MDA-MB-361	>1000	765 ± 8.62	<0.001
MDA-MB-468	15.8 ± 3.09	19.16 ± 3.06	<0.001

exhibited potent anti-viability properties in this breast cancer subtype, with IC_{50} values of >1000 μmol/L ($P < 0.001$) and 765 ± 8.62 μmol/L ($P < 0.001$), respectively. As shown in Table 3, these results were somewhat surprising, considering that previous studies demonstrated opposing viability effects compared with MDA-MB-361 cell lines.

The efficacy of short-term viability assays in predicting therapeutic potential has been disputed in the literature, with some researchers suggesting that long-term assays provide a better clinical model.[47] The ability of SKIs to block breast cancer colony formation in other breast cancer cell lines has been inconsistent. Therefore, we determined the effects of these inhibitors on long-term clonogenic survival in MDA-MB-361 cells. As predicted, both SKI-II and ABC294640 dose-dependently decreased breast cancer clonogenic survival (Figure 2b). SKI-II exhibited an IC_{50} value of 2.63 ± 1.25 μmol/L ($P < 0.001$), while ABC294640 had an IC_{50} value of 5.32 ± 1.13 μmol/L ($P < 0.001$). These low micromolar results are similar to the IC_{50} values in other breast cancer model systems (Table 4).

Although Sphk1 is known to inhibit apoptosis in breast cancer, the role of Sphk2 in programed cell death is less clear. Recent studies have demonstrated ABC294640 to have proapoptotic properties in ER-negative MCF-7TN-R cells.[26] Therefore, we determined whether Sphk2 plays a similar role in ER-positive breast cancers, which are phenotypically distinct from triple-negative breast cancer. Both endocrine-sensitive MCF-7 and endocrine-resistant MDA-MB-361 cells were treated with equivalent doses of ABC294640 and assessed for levels of fragmented DNA oligonucleotides as a measure of cell death. Interestingly, we found that ABC294640 increased apoptosis 12.24 ± 3.38-fold ($P < 0.05$) compared with the control in MCF-7 cells. The decrease in viability at the same dose suggests that induction of apoptosis may be an anti-viability mechanism of ABC294640 in ER-positive breast cancer. These results are similar to those we found in chemoresistant breast cancer.[37] Interestingly, pharmacological inhibition of Sphk2 does not alter apoptosis, or corresponding viability, in the MDA-MB-361 cell system. As ABC294640 is more effective in long-term clonogenic survival assays than in short-term viability assays, it is possible that pharmacological induction of apoptosis in the MDA-MB-361 cell system takes longer than 24 h. However, these data demonstrate that SKIs are not as efficacious in luminal, endocrine-resistant breast cancer compared with other breast cancer subtypes, such as luminal endocrine-sensitive and basal-like tumors.

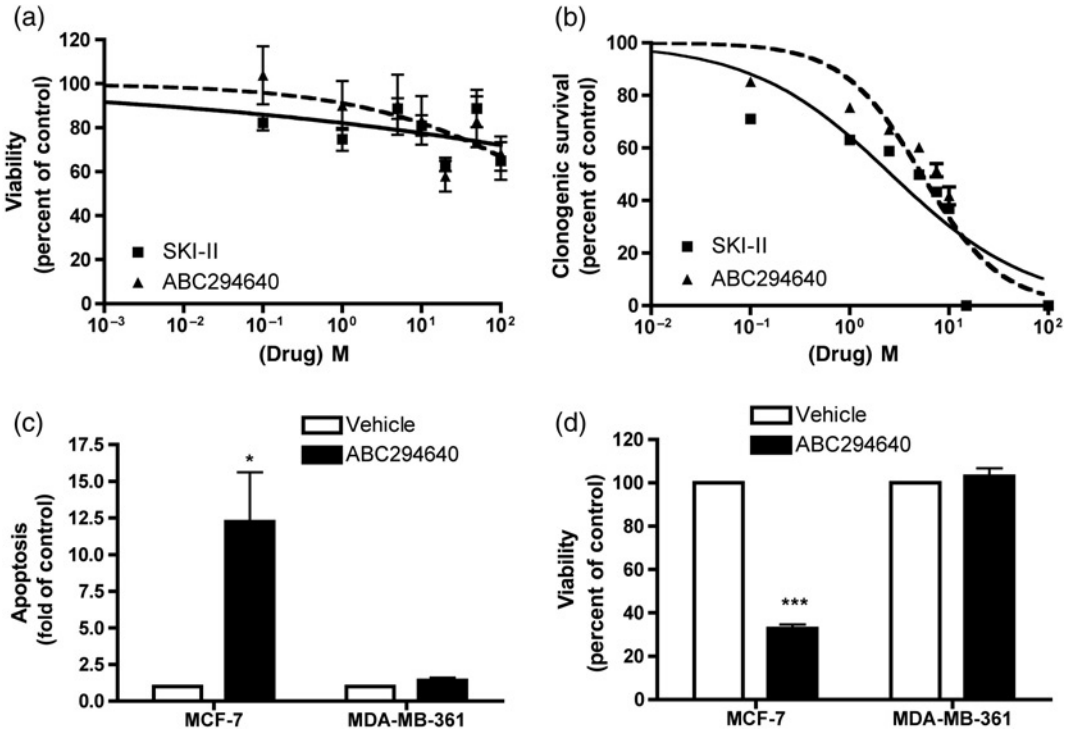

Figure 2 Effect of SKIs on MDA-MB-361 metastatic breast cancer. (a) Cells were plated at 7.5×10^5 cells per 96-well plate. The following day, cells were treated with indicated concentrations of SKI-II (solid line) or ABC294649 (dotted line) for 24 h. Data are presented as percentage of vehicle-treated samples. Mean values of $\pm$ SEM of five different experiments in quadruplicate are reported. (b) Cells were plated at 500 cells per 60 mm^2. The following day, cells were treated with SKI-II (solid line) or ABC294649 (dotted line) for 10–14 days. Data are presented as percent of vehicle treated samples. Mean values of $\pm$ SEM of three different experiments in duplicate are reported. (c) MCF-7 and MDA-MB-361 cells plated at 10,000 cells per well in a 96-well plate and treated with 50 μmol/L of ABC294640 for 24 h. Following treatment, cells were measured for defragmented oligonucleotides as a measure of apoptosis using enzyme-linked immunosorbent assay analyses and (d) cells were analyzed for viability using MTT analyses. Mean values of $\pm$ SEM of three different experiments in duplicate are reported. SKI, sphingosine kinase inhibitor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Table 4 Clonogenic survival IC50 values across breast cancer cells systems[35–38]

Cell system	SKI-II	ABC294640	*P* value
MCF-7	2.04 ± 1.08	3.15 ± 1.16	<0.001
MCF-7TN-R	2.70 ± 1.05	5.21 ± 1.10	<0.001
MDA-MB-231	2.51 ± 1.08	2.9 ± 1.16	<0.001
MDA-MB-361	>1000	765 ± 8.62	<0.001
MDA-MB-468	1.20 ± 1.20	4.88 ± 1.14	<0.001

Characterization of SKIs in basal-A, triple-negative breast cancer

Previous studies have demonstrated that SKIs can block viability and survival in basal-B, triple-negative, as well as chemoresistant basal-like, breast cancer systems.[26] However, the effect of these drugs on basal-A subtype breast cancer has not been investigated previously. Therefore, we utilized the MDA-MB-468 cell line to better characterize SKIs in this breast cancer system. As shown in Figure 3a, both SKIs dose-dependently blocked viability in these cells. SKI-II was slightly more effective, with an IC_{50} value of 15.8 ± 3.09 μmol/L ($P < 0.001$) compared with 19.16 ± 3.06 μmol/L ($P < 0.001$) with ABC294640 treatment (Figure 3a). Our results are similar to previously published values in other breast cancer systems (Table 3). We next determined the effect of these drugs on long-term clonogenic survival in these cells. Similar to other published results, treatment with both SKIs resulted in potent decreases in cancer colony formation, with IC_{50} values of 1.20 ± 1.20 μmol/L ($P < 0.001$) and 4.88 ± 1.14 μmol/L ($P < 0.001$) for SKII and ABC294640, respectively (Figure 3b).

The apoptotic role of Sphk2 in breast cancer is less clear than that of Sphk1. We determined whether pharmacologic inhibition of Sphk2 with ABC294640 would induce apoptosis in basal-A, triple-negative breast adenocarcinoma. Treatment with ABC294640 resulted in a 12.09 ± 3.47-fold ($P < 0.05$) increase in apoptosis, and a corresponding decrease in viability, in MDA-MB-468 cells (Figures 3c and d). These results suggest that Sphk2 plays a similar role in basal-B subtype breast cancer as it does with invasive ductal (MCF-7) and basal-like, chemoresistant (MCF-7TN-R) breast cancers.[35–37] Our findings are also in stark contrast to luminal breast cancer (MDA-MB-361) cells wherein inhibition of Sphk2 had no statistical effect on apoptosis.

The ability of SKIs to block both viability and survival in MDA-MB-468 cells suggests that pharmacologically targeting Sphk in basal-A breast cancer may be a promising therapeutic option. The ability of anti-cancer agents to block proliferation, in addition to inducing apoptosis, is an important biological characteristic. SKI-II and ABC294640 have varying anti-proliferative properties in other breast cancer cell systems. For example, ABC294640 has potent anti-proliferative properties in MCF-7 and MDA-MB-231 cells, whereas only marginally decreasing proliferation in

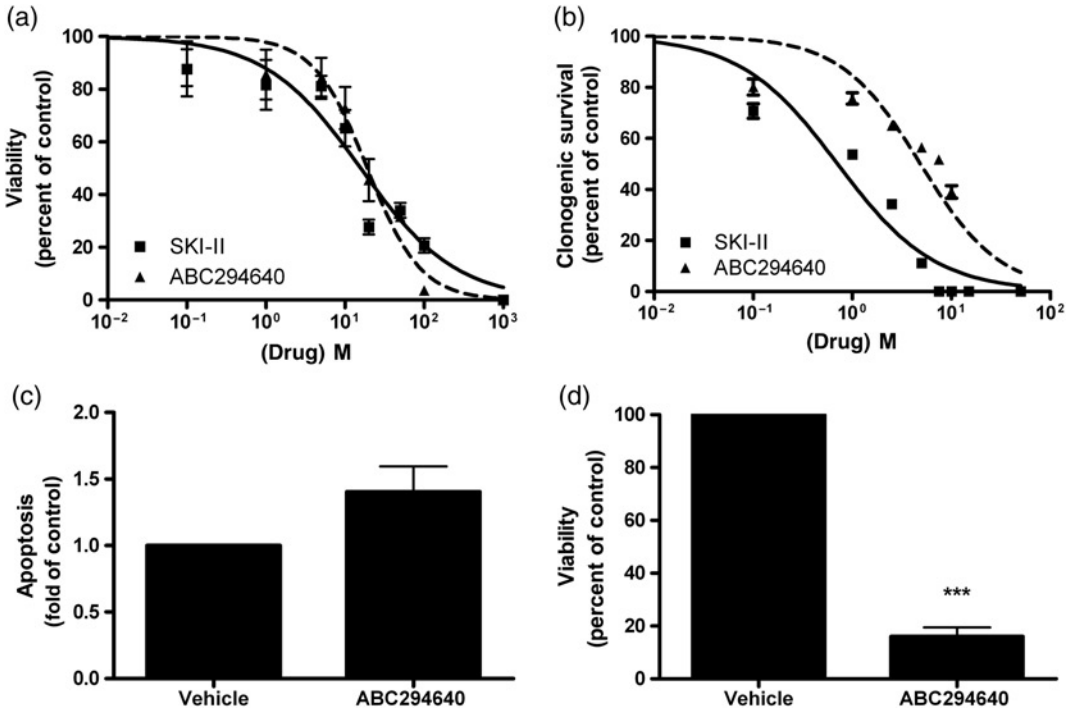

Figure 3 Effect of SKIs on endocrine resistant MDA-MB-468 breast cancer (a) Cells were plated at 7.5 × 105 cells per 96-well plate. The following day, cells were treated with indicated concentrations of SKI-II (solid line) or ABC294649 (dotted line) for 24 h. Data are presented as percent of vehicle-treated samples. Mean values of ± SEM of five different experiments in quadruplicate are reported. (b) Cells were plated at 500 cells per 60 mm^2. The following day, cells were treated with SKI-II (solid line) or ABC294649 (dotted line) for 10–14 days. Data are presented as percentage of vehicle-treated samples. Mean values of ± SEM of three different experiments in duplicate are reported. (c) Cells plated at 10,000 cells per well in a 96-well plate and treated with 50 μmol/L of ABC294640 for 24 h. Following treatment, cells were measured for defragmented oligonucleotides as a measure of apoptosis using enzyme-linked immunosorbent assay analyses and (d) cells were analyzed for viability using MTT analyses. Mean values of ± SEM of three different experiments in duplicate are reported. SKI, sphingosine kinase inhibitor; MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MCF-7TN-R cells.[26,35,36] Therefore, we determined the anti-proliferative properties of SKIs in MDA-MB-468 cells. We used nuclear Ki-67 staining as a measure of proliferation, similar to previously published experiments.[26,35,36] As seen in Figure 4, treatment with SKI-II and ABC204640 decreased cellular proliferation of MDA-MB-468 cells. SKI-II was slightly more potent, with treatment resulting in a 51.50 ± 5.81% ($P < 0.001$) reduction in Ki-67 staining compared with a 38.96 ± 8.87% reduction ($P < 0.001$) with ABC294640 (Figure 4b). Our results suggest that SKIs are more efficacious in the MDA-MB-468 than in the MCF-7TN-R cell system, but less than MCF-7 cell systems.

Anti-tumor activity of ABC294640 in basal-A, triple-negative breast cancer

ABC294640 is currently entering clinical trials for the treatment of advanced solid tumors. This drug was chosen over SKI-II because of the more desirable pharmacokinetic and pharmcodynamic properties of ABC294640.[48] There have been several recent studies demonstrating anti-tumor effects of ABC294640 in breast cancer xenograft models.[48,49] For example, treatment with the inhibitor decreased tumor growth by 68.4% in MCF-7 and 67.4% ($P < 0.001$) in MCF-7TN-R animal models.[26,46] As a result of the promising *in vitro* therapeutic properties of this drug, we studied the effect of ABC294640 on MDA-MB-468 tumor growth *in vivo*. Using well-established, immunocompromised mouse xenograft models for tumor growth, MDA-MB-468 cells were injected subcutaneously in female overiectomized mice and mice were subsequently measured for tumor growth. Treatment with ABC294640 (50 mg/kg) for 14 days decreased MDA-MB-468 tumor volume by 37.3% ($n = 10$, $P < 0.001$) (Figure 5). The anti-tumor properties of ABC294640 were somewhat less than those reported in previously published studies using other cells systems.

As a result of the anti-proliferative and pro-apoptotic properties of ABC294640 *in vitro*, we further investigated whether these properties contributed to the anti-tumor effects of the drug. Endpoint tumors from vehicle and ABC294640-treated mice were first analyzed for protein expression of Ki-67 using immunohistochemistry. As shown in Figure 5b, cellular proliferation is markedly decreased in mice exposed to ABC294640. Specifically, there was an 17.30 ± 2.96% ($P < 0.01$) decrease in Ki-67 expression in treatment tumors compared with vehicle control. We next analyzed expression of BCL-2 as a measure of programmed cell death. BCL-2 is known to mediate intrinsic breast cancer apoptosis through sequestration of the pro-apoptotic proteins Bax/Bid and decreased expression of BCL-2 correlates with increased cellular apoptosis.[50–52] Treatment tumors displayed a 93.11 ± 1.34% ($P < 0.01$) decrease in BCL-2 expression compared

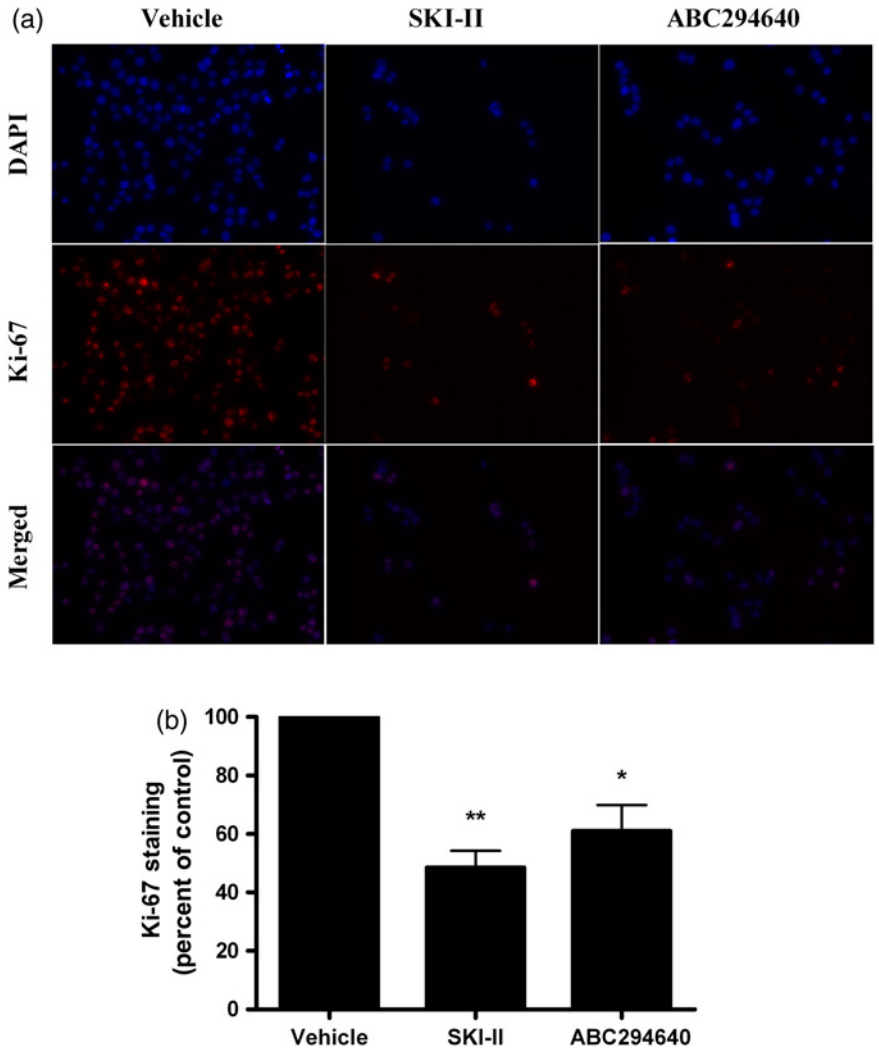

Figure 4 Anti-proliferative properties of ABC294640. (a) MDA-MB-468 cells treated with vehicle or ABC394640 for 48 h were fixed using 3.7% formaldehyde in phosphate-buffered saline (PBS), permeabilized using cold methanol and incubated with anti-Ki-67 antibody (red). Cells were washed (with PBS) and stained with 4′,6-diamidino-2-phenylindole (blue) nuclear stain before imaging. Three independent experiments were performed with representative pictures shown here. (b) Quantitation of Ki-67 staining was determined as a percentage of total positive cells/image. The vehicle control was set to 1 for comparison with ABC294640 treatment. (A color version of this figure is available in the online journal)

with control tumors (Figure 5c). These results suggest that ABC294640 inhibits tumor proliferation and induces apoptosis to exert its anti-tumor effects. Our results also correlate well with our cellular findings, thus providing proof of principle that the *in vitro* anti-cancer effects of ABC294640 translate *in vivo*. Taken together, our findings demonstrate that ABC294640 has therapeutic potential in the treatment of basal-A, triple-negative breast cancer.

Discussion

The sphingolipid pathway, particularly Sphk signaling, has become of increasing interest as a breast cancer therapeutic target.[1,4,5] The most commonly used SKIs, SKI-II and ABC294640, have demonstrated promising results in various breast cancer cell systems.[32,34–37] Specifically, both drugs have little effect on normal breast MCF10A cells, while proving to be highly effective in MCF-7 luminal, ER-positive breast adenocarcinoma cells.[26,36,45] These drugs exhibit biological activity in the basal-B, triple-negative MDA-MB-231 and basal-like, multidrug-resistant MCF-7TN-R cells.[26] However, pharmacologically inhibiting Sphk in luminal, endocrine therapy-resistant and basal-A, triple-negative breast cancers has not been investigated. Here, for the first time, we have characterized both SKI-II and ABC294640 in these breast cancer subtypes.

Unlike previously published studies, SKIs were not effective in luminal, endocrine therapy-resistant breast cancer compared with other cell systems. Both SKI-II and ABC294640 had little effect on short-term viability compared with endocrine-sensitive, ER-positive breast cancer.[35,46] Our laboratory previously demonstrated that SKIs can directly bind the ER and inhibit downstream estrogen signaling to promote its anti-cancer effects.[35,46] Because MDA-MB-361 cells are ER-positive, yet tamoxifen-resistant, it is possible that mutations in the ER pathway that result in endocrine resistance may also affect the binding of SKIs to the ER. This decreased anti-estrogenic effect of SKIs in

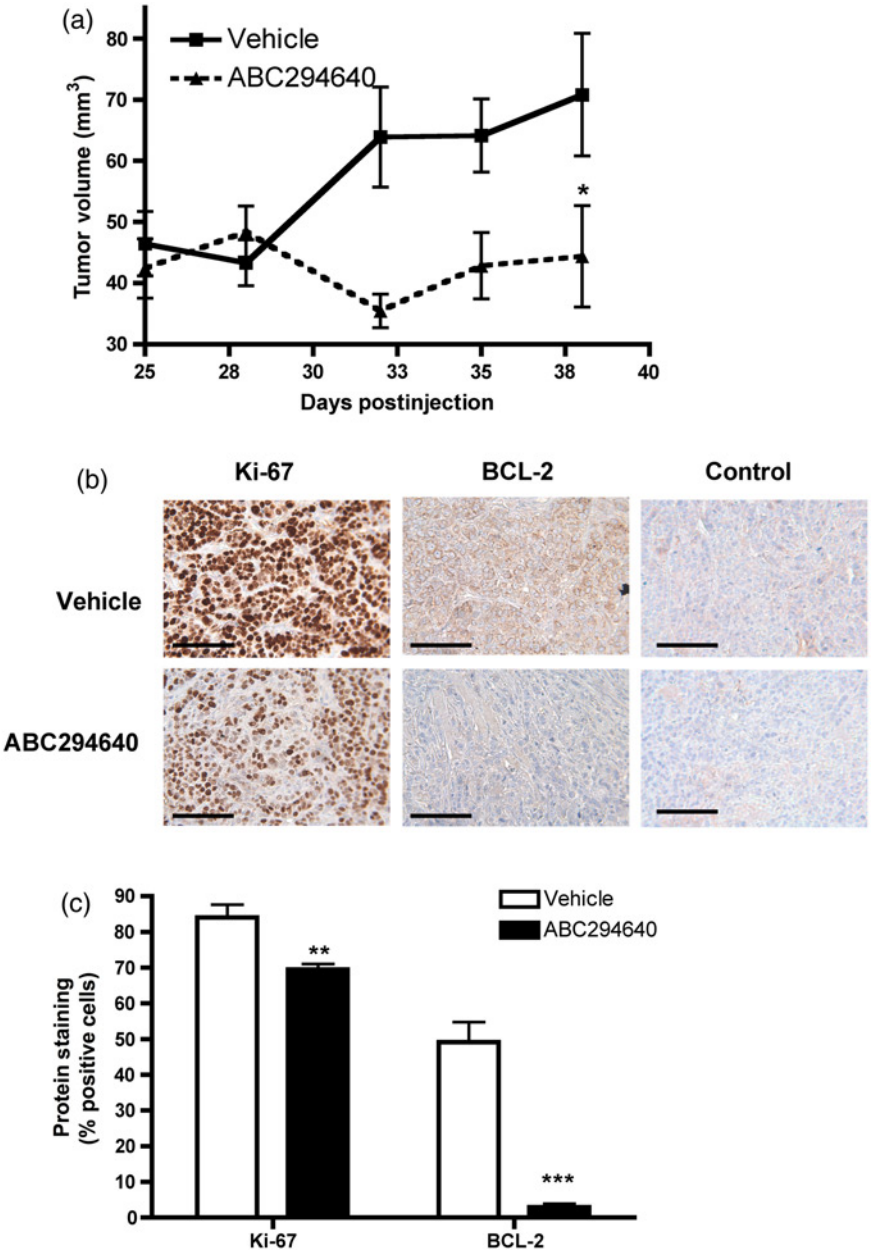

Figure 5 ABC294640 decreases endocrine therapy-resistant breast cancer tumor growth. (a) MDA-MB-468 cells were injected in the mammary fat pads of female mice. Tumors were allowed to form over nine days. Mice were treated intraperitoneally with 50 mg/kg of ABC294640 for 15 days. Tumor volume was measured every two days. Treatment tumors at endpoint were statistically significant from vehicle ($^{*}P < 0.05$). (b) Tumors from vehicle- and ABC294640-treated mice were processed and stained for Ki-67 and BCL-2. Representative images for staining and internal negative control in tumor sections are shown. Scale bar equal to 500 μm. (c) Quantitation of Ki-67 and BCL-2 staining is expressed as percent positive of total number of cells per field of view ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$). (A color version of this figure is available in the online journal)

MDA-MB-361 cells could account for the differential activity of these inhibitors across ER-positive cells lines. Furthermore, our finding that overexpression of Sphk promotes endocrine resistance in MCF-7 cells, suggests that alterations in Sphk expression between MCF-7 and MDA-MB-361 may contribute to short-term SKI resistance in these cells. Supporting this hypothesis, Hollestelle *et al.* performed gene expression profiling across a number of breast cancer cell lines, including MDA-MB-361 and MDA-MB-468. Array data from this study suggest that MDA-MB-361 cells exhibit increased mRNA expression of Sphk1, but not Sphk2, compared with MDA-MB-468 cells.[53] This hypothesis would correlate with our findings that long-term exposure dose-dependently blocked colony formation while short-term exposure had little effect on cell viability. Further study is needed to determine the mechanism of short-term SKI resistance in endocrine-resistant compared with endocrine-sensitive breast cancer.

Unlike MDA-MB-361 cells, both SKI-II and ABC294640 decreased viability, survival and proliferation in basal-A,

triple-negative breast cancer cells. Similar to recent studies, ABC294640 induced apoptosis and blocked proliferation *in vitro*, although these results were quantitatively less than luminal, endocrine-sensitive and basal-like, chemoresistant breast cancer cells.[26,36] The anti-proliferative and pro-apoptotic effects of ABC294640 translated into animal models, resulting in diminished tumor growth *in vivo*. Further studies are needed to better elucidate the differential effects of SKIs across the various cell lines. For example, if Sphk overexpression or activity is greater in MCF-7 and MCF-7TN-R cells compared with MDA-MB-468, then the presented results would be supported. However, another complicating factor may account for the disparity among breast cancer subtypes, such as differential location of Sphk2 within the cell. It is known that the subcellular localization of Sphk isoforms varies depending on tissue type and pathological state.[1] Sphk1 is found primarily in the cytoplasm of most cells, whereas Sphk2 can be cytoplasmic or nuclear. Sphk2 is found mainly in the nucleus of MCF-7 breast cancer cells but in the cytosol of HEK293 embryonic kidney cells and MDA-MB-453 breast cancer cells.[54–56] Currently, the cellular localization of Sphk2 in many breast cancer systems is unknown.

Alternatively, a proliferative pathway may increase Sphk activity in MDA-MB-361 compared with other cell systems. Sphk1 is stimulated by a number of extracellular and intracellular factors such as, EGF, vascular endothelial growth factor, TNF, phorbol esters, estrogen, calcium modulators and interleukins.[57–61] Sphk1 can also be directly phosphorylated by various growth factors such as EGF, protein kinase C and ERK which induce its translocation to the cell membrane, enhancing S1P generation.[62–65] In contrast, Sphk2 can be activated by EGF, protein kinase C and phorbol esters and is phosphorylated by ERK1/2 and protein kinase D, initiating its nuclear export.[54,66,67] Differential activity of growth pathways across breast cancer subtypes may alter the phosphorylation and activation profiles of Sphk, thus affecting the efficacy of our sphingosine kinase inhibitors. This possibility may account for the lack of effect on viability and apoptosis in MDA-MB-361 compared with MDA-MB-468 cells.

Although we and other researchers have investigated SKI-II and ABC294640 across the major breast cancer subtypes, most of these studies have been performed *in vitro*. Animal studies have been used to determine the pharmacokinetics and dynamics of these drugs, as well as characterize their anti-tumor properties.[36,37,48] Recent evidence suggests a role for Sphk in tumor and migration and metastasis in a variety of cancers, including the thyroid, kidney, esophageal, liver and breast.[68–72] Furthermore, increased Sphk expression correlates with increased tumor aggressiveness and decreased prognosis for clinical breast cancer patients.[6] To date, there have been few studies using SKI-II or ABC294640 analyzing their effects on invasion, migration or metastasis of breast cancer cell systems. More studies are necessary to better evaluate the clinical potential of these inhibitors. Taken together, our findings demonstrate the therapeutic promise of targeting sphingolipid signaling as a breast cancer treatment strategy.

Author Contributions: All authors participated in the design, interpretation of results and analysis of the data. JWA performed experiments and drafted the manuscript; MDW performed immunohistochemistry; and JLD performed animal experiments.

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