Pharmacology and Antitumor Activity of ABC294640, a Selective Inhibitor of Sphingosine Kinase-2


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Abbreviations: ABC294649, 3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide; BSA, bovine serum albumin; DMS, N,N-dimethylsphingosine; IC$_{50}$, concentration that inhibits by 50%; NBD-Sph, omega(7-nitro-2,1,3-benzoxadiazol-4-yl)(2S,3R,4E)-2-aminooctadec-4-ene-1,3-diol; PBS, phosphate-buffered saline; PEG, polyethylene glycol; S1P, sphingosine 1-phosphate; SK1, sphingosine kinase-1; SK2, sphingosine kinase-2.

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Abstract

Sphingolipid-metabolizing enzymes control the dynamic balance of the cellular levels of important bioactive lipids, including the apoptotic compound ceramide and the proliferative compound sphingosine 1-phosphate (S1P). Many growth factors and inflammatory cytokines promote the cleavage of sphingomyelin and ceramide leading to rapid elevation of S1P levels through the action of sphingosine kinases (SK1 and SK2). SK1 and SK2 are overexpressed in a variety of human cancers, making these enzymes potential molecular targets for cancer therapy. We have identified an aryladamantane compound, termed ABC294640, that selectively inhibits SK2 activity in vitro, acting as a competitive inhibitor with respect to sphingosine with a $K_i$ of 9.8 µM, and attenuates S1P formation in intact cells. In tissue culture, ABC294640 suppresses the proliferation of a broad panel of tumor cell lines, and inhibits tumor cell migration concomitant with loss of microfilaments. In vivo, ABC294640 has excellent oral bioavailability, and demonstrates a plasma half-time of clearance of 4.5 hr in mice. Acute and chronic toxicology studies indicate that ABC294640 induces a transient minor decrease in the hematocrit of rats and mice; however, this normalizes by 28 days of treatment. No other changes in hematology parameters, or gross or microscopic tissue pathology result from treatment with ABC294640. Oral administration of ABC294640 to mice bearing mammary adenocarcinoma xenografts results in dose-dependent antitumor activity associated with depletion of S1P levels in the tumors and progressive tumor cell apoptosis. Therefore, this newly developed SK2 inhibitor provides an orally-available drug candidate for the treatment of cancer and other diseases.
Introduction

Sphingolipids have become a focal point in biological research, with excellent rationale for their manipulation for the treatment of diseases, including cancer (reviewed in (Ogretmen, 2006; Cuvillier, 2007; Huwiler and Zangemeister-Wittke, 2007)). The parent lipid sphingomyelin is a structural component of cellular membranes, but also serves as the precursor for potent second messengers that have profound cellular effects. Stimulus-induced metabolism of these lipids is critically involved in cancer cell biology and inflammatory diseases, and so this metabolic pathway offers exciting new molecular targets for drug development.

In response to stimuli including growth factors and inflammatory cytokines, sphingomyelin is enzymatically hydrolyzed to ceramide, which can be further hydrolyzed by the action of ceramidase to produce sphingosine. Ceramide and sphingosine induce apoptosis in cancer cells by mechanisms that remain to be elucidated. Sphingosine is rapidly phosphorylated by sphingosine kinase (SK) to produce sphingosine 1-phosphate (S1P), which is mitogenic and anti-apoptotic. Through these conversions, a critical balance, i.e. a ceramide / S1P rheostat, has been hypothesized to determine the fate of the cell (Cuvillier et al., 1996). In this model, the balance between the cellular concentrations of ceramide and S1P determines whether a cell proliferates or undergoes apoptosis. Upon exposure to mitogens or activation of intracellular oncoproteins, the cells experience an increase in the intracellular levels of S1P and depletion of ceramide levels, and this situation promotes cell survival and proliferation. In contrast, activation of sphingomyelinase in the absence of activation of ceramidase and/or SK results in the accumulation of ceramide and subsequent apoptosis.

In spite of the high level of interest in sphingolipid-mediated signaling, there are very few known inhibitors of the enzymes of this pathway. In particular, the field suffers from a lack of potent and selective inhibitors of SK. Most pharmacological studies to date have used three compounds to inhibit SK activity: N,N-dimethylsphingosine (DMS), D,L-threo-dihydrosphingosine and N,N,N-trimethylsphingosine. However, these compounds are not
specific inhibitors of SK as they have been shown to affect protein kinase C (Igarashi et al., 1989), 3-phosphoinositide-dependent kinase (King et al., 2000), and casein kinase II (McDonald et al., 1991). Additionally, the compound phenoxodiol has been described as an SK inhibitor (Gamble et al., 2006); however, this isoflavone also inhibits several other enzymes. A few natural product inhibitors of SK have been isolated (Kono et al., 2001), but their selectivities remain unknown and their amenability to large-scale production is doubtful.

Clearly, inhibitors of SK that can be easily synthesized would be highly desirable for evaluating this enzyme as a therapeutic target. To address this problem, we previously identified and characterized structurally novel inhibitors of SK (French et al., 2003; French et al., 2006). These compounds, called SKI-I, -II and –V, inhibited S1P formation in intact cells, induced apoptosis, and demonstrated antitumor activity upon intraperitoneal administration (French et al., 2006), reinforcing the approach of targeting SK in cancer. We report here the pharmacologic characterization of a new orally-available SK inhibitor with \textit{in vivo} activity. Importantly, this compound is selective for SK2, thereby providing the first pharmacologic probe to evaluate the biological roles of this SK isozyme.
Methods

Materials. Unless otherwise noted, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Phalloidin -FITC were purchased from Invitrogen (Carlsbad, CA). Fibronectin and cell culture inserts were purchased from BD Biosciences (San Jose, CA). Recombinant human SK1 and SK2 were purchased from BPS Biosciences (San Diego, CA). C\textsubscript{17}-sphingosine and C\textsubscript{17}-S1P were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All cell lines were obtained from the American Type Culture Collection and grown in either DMEM or RPMI 1640 medium containing 10% fetal bovine serum and 50 µg/ml gentamycin sulfate. ABC294640, 3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide (Figure 1), was synthesized as described previously (Maines et al., 2008). The hydrochloride salt, ABC294640 • HCl, was prepared by dissolving 9.6 g of ABC294640 in 50 mL of CH\textsubscript{2}Cl\textsubscript{2}, and slowly adding an equimolar amount of 1M HCl in ether. After filtration and washing, 9.6 g (93%) of ABC294640 • HCl was recovered as fine crystals with a melting point of 204 - 206 °C.

Sphingosine kinase assays. The IC\textsubscript{50}s for ABC294640 and DMS were determined by a newly-developed HPLC-based SK activity assay. Briefly, the test compounds were incubated with recombinant SK1 or SK2 and omega(7-nitro-2-1,3-benzoxadiazol-4-yl)(2S,3R,4E)-2-aminooctadec-4-ene-1,3-diol (NBD-Sph) in the isozyme-selective assay buffers detailed below with 1 mg/ml fatty acid-free BSA, 100 µM ATP and 400 µM MgCl\textsubscript{2}. The product, i.e. NBD-S1P, was separated from NBD-Sph by HPLC as follows: Waters 2795 HPLC system with a Waters 2495 fluorescence detector, C8 Chromolith RP-8e column (100 × 4.6 mm, Merck KGaA, Germany), 1 ml/min mobile phase (acetonitrile : 20 mM (pH2.5) sodium phosphate buffer at 45 : 55). Fluorescence was monitored with excitation at 465 nm and emission at 531 nm. The ratio of NBD-S1P / (NBD-Sph + NBD-S1P) was used as a measure of SK activity. SK-izoyme selective assay buffers each contained 20 mM Tris pH7.4, 5 mM EDTA, 5 mM EGTA, 3 mM...
beta-mercaptoethanol, 5% glycerol, 1X protease inhibitors (Sigma, USA) and 1X phosphatase inhibitors (Roche, USA). For the SK1 assay buffer, 0.25% (final) Triton X-100 was added; and for the SK2 buffer, 1M (final) KCl was added. Assays were run for 2 hr at room temperature, and then a 1.5 volume of methanol was added to terminate the kinase reaction. After 10 min, the samples were centrifuged at 20,000 x g to pellet the precipitated protein, and the supernatants were analyzed by HPLC. In experiments to determine the $K_i$ for inhibition of SK2 by ABC294640, the ADP Quest assay system (DiscoveRx Corporation, Fremont CA) was used to measure kinase activity in the presence of varying concentrations of sphingosine and ABC294640.

To determine the effects of ABC294640 on cellular SK activity, near-confluent MDA-MB-231 cells were serum-starved overnight, and then treated with varying concentrations of ABC294640. The cells were then incubated with $[^3H]$sphingosine at a final concentration of 1 μM as previously described (French et al., 2006). The cells take up the exogenous sphingosine, which is converted to SIP via SK activity, and $[^3H]$SIP is separated from $[^3H]$sphingosine by extraction and quantified by scintillation counting.

Sphingolipid analyses. Biochemical analyses of ceramide species, sphingoid bases and their phosphates were performed by the Lipidomics Shared Resource at the Medical University of South Carolina on a Thermo Finnigan TSQ 7000, triple-stage quadrupole mass spectrometer operating in a Multiple Reaction Monitoring positive ionization mode. Quantitative analyses of the cellular sphingolipids were based on the 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 to internal standard peak area ratios from the samples were similarly normalized to their respective internal standards and compared to the calibration curves by linear regression. Final results were expressed as the level of the particular sphingolipid
normalized the total phospholipids phosphate levels determined from the Bligh & Dyer lipid extract (Bielawski et al., 2006).

**Cytotoxicity assays.** To determine the effects of the test compounds on proliferation, cells were plated into 96-well microtiter plates and allowed to attach for 24 h. Varying concentrations of ABC294640 were added to individual wells and the cells were incubated for an additional 72 h. At the end of this period, the number of viable cells was determined using the sulforhodamine-binding assay. The percentage of cells killed was calculated as the percentage decrease in sulforhodamine-binding compared with control cultures. Regression analyses of inhibition curves were performed using GraphPad Prism (San Diego, CA).

**Chemotaxis and cytoskeletal assays.** Chemotaxis assays were performed as follows: Transwell inserts (8 µm pore size) were pre-coated with fibronectin (5 µg/ml) for 1 h at room temperature. A-498 cells were trypsinized and resuspended in serum-free media at a concentration of 10^5 cells/ml. A total volume of 500 µl of this cell suspension was added to the top of the insert and medium containing 10 % fetal bovine serum was placed at the bottom to act as a chemoattractant. Equal concentrations of ABC294640 were added to the top and the bottom of the chamber. After 4 h at 37 °C, cells that migrated through the membrane were fixed in 4 % formaldehyde and stained with crystal violet. The number of migrating cells was established by counting ten random microscope fields. Experiments were performed in duplicate and repeated three times.

Microfilaments were stained with FITC-conjugated phalloidin (Invitrogen), according to the manufacturer’s directions. Briefly, cells in chamber slides were exposed to the vehicle, ABC294640 or cytochalasin B for various times, fixed in 3.7 % paraformaldehyde, permeabilized with 0.1% Triton-X 100, blocked with 2% BSA in TBST, and stained with 20 µl of phalloidin...
methanol solution per ml of blocking solution. After 12-16 h at 4 °C, the excess phalloidin was removed by washing in TBST, cells were mounted and observed with a Zeiss LSM 510 laser scanning confocal microscope. Cells were randomly selected and at least five different images with multiple cells were taken per experimental point.

The G (globular) to F (fibrous) actin ratio was quantified to assess the status of microfilaments. Briefly, cells were exposed to the drug for various times and actin was extracted by washing the cells with 1% Triton X-100 solution in PBS. The two forms of actin were separated by centrifugation at 14,000 x g. The supernatant (containing G actin) was mixed 1:1 with Laemmli buffer and the pellet (containing F actin) was dissolved in Laemmli buffer. Samples were subjected to SDS-PAGE and the actin content of each fraction was quantified by immunoblotting with anti-actin antibody and analyses using the ImageJ (NIH) program.

Quantification of ABC294640 in plasma and tumors. Plasma samples were prepared by centrifugation (5000 x g, 5 min at 4 °C) of whole blood that was collected into syringes containing EDTA as an anticoagulant. Samples were spiked with 10 μg of an internal standard (3-(4-chlorophenyl)adamantane-1-carboxylic acid [2-(3,4-dihydroxyphenyl)ethyl]amide), brought to 1 mL with water and extracted three times with 2 mL of ethyl acetate. Extracts were dried over nitrogen at 35 °C, reconstituted in 0.2 mL of 0.1% formic acid in water/methanol (50:50, Phase A), filtered and transferred to vials. Analyses were performed using an Agilent 1100 binary pump HPLC system coupled to a Finnigan LCQ Classic ion trap quadrupole mass spectrometer running in ESI positive ion mode. Sample (10 μL) was injected and resolved using a Supelco Discovery C18 column (2.1 x 20 mm, 5 mm particle size) connected to a Zorbax precolumn (Agilent) with a mobile phase consisting of 0.1% formic acid in water/methanol (50:50). The flow rate was 0.3 mL/min, and samples were eluted by a linear gradient increasing from 50% to 100% methanol over 3 min. ABC294640 and the internal standard were detected at 5.1 min and 5.5 min, respectively, using selected ion mode (m/z =
381 and 426, respectively). Peak areas were integrated using Xcalibur software, and
ABC294640 concentrations determined from a standard curve, which was linear in range of all
plasma levels observed in these studies.

**Oral bioavailability and pharmacokinetic studies.** Formulations of ABC294640 • HCl were
administered orally or intravenously to fasted female Swiss-Webster mice at a dose of 100
mg/kg in 0.1 mL of the indicated solvents. Blood samples were removed at 1 and 7 hr after
dosing, and the plasma concentration of ABC294640 was determined by reverse-phase LC/MS
running in SIM mode as described above. For pharmacokinetic studies, female Swiss-Webster
mice (6-8 weeks old) were fasted overnight and administered a bolus dose of 0.1 mL of
ABC294640 • HCl either orally or intravenously. After dosing, mice were anesthetized with
halothane and blood was removed via intracardiac puncture at the indicated times. Plasma
samples were processed and ABC294640 levels were determined as described above.
Noncompartmental pharmacokinetic analyses were performed using WINNONLIN (Pharsight).

**Toxicology studies.** Acute (7-day) and chronic (28-day) toxicology studies were conducted
with ABC294640 • HCl. In the first study (which was conducted by Eurofins | Product Safety
Laboratories, Dayton, NJ), Sprague-Dawley male rats (7-8 weeks old) were orally dosed with 0,
100 or 250 mg of ABC294640 • HCl /kg in 0.375% Polysorbate-80 in PBS daily for 7 days. The
animals were observed daily for viability, signs of gross toxicity, and behavioral changes, and a
battery of detailed observations were performed on study Days 1 and 7. Blood was sampled
from all animals on Day 8 of the study for hematology, clinical biochemistry and serology
assessments, and the animals were sacrificed. Gross necropsies were performed on all study
rats, and selected organs and tissues were evaluated in the control and high-dose level groups.
In the second study, C57BL/6 mice were orally dosed with 0, 100 or 250 mg of ABC294640 •
HCl /kg daily exactly as indicated above, and sacrificed at either Day 7 or Day 28 for hematology studies.

**Antitumor evaluation.** A syngeneic mouse tumor model that uses a transformed murine mammary adenocarcinoma cell line (JC, ATCC Number CRL-2116) and Balb/C mice (Charles River, Wilmington, MA) was performed as previously described (Lee et al., 2003). Animal care and procedures were in accordance with guidelines and regulations of the IACUC of the Penn State College of Medicine. Animals were housed under 12 hr light/dark cycles, with food and water provided *ad libitum*. Tumor cells (1 x 10^6) were implanted subcutaneously, and tumor volume was calculated using the equation: (L x W^2)/2. Upon detection of tumors, mice were randomized into treatment groups. Treatment was then administered every other day thereafter consisting of oral doses of 3.5, 10, 35 or 100 mg ABC294640 • HCl / kg body weight or vehicle (0.375% Polysorbate-80). Whole body weights and tumor volume measurements were performed each day of treatment. On Day 15, mice were dosed, euthanized 1 hr later and tumors were excised and immediately frozen. P-values were determined using one-way ANOVA using GraphPad InStat (San Diego, CA).

**Pharmacodynamic studies and tumor accumulation of ABC294640.** Apoptosis was measured in sections from tumors treated with ABC294640 • HCl using a TUNEL detection kit according to the manufacturer’s instructions (*In situ* cell death detection kit, Roche, Germany). Briefly, tumor sections were incubated with permeabilization solution (0.1% Triton X–100, 0.1% sodium citrate, freshly prepared) for 8 min at room temperature and then washed twice with PBS. Sections were incubated with TUNEL reaction mixture in a humid atmosphere at 37 °C for 60 min and mounted with crystal mounting medium. The amount of apoptosis was calculated for an average of 10 microscopic fields in each sample (magnification ×100) and expressed as the percentage of cells that were TUNEL-positive. For the analyses of sphingolipids, frozen
tumor slices were homogenized in ice-cold PBS to a final concentration of 10 mg/mL. A 0.5 mL aliquot of the homogenate was combined with 0.5 mL of methanol, 0.25 mL of chloroform, and 375 pmol each of internal standards C_{17}-sphingosine and C_{17}-S1P. Blank samples spiked with known amounts of sphingosine, S1P and the internal standards were processed in parallel to provide a standard curve for quantification. After sonication, samples were incubated overnight at 48 °C, followed by addition of 75 μL of 1N potassium hydroxide in methanol. The samples were then sonicated and incubated at 37 °C for 2 hr. A portion (0.4 mL) of each sample was then transferred to a new tube, dried, reconstituted in 0.25 mL of Phase A, filtered and transferred to a vial. HPLC was performed as described above. Elution was performed at 0.45 mL/min with 65% Phase B for 2 min followed by a linear gradient to 100% Phase B over 5 min. Ions for C_{17}-sphingosine, sphingosine, C_{17}-S1P and S1P were monitored at m/z 286 (parent ion) → 268 (daughter ion), 300 → 282, 366 → 250 and 380 → 264, respectively. Similarly, extracts of tumors from ABC294640-treated mice were prepared and levels of ABC294640 were quantified by LC/MS as described above.
Results

Inhibition of sphingosine kinase activity by ABC294640. Screening of a chemical library identified SK inhibitors containing an aryladamantane scaffold, and approximately 140 congeners were synthesized and tested for their ability inhibit a recombinant fusion protein of GST and human SK1 (Smith et al., 2008). The structure-activity properties of this series will be reported elsewhere. Although ABC294640 is not the most potent SK inhibitor in this series, its biologic and pharmacologic properties were characterized because of its excellent solubility and oral absorption (described below), and in vivo activity in models of diabetic retinopathy (Maines et al., 2006) and ulcerative colitis (Maines et al., 2008).

In evaluating the potencies of these compounds, we developed an HPLC-based assay for SK activity that avoids the use of radiolabeled substrate as we have previously described (French et al., 2003). Using recombinant human SK1 and SK2, ABC294640 demonstrated dose-dependent inhibition of SK2 with an IC\(_{50}\) of approximately 60 µM without affecting the activity of SK1 at concentrations up to at least 100 µM (Figure 2A). In contrast, DMS inhibited both SK1 and SK2 with IC\(_{50}\)s of approximately 60 and 20 µM, respectively. Additional studies demonstrated that both ABC294640 and DMS act as competitive inhibitors with respect to sphingosine, making the IC\(_{50}\) strongly affected by the concentration of sphingosine used in the assay. Kinetic analyses of varying concentrations of ABC294640 in the presence of 2.5 – 25 µM sphingosine indicated a K\(_i\) of 9.8 ± 1.4 µM for the inhibition of SK2 (Figure 2B).

To assess its selectivity, ABC294640 was tested against a panel of serine / threonine and tyrosine protein kinases, several of which are regulated by interactions with lipids. Each kinase was incubated with its appropriate peptide substrate, ATP and 50 µM ABC294640. Activities were normalized to vehicle-treated (DMSO) control reactions. As indicated in Table 1, none of the other 20 diverse kinases tested were significantly inhibited by the compound. These data suggest that the biological effects of this compound are not mediated by off-target inhibition of protein kinases, consistent with its targeting of the sphingosine binding site of SK2.
It was also important to determine the ability of ABC294640 to inhibit endogenous SK activity in an intact cell model. Therefore, we used our previously described method in which MDA-MB-231 cells are incubated with $[\text{H}]$sphingosine at a final concentration of 1 $\mu$M (French et al., 2003). The cells take up the exogenous sphingosine, which is converted to S1P via SK activity, and the cells are harvested and $[\text{H}]$S1P is separated from $[\text{H}]$sphingosine by extraction and quantified by scintillation counting. In this assay, ABC294640 decreased $[\text{H}]$S1P formation in a dose-dependent fashion with an $IC_{50}$ value of 26 $\mu$M. To confirm the affects of ABC294640 on endogenous SK activity, the time-dependence of alteration of the sphingolipid profile in JC murine adenocarcinoma cells was assessed by lipidomic profiling. As demonstrated in Figure 3, the predominant molecular species of ceramide in untreated cells included $C_{16}$-ceramide, $C_{22}$-ceramide, $C_{24}$-ceramide and $C_{24:1}$-ceramide. Each of these molecular species of ceramide was elevated after 12 and 24 hr of ABC294640-treatment, but these changes were largely normalized by 48 hr of treatment. Interestingly, the cellular levels of dihydro-$C_{16}$-ceramide were dramatically and persistently elevated by treatment with ABC294640, as were levels of dihydro-sphingosine. Importantly, cellular S1P levels were decreased at all times of ABC294640-treatment, particularly in cells treated for 48 hr. Therefore, ABC294640 markedly alters the ratio of ceramide : S1P consistent with inhibition of SK activity.

Inhibition of tumor cell proliferation and migration by ABC294640. The effects of ABC294640 on the proliferation of human tumor cell lines representing major tumor types were determined using the sulforhodamine B assay for quantifying cell number. As indicated in Table 2, ABC294640 inhibited tumor cell proliferation with $IC_{50}$s ranging from approximately 6 – 48 $\mu$M with Hep-G2 and HT-29 cells being the most and least sensitive, respectively. It is notable that the $IC_{50}$ for inhibition of the proliferation of MDA-MB-231 closely matches the $IC_{50}$ for suppression of SK activity in this cell line, i.e. 29 and 26 $\mu$M respectively, supporting the
hypotheses that the antiproliferative effects of ABC294640 are mediated by inhibition of SK activity, and that SK2 in particular is important for cell proliferation.

Binding of S1P to G protein-coupled S1P receptors induces a plethora of biological effects, including rapid reorganization of the actin cytoskeleton and stimulated migration of the cells (Hait et al., 2006). Therefore, we hypothesized that inhibition of SK activity by ABC294640 will affect microfilament structure and the ability of cells to migrate. A-498 cells are highly metastatic and mobile, which allowed us to perform migration assays using Boyden chambers for a short time (4 hr). As shown in Figure 4A, even short exposure to ABC294640 affected the ability of A-498 cells to migrate through a filter. This was confirmed using the “scratch assay”, which measures haptokinetic (random) cell movement (data not shown). Since cell migration is dependent on the microfilament cytoskeleton, A-498 cells were treated with ABC294640 and actin fibers were stained with FITC-phalloidin and observed by confocal microscopy. Representative images from those experiments are presented in Figure 4C, in which actin fibers are stained green, and nuclei are stained blue with DAPI. Cytochalasin D was used as a positive control for microfilament depolymerization. At the 24 h time point and concentrations of 50 µM or higher, the most noticeable phenotypes were a decrease in the number of stress fibers in the cells and a decrease in the number of lamellipodia protruding from the cell surface. Reorganization of actin structure was confirmed by separating and measuring the globular (G) and fibrous (F) actin levels. As shown on Figure 4B, the increase in G/F actin ratio is consistent with the changes in actin structure observed by confocal microscopy.

Absorption of ABC294640. The HCl salt of ABC294640 (ABC294640 • HCl) has been synthesized in multigram amounts for characterizations of its toxicity, pharmacokinetics and in vivo efficacy. Formulation analyses were conducted to identify a suitable pharmaceutical composition for in vivo studies. We chose five different oral formulations from the Division of Drug Information Resources’ Inactive Ingredient Guide, a compendium of all inactive ingredients.
in approved drug products currently marketed for human use, to assess their ability to support oral absorption of ABC294640. Solutions of ABC294640 • HCl in water, 90% propylene glycol (PG), 100% polyethylene glycol 400 (PEG400), 50% PEG400 or 0.375% Polysorbate-80 did not precipitate (measured as turbidity at 590 nm), and so were administered to fasted female Swiss-Webster mice at a dose of 100 mg/kg. Blood samples were removed at 1 and 7 hr, and plasma levels of ABC294640 were determined using an internal standard and reverse-phase HPLC coupled to an ion-trap quadrupole mass spectrometer running in positive SIM detection mode. As shown in Table 3, substantial amounts of ABC294640 were detected in the blood 1 hr after oral dosing, with the highest levels attained in the samples formulated in 90% PG. It should be noted that these ABC294640 concentrations are sufficient to inhibit SK activity and proliferation of tumor cells. By 7 hr, the plasma concentrations decreased by approximately 50% in most cases. Effective absorption was observed in the sample formulated in 0.375% Polysorbate-80, and this solvent for ABC294640 • HCl was used in further pharmacokinetic and efficacy analyses because of its low toxicity.

To further understand the absorption properties of ABC294640 • HCl, the relationship between plasma concentration and dose was examined. Mice were orally dosed with 10, 35 or 100 mg/kg of ABC294640, and the plasma levels were determined at 30 min. As shown in Figure 5, the plasma ABC294640 values demonstrated a good linear response with doses up to at least 100 mg/kg.

**Pharmacokinetics of ABC294640.** Detailed pharmacokinetic studies were performed on ABC294640 • HCl in 0.375% Polysorbate-80. Female Swiss-Webster mice were dosed with 50 mg/kg ABC294640 either intravenously or orally. Groups of mice (3 per group) were anesthetized and blood removed via cardiac puncture at time points ranging from 1 min to 7 hr. Plasma concentrations of ABC294640 were determined by LC/MS and pharmacokinetic parameters were calculated using the WINNONLIN software package (Table 4). Intravenous
administration of ABC294640 resulted in high plasma concentrations that were eliminated with a half-time of clearance of 1.4 hr. Although the peak plasma level of ABC294640 was lower when the compound was administered by oral gavage, the compound was much more persistent, likely reflecting continued absorption from the gastrointestinal tract, such that the calculated half-time for clearance was 4.5 hr. Importantly, comparison of the oral versus the intravenous pharmacokinetics of ABC294640 indicated an excellent oral bioavailability of 66% (F = AUC\textsubscript{oral} / AUC\textsubscript{iv}).

**Toxicity of ABC294640.** Preliminary toxicity studies were performed to determine the appropriate dose for in vivo efficacy testing. No immediate or delayed toxicity was observed in female Swiss-Webster mice treated with intraperitoneal doses of ABC294640 • HCl up to at least 250 mg/kg. Repeated injections in the same mice every other day over 15 days showed a similar lack of toxicity at doses up to at least 250 mg/kg. Dose-escalation toxicity testing was performed via oral gavage with ABC294640 • HCl dissolved in 0.375% Polysorbate-80, and no toxic effects were observed after administration of doses up to 1000 mg/kg. Therefore, the compound was considered to be suitable for more detailed in vivo studies.

Non-GLP acute toxicology studies were contracted to Eurofins | Product Safety Laboratories, in which ABC294640 • HCl was given orally in 0.375% Polysorbate-80 to rats at doses of 0, 100 or 250 mg/kg daily for 7 days. There were no clinical observations or gross findings that were considered to be the result of ABC294640 • HCl administration or otherwise. There were no significant changes in total body weight of the treated animals, although there was a slight decrease in body weight gain, consistent with a small decrease in food consumption, in the high-dose rats. Hematology studies (Table 5) indicated decreases in red blood cell number and hematocrit of approximately 20% in animals given either 100 or 250 mg/kg/day; and a slight increase in neutrophils and decrease in basophils in the treated rats. These changes would be scored as grade 0 toxicities on the standard NCI scale for evaluating
toxicity in clinical trials. Importantly, no decreases in lymphocyte, platelet or granulocyte counts were observed, indicating that the compound does not induce immunologic toxicities that are common with other anticancer drugs. Similarly, there were no drug-induced alterations of a broad panel of clinical chemistry or coagulation parameters. No gross abnormalities were noted for any of the euthanized animals when necropsied at the end of the 7-day observation period. Similarly, there were no treatment-related microscopic changes in any organ examined in the high-dose group, except for a slight decrease in the background level of extramedullary hematopoiesis in the spleen which may underlie the small decreases in the hematocrit.

To further characterize the hematologic changes observed in the acute study, mice were treated with 0, 100 or 250 mg ABC294640 • HCl / kg daily for 28 days. As indicated in Figure 6A, mice treated with 250 mg/kg experienced a 20% decrease in red blood cell count and hematocrit, and a modest increase in the number of circulating neutrophils on Day 7, essentially identical to the previous study with rats. However, after 28 days of treatment (Figure 6B), these parameters were restored to normal levels, indicating that the animals had fully recovered from any transient impairment of hematopoiesis. Additionally, there were no changes in the brain or spleen weights of treated mice, whereas a slight decrease (12%) in liver weight was observed in mice treated with 250 mg/kg.

**Antitumor activity of ABC294640.** The antitumor activity of ABC294640 • HCl was tested in a syngeneic tumor model that uses the mouse JC mammary adenocarcinoma cell line growing subcutaneously in immunocompetent Balb/c mice (Lee et al., 2003). Because of the excellent oral absorption described above, we determined the ability of orally-delivered ABC294640 • HCl to reduce tumor growth *in vivo*. The SK inhibitor was administered to fasted mice on odd days at doses ranging from 3.5 to 100 mg/kg. Body weights and tumor volumes were monitored daily. As demonstrated in Figure 7, ABC294640 • HCl caused dose-dependent reductions in the growth of the mammary adenocarcinoma xenografts. Body weights in each treatment group
remained unchanged from vehicle-treated mice during the course of the study (data not shown). Comparison with the potencies in the tumor studies with the toxicity data described above reveals that ABC294640 • HCl has a therapeutic index of greater than 7 (250 mg/kg nontoxic dose / 35 mg/kg antitumor activity). Thus, this SK2 inhibitor has an excellent therapeutic index.

To ensure that the antitumor effect of ABC294640 • HCl administration is mediated by the compound, its accumulation in tumors was quantified by LC/MS. In these experiments, mice bearing JC tumor xenografts were treated with 100 mg/kg of ABC294640 • HCl by intraperitoneal injection, and the concentrations of the compound in the plasma and the tumor were measured at 2 and 5 hr. As indicated in Figure 8A, approximately 75 μg/mL (197 μM) ABC294640 was present in the plasma at 2 hr, and this decreased to 56 μg/mL (147 μM) at 5 hr. The amounts of ABC294640 in the tumor at 2 and 5 hr were determined to be 36 and 54 μg/g wet weight, respectively, corresponding to approximately 94 and 140 μM (assuming that 1 g approximately equals 1 mL). Therefore, amounts of ABC294640 well above those needed to block cell proliferation are accumulated in the tumors of intact mice.

As a global pharmacodynamic endpoint for ABC294640 • HCl treatment, tumors from mice given a single dose of the compound were scored for their apoptotic indices using the TUNEL method. As shown in Figure 8B, untreated tumors had focal areas of apoptosis such that the overall tumor apoptosis index was approximately 12%. Treatment of the mice with 100 mg/kg ABC294640 • HCl enhanced tumor cell apoptosis in a time-dependent manner, such that nearly 50% of the tumor cells expressed fragmented DNA by 5 days.

To determine a mechanistic pharmacodynamic endpoint for ABC294640 • HCl, we quantified sphingolipid metabolites in the tumors by LC-MS/MS. Samples of frozen tumors removed at the end of the study in Figure 7 were homogenized in cold PBS, spiked with internal standards and processed by liquid-liquid extraction. Ratios of sphingosine and S1P to internal standards were determined and compared with vehicle-treated tumor samples. As shown in Figure 8C, treatment of the mice with ABC294640 • HCl at the 100 mg/kg had no effect on
sphingosine levels in the tumors. This is likely due to rapid conversion of sphingosine to ceramide in the presence of the SK inhibitor. Importantly, S1P levels were significantly reduced in the tumors of mice treated with ABC294640 • HCl. These findings provide further evidence that the antitumor activity of this compound is linked to SK inhibition and decreased S1P formation.
Discussion

Accumulating evidence demonstrates that S1P is a critical second messenger that exerts proliferative, antiapoptotic and inflammatory actions. An oncogenic role of SK has been directly demonstrated since transfection into NIH/3T3 fibroblasts was sufficient to promote foci formation and cell growth in soft-agar, and to allow these cells to form tumors in NOD/SCID mice (Xia et al., 2000). Additionally, inhibition of SK by transfection with a dominant-negative SK mutant or by treatment of cells with the nonspecific SK inhibitor DMS blocked transformation mediated by oncogenic H-Ras. As abnormal activation of Ras frequently occurs in cancer, these findings suggest a significant role of SK in this disease. SK has also been linked to estrogen signaling (Sukocheva et al., 2003), and estrogen-dependent tumorigenesis in MCF-7 cells (Nava et al., 2002). Other pathways or targets to which SK activity has been linked in cancer include VEGF signaling via the Ras and MAP kinase pathway (Shu et al., 2002), protein kinase C (Nakade et al., 2003), TNFα (Vann et al., 2002) and caspase activation (Edsall et al., 2001). Angiogenic factors and processes, such as cell motility, mitogenesis in smooth muscle cells, endothelial cell differentiation and growth factor signaling are also affected by SK and S1P (Lee et al., 1999). While the elucidation of downstream targets of S1P remains an interesting problem in cell biology, sufficient validation of these pathways has been established to justify their evaluation as targets for new types of anticancer drugs. As S1P appears to be the most direct mitogenic messenger, inhibition of its production should have useful antiproliferative effects on tumor cells.

Two isozymes of SK (termed SK1 and SK2) exist (Kohama et al., 1998), with SK2 being cloned in 2000 (Liu et al., 2000). However, the roles of these SK isozymes remain to be defined as they demonstrate different kinetic parameters and expression patterns in normal tissue. The majority of studies linking SK activity to cancer and growth control have focused on SK1 (Nava et al., 2002; Johnson et al., 2005; Le Scolan et al., 2005; Pchejetski et al., 2005; Sarkar et al., 2005; Kawamori et al., 2006). For example, EGF rapidly induces the expression of SK1, but not
SK2, in MCF-7 cells (Doll et al., 2005); however, it has also been reported that EGF activates SK2 in MDA-MB-453 cells (Hait et al., 2005). Overexpression of SK1 has been shown to be oncogenic in a variety of cells (Xia et al., 2000; French et al., 2003; Le Scolan et al., 2005); whereas overexpression of SK2 is reported to inhibit cell growth and to induce apoptosis (Maceyka et al., 2005). However, these effects of SK2 are only partially dependent on its catalytic activity, suggesting that the antiproliferative effects may be mediated by its BH3 domain (Maceyka et al., 2005). Consistent with this, are the observations that physiological levels of SK2 do not inhibit DNA synthesis (Okada et al., 2005). SK1-deleted transgenic mice have normal phenotypes, and serum concentrations of S1P are reduced by 50%. However, levels of S1P within a variety of tissues are not different from those of control mice, indicating functional replacement of SK1 by SK2 in normal tissues (Allende et al., 2004). Similarly, genetic ablation of SK2 results in mice with normal fertility and life-span, and 25% reduced serum S1P levels (Kharel et al., 2005). Our data (French et al., 2003), and that of others (Johnson et al., 2005; Van Brocklyn et al., 2005; Kawamori et al., 2006), demonstrate that SK1 is marked overexpressed in a variety of human cancers, while SK2 is typically not (Van Brocklyn et al., 2005), although several types of cancer do have elevated levels of SK2 message (unpublished observations). Therefore, it was unclear if selective targeting of SK1 or SK2 by SK inhibitors will be sufficient to inhibit tumor growth and induce apoptosis, or whether simultaneous inhibition of SK1 and SK2 will be necessary to prevent the functional redundancy of these isozymes.

Based upon this growing body of knowledge implicating SKs in the abnormal growth of cancer cells, we sought to identify novel inhibitors of SK1 and/or SK2 that may serve as effective cancer therapeutics. Screening a chemically diverse small molecule library using a recombinant human SK1-fusion protein resulted in the identification of multiple chemotypes (French et al., 2003). Follow up hit-to-lead efforts resulted in the discovery of ABC294640, an aryladamantane compound with SK inhibitory activity (Smith et al., 2008). The potency of ABC294640 is similar to other SK inhibitors described in the literature (Kono et al., 2001);
however, these natural products have unknown selectivities and potential for development. Furthermore, analyses of the kinetics of inhibition of SK2 by ABC294640 clearly indicate that this compound acts as a competitive inhibitor with respect to sphingosine, with a $K_i$ of approximately 10 µM (Figure 2B). Since the levels of sphingosine in a cell are very low, e.g. in comparison with ceramide levels (Figure 3), inhibition of SK2 by ABC294640 is likely to be very efficient. The selective targeting of SK2 was unexpected; however, our studies using siRNAs directed at either SK1 or SK2 demonstrate that ablation of SK2 from tumor cells has greater antiproliferative and anti-migratory affects than does depletion of SK1. Since many kinase inhibitors lack specificity, we tested the ability of ABC294640 to inhibit several other kinases. These assays indicated that ABC294640 is highly specific for SK versus protein kinases, making it a viable candidate chemotherapeutic agent.

Our studies on the chemicophysical properties of ABC294640 further support its development. For example, the presence of the pyridine moiety permits protonation of the nitrogen atom, and the formation of a hydrochloride salt. This greatly improves the solubility, such that ABC294640·HCl was quite soluble in almost all tested carriers. These broad solubility properties enabled testing multiple formulations to evaluate their respective in vivo absorption properties. While all formulations showed encouraging results, we chose 0.375% Polysorbate-80 due to its ease of formation and lower viscosity, which facilitates oral dosing. Additionally, long term stability studies revealed that ABC294640·HCl was highly stable in 0.375% Polysorbate-80 versus the other formulations (data not shown).

Identification of an optimal oral formulation permitted acute and chronic toxicology studies, where we observed excellent toxicity profiles for ABC294640·HCl. A dose of 100 mg/kg, which was determined to be at least 10-fold below than the lowest observed adverse effect level, resulted in ABC294640 plasma levels that exceeded the IC$_{50}$ towards human SK2 and are sufficient for suppression of tumor cell proliferation and migration. Biodistribution
studies demonstrated that high concentrations of ABC294640 accumulate in the tumors of intact mice, indicating that the drug does effectively reach its target tissue.

To determine the therapeutic efficacy of the compound, we evaluated ABC294640 • HCl in a syngeneic murine breast cancer model and demonstrated significant dose-dependent inhibition of tumor growth. Previous studies have demonstrated this model is sensitive to SK inhibitors (French et al., 2003). Furthermore, tumor growth inhibition correlated with progressive tumor cell apoptosis and decreases in S1P levels in the tumors when compared to vehicle-treated mice. This is the first pharmacodynamic evidence of S1P modulation linked to antitumor activity. We believe that this finding, in conjunction with low toxicity of ABC294640 • HCl, provides validation of SK2 as a chemotherapeutic target.

Current studies are underway to determine the mechanism of antitumor activity, since SK activity has been linked to survival, inflammatory and angiogenic pathways. Ideally, SK2 inhibition could lead to modulation of all of these pathways, resulting in tumor inhibition through multiple mechanisms. We also determined that sphingosine levels remained unchanged despite decreases in S1P. This likely relates to an increase in synthesis of ceramide from sphingosine, resulting in a constant sphingosine level. This would be beneficial in a tumor environment, as increases in ceramide levels cause apoptosis in cancer cells (Kolesnick and Fuks, 2003), and this does occur in the ABC294640-treated tumor cells. Interestingly, we found that SK2 inhibition affects actin structure at time points and concentrations that are non-toxic, suggesting that the compound may also have anti-metastatic activity in vivo.

In conclusion, ABC294640 • HCl is a first-in-class inhibitor of SK with good pharmacologic properties, low toxicity and anticancer activity. These data support our previous demonstrations that ABC294640 • HCl has therapeutic activity in inhibiting diabetes-induced retinal vascular leakage in rats (Maines et al., 2006) and dextran sulfate sodium-induced ulcerative colitis in mice (Maines et al., 2008). Additional unpublished studies also demonstrate
that ABC294640 • HCl is efficacious in models of Crohn’s Disease, rheumatoid arthritis and ischemia-reperfusion injury. In all, the data indicate that ABC294640 • HCl warrants further developmental efforts to fully determine its potential as an anticancer and anti-inflammatory drug.
References


Footnotes

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Legends for Figures

Figure 1. Structure of ABC294640. 3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide (CAS 915385-81-8).

Figure 2. Inhibition of sphingosine kinases. Panel A. Recombinant SK1 (circles) or SK2 (squares) was incubated with the indicated concentrations of ABC294640 (filled symbols) or DMS (open symbols), and the kinase activity was measured using the HPLC assay described in the Methods section. Values represent the mean ± SEM for three experiments. Panel B. Recombinant SK2 was incubated with the indicated concentration of ABC294640 and kinase activity was determined in assays containing 2.5 (■), 5 (▲), 10 (▼) or 25 (◆) µM sphingosine using the ADP Quest assay as described in the Methods section. The reciprocal of the velocity is plotted against the ABC294640 concentration providing a Dixon plot of the results.

Figure 3. Time course of sphingolipid alterations by ABC294640. JC murine adenocarcinoma cells were exposed to 40 µM ABC294640 for 0 (blue), 12 (red), 24 (yellow) or 48 (green) hr. Cells were harvested and the masses of the indicated sphingolipid species were quantified by mass spectrometry in the Lipidomics Core Facility as described in the Methods section. Labels indicate the chain-length and saturation of molecular species of ceramide (Cer). Other labels refer to dihydro-C16-ceramide (DHC16-Cer), dihydrospingosine (DHSph), dihydrospingosine 1-phosphate (DhSph-1P), sphingosine (Sph) and S1P.
Figure 4. Disruption of tumor cell migration and microfilament structure by ABC294640.

Panel A. A-498 cells were plated into a Boyden chamber and treated with the indicated concentration of ABC294640 for 4 hr as described in the Methods section. The number of cells migrating to the opposite side of the filter was then quantified. Panel B. A-498 cells were treated with the indicated concentration of ABC294640 for 24 hr, and the amounts of G-actin and F-actin were determined as described in the Methods section. Cytochalasin D (cch) was used as a positive control at a concentration of 1 µM. Panels C and D. A-498 cells were treated with 0 (Panel C) or 50 µM ABC294640 (Panel D) for 24 hr, and then stress fibers were visualized with FITC-phalloidin as described in the Methods section.

Figure 5. Relationship between dose and plasma ABC294640 level. Mice were orally dosed with the indicated amounts of ABC294640 • HCl in 0.375% Polysorbate-80, bled at 30 minutes, and the concentration of ABC294640 in the plasma was determined as described in the Methods section. Values represent mean ± SEM (n =3 mice per group).

Figure 6. Hematologic parameters in ABC294640-treated mice. Mice were orally dosed with 0 (Veh), 100 or 250 mg/kg of ABC294640 • HCl in 0.375% Polysorbate-80 daily for either 7 (Panel A) or 28 (Panel B) days. Blood was harvested by cardiac puncture and a Complete Blood Count was performed. Values represent mean ± standard error (n = 3 or 4 mice per group) for the red blood cells (RBC), hematocrit (HCT), total white blood cell (WBC), and neutrophil (Neutr) values.
Figure 7. Antitumor activity of orally-administered ABC294640. Female Balb/C mice were injected subcutaneously with JC cells suspended in PBS. After palpable tumor growth, animals were treated every-other day by oral gavage with 0 (□), 3.5 (◆), 10 (▼), 35 (▲), or 100 (■) mg/kg of ABC294640 • HCl in 0.375% Polysorbate-80. Values represent the mean ± SEM (n = 5 mice per group) tumor volume normalized to treatment Day 1 for each mouse. *, p < 0.05; **, p < 0.01.

Figure 8. Pharmacodynamic effects of ABC294640. Panel A. Accumulation of ABC294640 in tumors. Mice bearing JC tumor xenografts were treated by intraperitoneal injection of 100 mg/kg of ABC294640 • HCl and tumors were harvested at the times 2 or 5 hr. The tumors were homogenized and the amount of ABC294640 was quantified (n = 4 mice per group). Values represent the mean ± SEM in μg/mL for plasma samples (filled bars) and μg/g wet weight for tumor samples (open bars). Panel B. Induction of tumor apoptosis by ABC294640. Mice bearing JC tumor xenografts were treated by intraperitoneal injection of 0 or 100 mg/kg of ABC294640 • HCl. Tumors were harvested either 3 or 5 days after drug treatment, fixed and sectioned, and the amount of apoptosis was quantified as TUNEL staining. Values represent the mean ± SEM percent of tumor cells that were TUNEL-positive (n = 4 mice per group). Panel C. Alteration of S1P levels by ABC294640. Tumors were harvested at the end of the experiment described in Figure 7. The levels of sphingosine (filled bars) or S1P (open bars) were determined by LC/MS/MS. Values represent the mean ± sd levels compared with the vehicle-treatment group. **, p < 0.01 versus vehicle-treated mice.
Table 1. **Kinase selectivity of ABC294640.** The affects of 50 μM ABC294640 on the activities of the indicated protein kinases was assessed by Upstate Cell Signaling. Values represent percent of activity of the indicated kinase remaining in the presence of ABC294640, and are the mean ± sd of duplicate measurements.

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Activity (% of control)</th>
<th>Kinase</th>
<th>Activity (% of control)</th>
</tr>
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<tr>
<td>Ca(^{2+})/calmodulin PK IV</td>
<td>81 ± 3</td>
<td>cRaf</td>
<td>96 ± 5</td>
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<tr>
<td>Abl</td>
<td>98 ± 0</td>
<td>MEK kinase 1</td>
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<td>Aurora-A</td>
<td>103 ± 1</td>
<td>CHK1</td>
<td>142 ± 13</td>
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<td>Protein kinase C α</td>
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<td>EFGR</td>
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<td>CDK1/cyclinB</td>
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<td>CDK2/cyclinE</td>
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<tr>
<td>PDK1</td>
<td>116 ± 3</td>
<td>PKBγ</td>
<td>105 ± 8</td>
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Table 2. Effects of ABC294640 on tumor cell proliferation. Sparsely plated cells were treated with ABC294640 for 48 h, and the number of viable cells was determined and compared to vehicle (DMSO)-treated cells as described in the Methods section. Values are the mean ± SEM for at least three separate experiments.

<table>
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<tr>
<th>Cell Line</th>
<th>Tissue</th>
<th>IC50 (μM)</th>
<th>Cell Line</th>
<th>Tissue</th>
<th>IC50 (μM)</th>
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<tr>
<td>1025LU</td>
<td>melanoma</td>
<td>33.7 ± 2.7</td>
<td>Hep-G2</td>
<td>liver</td>
<td>6.0 ± 2.6</td>
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<tr>
<td>A-498</td>
<td>kidney</td>
<td>12.2 ± 6.0</td>
<td>MCF-7</td>
<td>breast, ER+</td>
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<tr>
<td>Caco-2</td>
<td>colon</td>
<td>11.8 ± 5.6</td>
<td>MDA-MB-231</td>
<td>breast, ER-</td>
<td>29.1 ± 11.1</td>
</tr>
<tr>
<td>HT-29</td>
<td>colon</td>
<td>48.1 ± 7.6</td>
<td>Panc-1</td>
<td>pancreas</td>
<td>32.8 ± 0.1</td>
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<tr>
<td>DU145</td>
<td>prostate</td>
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<td>T24</td>
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<tr>
<td>SK-OV-3</td>
<td>ovary</td>
<td>10.5 ± 2.6</td>
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Table 3. Effects of formulation on the oral absorption of ABC294640. Mice were dosed with 100 mg/kg of ABC294640 dissolved in the indicated carriers, and plasma levels of the compound were determined at indicated times. Values represent the mean ± SEM (n = 3 per group) concentration of plasma ABC294640.

<table>
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<tr>
<th>Formulation</th>
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<tr>
<td></td>
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<tr>
<td>Water</td>
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<tr>
<td>90% Propylene Glycol</td>
<td>62.0 ± 11.8</td>
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<tr>
<td>100% PEG400</td>
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</tr>
<tr>
<td>50% PEG400</td>
<td>31.3 ± 0.5</td>
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<tr>
<td>0.375% Polysorbate-80</td>
<td>51.6 ± 0.9</td>
</tr>
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</table>
Table 4. Pharmacokinetics of ABC294640. Mice were given 50 mg/kg of ABC294640 • HCl in 0.375% Polysorbate-80 intravenously or by oral gavage. Plasma was harvested at times between 1 min and 7 hr, and ABC294640 levels were determined as described in the Methods section. Values represent the mean ± SEM (n = 3 per group).

<table>
<thead>
<tr>
<th>Route</th>
<th>( \text{AUC}_{0\rightarrow\infty} )</th>
<th>( \text{AUC}_{0\rightarrow\infty} )</th>
<th>( T_{\text{max}} )</th>
<th>( C_{\text{max}} )</th>
<th>( C_{\text{max}} )</th>
<th>( T_{1/2} )</th>
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<tbody>
<tr>
<td></td>
<td>(μg•h/ml)</td>
<td>(μM•h)</td>
<td>(h)</td>
<td>(μg/ml)</td>
<td>(μM)</td>
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<tr>
<td>IV</td>
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<tr>
<td>Oral</td>
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<td>90.1</td>
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<td>19</td>
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Table 5. Summary of hematologic effects of ABC294640. Sprague-Dawley rats were treated with 0, 100 or 250 mg ABC294640 • HCl / kg for 7 days as described in the Methods section. Values represent the mean ± SD for 5 rats per group.

<table>
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<th>250</th>
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<tbody>
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<td>Erythrocyte Count</td>
<td>$10^6/\mu$L</td>
<td>7.52 ± 0.23</td>
<td>6.00 ± 0.20*</td>
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</tr>
<tr>
<td>Hematocrit</td>
<td>%</td>
<td>41.9 ± 0.8</td>
<td>33.8 ± 0.8*</td>
<td>32.7 ± 1.8*</td>
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<tr>
<td>Absolute Reticulocyte Count</td>
<td>$10^3/\mu$L</td>
<td>350 ± 59</td>
<td>431 ± 14*</td>
<td>355 ± 41</td>
</tr>
<tr>
<td>Platelet Count</td>
<td>$10^3/\mu$L</td>
<td>734 ± 181</td>
<td>1201 ± 300*</td>
<td>950 ± 301</td>
</tr>
<tr>
<td>Total Leukocyte Count</td>
<td>$10^3/\mu$L</td>
<td>14.48 ± 0.80</td>
<td>12.74 ± 2.09</td>
<td>15.24 ± 3.23</td>
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<tr>
<td>Absolute Neutrophils</td>
<td>$10^3/\mu$L</td>
<td>1.75 ± 0.43</td>
<td>2.09 ± 0.33</td>
<td>2.98 ± 0.50</td>
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<tr>
<td>Absolute Lymphocytes</td>
<td>$10^3/\mu$L</td>
<td>11.99 ± 0.86</td>
<td>9.96 ± 1.67</td>
<td>11.69 ± 2.81</td>
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<tr>
<td>Absolute Monocytes</td>
<td>$10^3/\mu$L</td>
<td>0.35 ± 0.12</td>
<td>0.39 ± 0.21</td>
<td>0.28 ± 0.08</td>
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<tr>
<td>Absolute Eosinophils</td>
<td>$10^3/\mu$L</td>
<td>0.27 ± 0.14</td>
<td>0.17 ± 0.07</td>
<td>0.15 ± 0.04</td>
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<tr>
<td>Absolute Basophils</td>
<td>$10^3/\mu$L</td>
<td>0.06 ± 0.01</td>
<td>0.04 ± 0.01*</td>
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<tr>
<td>Absolute Large Unstained Cells</td>
<td>$10^3/\mu$L</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>0.08 ± 0.02</td>
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</table>

* = Statistically significant difference from control at p < 0.05 by Dunnett/Tamhane-Dunnett test.
Figure 1

ABC294640
Figure 2

A

SK Activity (% of control)

[SK Inhibitor] (μM)

B

1/Velocity

[ABC294640] (μM)
Figure 3
Figure 4

**A**

Cell migration (% of control)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
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<th>50</th>
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<tr>
<td>[ABC294640] (μM)</td>
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</table>

**B**

G-actin/F-actin (normalized to control)

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<thead>
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<th></th>
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<th>30</th>
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<td>[ABC294640] (μM)</td>
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</table>

**C**

Vehicle

**D**

50 μM ABC294640

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Figure 5
Figure 6
Figure 7
Figure 8