Published in final edited form as:

Cancer Transl Med. 2017 ; 3(4): 109–121. doi:10.4103/ctm.ctm_7_17.

SKI-178: A Multitargeted Inhibitor of Sphingosine Kinase and Microtubule Dynamics Demonstrating Therapeutic Efficacy in Acute Myeloid Leukemia Models

Jeremy A. Hengst^{1,2}, Taryn E. Dick^{1,2}, Arati Sharma¹, Kenichiro Doi³, Shailaja Hegde⁴, Su-Fern Tan⁵, Laura M. Geffert^{1,2}, Todd E. Fox⁵, Arun K. Sharma¹, Dhimant Desai¹, Shantu Amin¹, Mark Kester⁵, Thomas P. Loughran⁵, Robert F. Paulson⁴, David F. Claxton⁶, Hong-Gang Wang³, and Jong K. Yun^{1,2}

¹Department of Pharmacology, Penn State Hershey College of Medicine, Hershey, PA, USA

²The Jake Gittlen Laboratories for Cancer Research, The Pennsylvania State University College of Medicine, Hershey, PA, USA

³Department of Pediatrics, Penn State Hershey College of Medicine, Hershey, PA, USA

⁴Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA, USA

⁵University of Virginia Cancer Center, University of Virginia, Charlottesville, VA, USA

⁶Department of Hematology, Penn State Hershey Cancer Institute, Hershey, PA, USA

Abstract

Aim—To further characterize the selectivity, mechanism-of-action and therapeutic efficacy of the novel small molecule inhibitor, SKI-178.

Methods—Using the state-of-the-art Cellular Thermal Shift Assay (CETSA) technique to detect “direct target engagement” of proteins intact cells, *in vitro* and *in vivo* assays, pharmacological assays and multiple mouse models of acute myeloid leukemia (AML).

Results—Herein, we demonstrate that SKI-178 directly target engages both Sphingosine Kinase 1 and 2. We also present evidence that, in addition to its actions as a Sphingosine Kinase Inhibitor, SKI-178 functions as a microtubule network disrupting agent both *in vitro* and in intact cells. Interestingly, we separately demonstrate that simultaneous SphK inhibition and microtubule disruption synergistically induces apoptosis in AML cell lines. Furthermore, we demonstrate that SKI-178 is well tolerated in normal healthy mice. Most importantly, we demonstrate that SKI-178 has therapeutic efficacy in several mouse models of AML.

Conclusion—SKI-178 is a multi-targeted agent that functions both as an inhibitor of the SphKs as well as a disruptor of the microtubule network. SKI-178 induced apoptosis arises from a synergistic interaction of these two activities. SKI-178 is safe and effective in mouse models of AML, supporting its further development as a multi-targeted anti-cancer therapeutic agent.

Keywords

Sphingosine kinase; sphingosine kinase inhibitor; microtubule disrupting agent; multi-targeted agent; polypharmacology; acute myeloid leukemia

INTRODUCTION

Numerous studies have demonstrated that the Sphingosine Kinases (SphK1 and/or SphK2) are over-expressed in cancer.^{1–7} Elevated expression of SphK1 is recognized as a risk factor for decreased 5-year survival and decreased progression-free survival in numerous solid and hematological cancers (summarized in a recent meta-analysis).⁸ Over-expression of SphK1 and, more recently SphK2, have been associated with oncogenic transformation and growth in soft agar.^{9–11} In a cancerous setting, over-expression of the SphKs deregulates the “sphingolipid rheostat”, increasing Sphingosine-1-Phosphate (S1P) production at the expense of Ceramide/Sphingosine. Thus, S1P driven pro-mitogenic/pro-survival signaling predominates and cancer cells become dependent upon S1P signaling (“non-oncogene” addiction).¹² Sph/Cer are thought to be important for induction of apoptosis, and SphK over-expression also effectively depletes Sph/Cer levels in cancer cells, making them more resistant to chemotherapies. Hence, it was proposed that inhibition of SphK accomplished two effects simultaneously; decreasing pro-mitogenic/pro-survival S1P signaling and increasing levels of pro-apoptotic sphingolipids (Sph/Cer), providing a strong rationale for the development of Sphingosine Kinase Inhibitors (SKIs) as anti-cancer therapeutic agents.

In 2002, we were the first group to identify four classes of potent, non-lipid based, “drug-like” small molecule inhibitors of SphK (SKI-I-IV).¹ One of these compounds, SKI-II has been used extensively in the literature and was recently co-crystalized with SphK1.¹³ Like SKI-II, other “first-generation” SKIs, such as, N,N-dimethyl sphingosine (DMS), SK1-I, ABC294640, *etc.*, induced apoptosis, reduced tumor volume in numerous animal studies and provided encouraging results that SKIs would be effective therapeutic agents for multiple cancer types.^{1,14–17} Based upon these early successes, several groups have separately developed “second-generation”, isoform selective, nanomolar potency SphK inhibitors (based on *in vitro* studies) that, unlike the “first-generation” SKIs, were unable to induce apoptosis of an array of cancer cell lines, although they too inhibited S1P production and induced intracellular Cer accumulation.^{18–23} The discouraging results with these “second-generation” inhibitors have called into question whether the SphKs are therapeutic targets for cancer.¹⁹

Concurrent with the development of these “second-generation” inhibitors, we initiated studies to optimize our SKI-I chemotype for further development as an anti-cancer therapeutic strategy due to the fact that SKI-I was the most potently cytotoxic of the four originally identified chemotypes. Through a series of Structure-Activity Relationship (SAR) studies, we identified a refined SKI-I analog (SKI-178) that competes for the Sph binding site in SphK1. SKI-178 effectively reduces S1P formation while inducing Cer accumulation and, in contrast to other “second-generation” SKIs, is potently cytotoxic against a broad range of cancer cell lines.²⁴ Further investigation of the apoptotic mechanism-of-action of

SKI-178 demonstrated that apoptotic cell death is the direct result of prolonged cyclin-dependent kinase 1 (CDK1) activation during mitotic arrest.²⁵ This prolonged activation of CDK1 leads to the sustained phosphorylation and inhibition of various anti-apoptotic Bcl-2 family members including Bcl-2, Bcl-xl, and Mcl-1.²⁵ While there is some evidence in the literature to suggest a link between SphK1 inhibition and mitotic arrest,^{26,27} the apparent discrepancy between the actions of SKI-178 and the other non-cytotoxic SphK1 inhibitors has led us to suspect that SKI-178 has multiple cellular targets that account for the ability of SKI-178 to induce apoptosis.^{26,27} Thus, the goal of these studies is to clarify the target selectivity of SKI-178 and to evaluate the therapeutic potential of SKI-178 as a multi-targeted agent for the treatment of solid and hematological cancers.

METHODS

Animal Studies

All animal studies reported in this manuscript were conducted ethically under the guidance and approval of the Penn State University Park and Penn State Hershey IACUC committees.

Reagents

SKI-178 (N'-[(1E)-1-(3,4-dimethoxyphenyl)ethylidene]-3-(4-methoxyphenyl)-1H-pyrazole-5-carbohydrazide) was synthesized as described previously.²⁴ Reagents were purchased as follows: vincristine (Thermo Fisher Scientific, Waltham, MA, USA), Colchicine (Enzo Life Sciences, Farmingdale, NY, USA), Paclitaxel (Sigma-Aldrich, St. Louis, MO, USA), MG132 (Sigma-Aldrich, St. Louis, MO, USA) and PF-543 (Selleck Chemicals, Houston, TX, USA), antibodies against SphK1, pBcl-2(Ser70), Cleaved caspase-7, and Tubulin (Cell Signaling Technology, Danvers, MA, USA), SphK1 (pSer225) (ECM Biosciences, Versailles, KY), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β -hydroxy-propyl-cyclodextrin was from Acros (NJ, USA). 1,2-Propanediol, Tween-20, and dextrose were from Sigma Aldrich (MO, USA).

Cell lines, constructs, and culture conditions

The human acute myeloid leukemia (AML) derived cell lines, HL-60 (CCL-240), was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in IMDM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Denville Scientific, South Plainfield, NJ, USA). Molm-13 (ACC 554) cells were obtained from DSMZ (Braunschweig, Germany) and cultured in IMDM (Hyclone, GE Healthcare, UT, USA) supplemented with 10% FBS (Denville Scientific, South Plainfield, NJ, USA). His_{6X}-SphK1 overexpressed in HEK293 cells were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS.²⁸ HEK293 cells over-expressing His_{6X}-SphK2 were generated and cultured as described.²⁸ All cell lines were maintained at 37°C with 5% CO₂ in a humidified incubator.

Whole cell analysis of tubulin polymerization

Microtubules were fixed and stained as previously described.²⁹ Cells were treated in 24 well plates with vincristine, paclitaxel, SKI-178, or PF-543 for 18 h. Cells were pelleted by centrifugation at 600 × g for 5min. Cell pellets were resuspended and fixed with 1mL 0.5%

glutaraldehyde in microtubule stabilizing buffer (80 mM PIPES (pH 6.8) 1 mM MgCl₂, 5 mM EDTA, and 0.5% Triton X-100) for 10 min at room temperature. Glutaraldehyde was quenched with 0.7 mL of 1 mg/mL NaBH₄ in 1× PBS and cells were subsequently pelleted at 1,000 × g for 7 min. Cell pellet was re-suspended in 20 µL of 50 µg/mL RNase A in antibody dilution solution (1× PBS [pH 7.4], 0.2% Triton X-100, 2% bovine serum albumin [BSA], 0.1% NaN₃) and incubated at 4°C overnight. The next morning, 5 µL of a 1:50 dilution of anti-α-tubulin-FITC antibody in antibody dilution solution was added to the 20 µL suspension to achieve a final antibody dilution of 1:250 and incubated in for 3h protected from light. Samples were further diluted in 200 µL of 50 µg/mL Propidium Iodide in 1× PBS. Cells were analyzed by flow cytometry on a BD FACSCalibur (BD Biosciences, San Jose, CA, USA) and results were analyzed by ModFit LT (Verity Software House, Topsham, ME, USA).

***In vitro* tubulin polymerization assay**

Inhibition of tubulin polymerization was measured using the Cytoskeleton HTS-Tubulin Polymerization Assay Kit (Cytoskeleton, Inc., Denver, CO, USA) as per manufacturer instructions. The assay contained 100 µL of 4 mg/mL tubulin in G-PEM buffer (80 mM PIPES, pH 6.9, 0.5 mM EGTA, 2 mM MgCl₂, and 1 mM GTP). Totally, 10 µL of 10X PF-543, vincristine, and SKI-178 were pre-warmed to 37 °C in a half area 96-well plate (10 µL DMSO was used as control). The polymerization of purified tubulin into microtubules was carried out at 37 °C, and light scattering was recorded at 340 nm every minute for 60 min.

Generating U251-MG cells with GFP-tagged tubulin

U251-MG human glioblastoma cell lines were provided by Dr. James Connor (Penn State College of Medicine, Hershey, PA, USA) and were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Denville Scientific, South Plainfield, NJ, USA). U251-MG cells were transfected with pAcGFP1-αTubulin (Clontech; provided by Dr. Christopher Yengo [Penn State College of Medicine, Hershey, PA, USA]) and selected in 500 µg/mL G-418 select for resistant cells.

Western blot analysis

Briefly, whole cell lysates were harvested in 100 µL 1× RIPA buffer (20 mM Tris-HCL [pH 7.5], 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 5 mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1 mM sodium orthovanadate, 30 mM sodium fluoride, and complete protease inhibitor cocktail tablet [Roche Diagnostics, Mannheim, Germany]). Lysates were centrifuged (20,000 ×g) for 15 min at 4°C to remove cell debris. Total protein concentrations were quantified using the BCA assay from Pierce (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of denatured total protein were resolved by NuPAGE 4–12% Bis-Tris gel electrophoresis (Life technologies Carlsbad, CA, USA) and transferred to PVDF membranes (Life technologies Carlsbad, CA, USA). Membranes were blocked for 1 h at room temperature in 5% milk/TBS-T, incubated overnight at 4°C with primary antibodies (1:1000), and immunodetection was done with corresponding secondary IgG HRP-linked antibodies (1:5000) using the ECL chemiluminescence reagents (Thermo Fisher Scientific, Waltham, MA, USA).

Analysis of apoptosis

HL-60 cells were treated with PF-543, vincristine, colchicine, or combinations thereof, for 48h. Stages of apoptosis were assayed using the MUSE™ Annexin V & Dead Cell kit combined with laser-based fluorescence detection using a MUSE™ cell analyzer (EMD Millipore, Billerica, MA, USA) according to manufacturer recommendations.

Cellular thermal shift assay (CETSA)

Direct target engagement of SphK1, SphK2 and β -Tubulin were performed as described in with some minor modification.³⁰ Briefly, nearly confluent 10 cm culture dishes of HEK293 cells, overexpressing either His_{6X}-SphK1 or His_{6X}-SphK2, were treated with either vehicle control or SKI-178 for 16h. Cells were collected by trypsinization, neutralized by the addition of DMEM containing 10% FBS, pelleted at $300 \times g$ for 5 min, and washed with $1 \times$ PBS. After washing, cells were pelleted again at $300 \times g$ for 5 min and resuspended in 1 mL of 20 mM Tris pH 7.4 containing a complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Totally, 100 μ L aliquots of the cell suspension were heated to designated temperatures for 3 min. After cooling for 3 min at room temperature, cells were freeze-thawed three times with liquid nitrogen, and the soluble fraction (lysate) was separated from cell debris as well as the precipitated and aggregated proteins by centrifugation at $20,000 \times g$ for 20 min at 4°C. The supernatant was transferred to a new tube and quantification of the remaining soluble protein was achieved by Western blot analysis.

MLL-AF9 retro-viral transduction model of AML

Viral stocks were generated in HEK293T cells as described previously.³¹ In the MLL-AF9 model, BM cells from B6.SJL*Ptprca²Pep3b*/BoyJ (CD45.1⁺) donor mice pretreated with 5-fluorouracil (150 mg/kg; Sigma-Aldrich) were harvested and infected with mouse stem cell virus-green fluorescent protein (MSCV-GFP) control retrovirus or MSCV-IRES-GFP-retrovirus (MIGR)-MLL-AF9retrovirus overnight in IMDM containing 5% FBS and supplemented with 2.5 ng/mL of IL-3 and 15 ng/mL of SCF (R&D Systems).³² Transduced cells (0.5×10^6) were transplanted by retro-orbital injection into C57BL/6 (CD45.2⁺) recipient mice that were preconditioned with 950 rads of irradiation. Six to eight weeks after transplantation, engraftment was confirmed by measurement of WBC counts in peripheral blood (WBCs $10^4/\mu$ L were indicative of AML). SKI-178 dissolved in 45% β -HPCD or vehicle control was administered by retro-orbital injection under isoflurane anesthesia every-other-day for the time periods indicated in the figure legends. For secondary transplantation experiments, mice treated with SKI-178 for 10 weeks were euthanized; spleen cells were isolated and transplanted into irradiated secondary C57BL/6 (CD45.2⁺) recipients. Six weeks after secondary transplantation, mice were analyzed for WBC counts.

MOLM-13 xenotransplantation leukemia model in NOD.Cg-*Prkdc^{scid}Il2rg^{tm1}Wjl/SzJ* (NSG) mice

The luciferase expressing MOLM-13 cell line (MOLM-13/Luc2) was generated by retroviral transduction of the MSCV-Luc2-IRES-YFP vector (kindly provided by Dr. Gerard Grosveld

at St. Jude Children's Research Hospital) followed by FACS sorting of YFP-positive cells. The MOLM-13/Luc cells were intravenously transplanted to 8-week male NSG mice at 2×10^6 cells in 100 μ L 1 $\times$ PBS per mouse. Mice were treated with SKI-178 beginning day 4 by retro-orbital injection every other day. Luciferase imaging was conducted on a Kodak IVIS FX system on the indicated days. Mice were anesthetized by intraperitoneal (i.p.) injection of ketamine/xylazine mixture (100 μ L/20 g mouse body wt.) and also i.p. injected with sterilized D-luciferin (LUCK, Gold Biotechnology) in PBS at 30 mg/mL (100 μ L/20g body wt.). *In vivo* human tumor cells population in the mouse body was measured by luciferase expression and quantified as photons/second/cm²/steradian as described previously.³³ SKI-178 dissolved in 30% v/v 1,2-Propanediol, 5% v/v Tween-20, 65% v/v of 5% dextrose in DDW (PTD) or vehicle control was administered to mice at 5 mg/kg body wt. beginning day 4 by retro-orbital injection every other day under isoflurane anesthesia.

RESULTS

SKI-178 directly target engages SphK1 and SphK2 protein in cells

Our initial characterization of the SphK inhibitory activity of SKI-178 relied on *in vitro* SphK activity assays using recombinant SphK1 and SphK2 proteins and isoform selective biochemical buffers using standard published assay conditions.³⁴ Additionally, we employed LC-MS/MS techniques to measure the depletion of sphingosine-1-phosphate (S1P)/accumulation of ceramide species. However, these techniques are fundamentally lacking in that they do not demonstrate that SKI-178 was able to bind to and directly “target engage” either SphK protein in intact cells. Demonstration of “direct target engagement” has taken on a heightened importance in recent years due to the Phase III Clinical Trial failure of Iniparib.³⁵ To confirm that SKI-178 directly binds SphK1 and SphK2, we employed the recently developed cellular thermal shift assay (CETSA) to monitor direct binding of SKI-178 to SphK1 and SphK2 in the context of intact cells.^{30,36} With this assay, the binding of a ligand, such as SKI-178, to target proteins alters their thermal stability which can be monitored by comparing the amount of protein remaining in solution after temperature-induced protein aggregates have been removed by centrifugation. The outputs from this assay allow for the comparison between temperature-induced protein aggregation curves and are used to evaluate drug target engagement in cells.

To easily and reliably demonstrate “direct target engagement” of SphK1 and SphK2, we employed HEK293 cells that stably over-expressed either NH₂-terminally His₆X-tagged SphK1 or SphK2 protein. The corresponding stable over-expression cell lines were treated with SKI-178 for 16 h, after which multiple aliquots of the cell suspension were heated over a temperature gradient that was empirically determined for both SphK1 and SphK2. Cells were subsequently lysed and centrifuged to separate soluble fractions from precipitated denatured proteins. The binding of SKI-178 to SphK1 and/or SphK2 should alter their melting curves by either stabilizing or destabilizing the proteins. The presence of SphK1 and SphK2 in the soluble fraction after thermal denaturation was examined by Western blotting. As shown in Figure 1a, relative to control, cells treated with SKI-178 show a marked increase in the accumulation of SphK1 protein in the soluble fraction at all temperatures examined, indicating the binding of SKI-178 stabilized SphK1 protein. Since SphK1 is

known to be activated by phosphorylation at Ser225 by ERK1/2 activity, we confirmed that SKI-178 is also able to bind to active pSphK1 (Ser225) at all temperatures examined.³⁷ These data provide direct evidence that in cells, SKI-178 is able to target engage SphK1 including its Ser225 phosphorylated active form. Similar results were obtained for SphK2 [Figure 1b] indicating that SKI-178 is capable of direct target engagement of SphK2. Therefore, these data indicate that SKI-178 is not SphK1 selective, as we had previously reported, but is instead a non-isoform selective SphK1/2 inhibitor.

Knowing the temperature at which SphK1 denatures and precipitates allows us to employ a variation of these studies termed isothermal dose-response fingerprint (ITDRF_{CETSA}).³⁶ With this experimental design, the stabilization or destabilization of a protein can be monitored as a function of increasing drug concentration.³⁰ This allows for determination of the IC₅₀ of SKI-178 stabilization of both SphK1 and SphK2 in cells. To this end, His_{6X}-SphK1 and His_{6X}-SphK2 cells were again treated for 16h with a 1:2 serial dilution of SKI-178 and subsequently heated to 60°C and 63°C respectively. As seen in Figure 1c, SKI-178 dose dependently binds and significantly stabilizes both SphK1 and SphK2 protein. The concentrations of SKI-178 required to bind both SphK1 and SphK2 correlate well with the published cytotoxic IC₅₀ of SKI-178 which ranges from about 400–800nM depending on the cell line.²⁵ Therefore, SKI-178 directly binds and inhibits SphK1 and SphK2 at concentrations required to induce apoptotic cell death. Together, these results further establish SKI-178 as a relevant inhibitor of SphK kinase activity in intact cells.

SKI-178 directly impairs microtubule assembly

The data presented above corroborate the SphK inhibitory potential of SKI-178, however, given the recent studies that suggest that SphK inhibition does not induce apoptosis; it would be imprudent to ignore the possibility of off-target effects leading to SKI-178 induced apoptosis. As stated above, we have previously determined that SKI-178 induced apoptosis is dependent upon sustained CDK1 activation and sustained mitotic arrest. Examination of the literature revealed that, similar to SKI-178, a group of anticancer drugs, termed microtubule-targeting agents (MTA), disrupt the dynamic equilibrium of the mitotic spindles, leading to prolonged mitotic arrest and apoptotic cell death as a result of sustained activation of CDK1.³⁸ Based on the striking similarity between the MOA of SKI-178 and various agents that target microtubules, we investigated the possibility that SKI-178, in addition to being a SphK inhibitor, also works as a microtubule targeting agent.

To visually examine the effects of SKI-178 on microtubule dynamics, U-251-MG glioblastoma cells over-expressing GFP-tagged α -tubulin were treated with either SKI-178 or PF-543, a non-cytotoxic but highly potent inhibitor of SphK1. As shown in Figure 2a, SKI-178, but not PF-543, significantly disrupts the microtubule network relative to a vehicle treated control. To further assess the effects of SKI-178 on microtubule polymerization, a previously described whole cell microtubule assay was performed to quantify the effects of SKI-178 on tubulin polymerization, in intact cells, using flow cytometry.²⁹ As would be expected, relative to a vehicle control, the MTA vincristine decreased tubulin fluorescence, while the microtubule stabilizing agent, paclitaxel, increased fluorescence. SKI-178 destabilizes microtubule polymerization similar to that of vincristine [Figure 2b], whereas,

PF-543 had no effect on microtubule polymerization. The fact that PF-543 has no effect on microtubule polymerization indicates that SphK inhibition, by SKI-178, does not contribute to its effects on microtubule disruption.

To determine whether this was a direct or indirect effect on tubulin polymerization, an *in vitro* tubulin polymerization assay was employed. As shown in Figure 2C, SKI-178 dose dependently decreased the rate of tubulin polymerization relative to vehicle treated control, while PF-543 had little to no effect. These results confirm that, in a cell free system, SKI-178 can directly bind to and inhibit tubulin polymerization similar to that of vincristine. These results, in combination with the SphK CETSA data shown in Figure 1 provide direct evidence that SKI-178 is able to target engage both SphK and tubulin proteins in cells, suggesting that SKI-178 might be a multi-targeted inhibitor capable of binding to and disrupting the functions of two relevant chemotherapeutic targets.

SphK1 inhibition sensitizes cells to the effects of microtubule disruption

The data described above suggests that SKI-178 functions as a multi-targeted inhibitor of both the SphKs and microtubule dynamics. Interestingly, numerous studies have linked sphingolipid metabolites to the actions of MTAs such as paclitaxel, colchicine, and the vinca alkaloids. Indeed, SKIs, other inhibitors of Cer metabolism and Cer itself all synergistically enhance the apoptotic efficacy of MTAs.^{39–49} More recently, it has been suggested that Cer may actually be required for paclitaxel induced mitochondrial outer membrane permeabilization (MOMP) and apoptosis.⁵⁰ Mechanistically, sphingolipids have also been shown to activate the pro-apoptotic Bcl-2 family proteins Bak and Bax.⁵¹ Other studies have shown that upon activation, Bax and Bak bind to mitochondrial Cer and organize the formation of Cer containing channels leading to MOMP and apoptosis.^{52–56} Given the established synergism between sphingolipid metabolites and MTAs, we reasoned that combined inhibition of the two major targets of SKI-178 (namely SphK and microtubules) either as a single agent (SKI-178) or using multiple agents (i.e. a SKI and a MTA, in combination) can synergistically act to induce apoptosis of cancer cells such as AML cell lines.⁵⁷

Therefore, to demonstrate that SphK inhibition could sensitize cells to microtubule disruption independently of SKI-178, we used the MTA vincristine in combination with PF-543, the most potent and selective inhibitor of SphK1 currently available. We hypothesized that SKI-178 alone exerts the same effect as these agents in combination. To determine whether SphK1 inhibition by PF-543 could sensitize cells to vincristine, HL-60 cells were treated with non-cytotoxic doses of either compound alone or in combination, and the percent of total apoptosis was quantified by Annexin V positive staining. Our results show that while there was no induction of apoptotic cell death with either 6 nM vincristine or 5 μ M PF-543, in combination we observed a statistically significant increase in the percentage of total apoptotic cells [Figure 3a and b]. As a control, we observed significant induction of apoptosis by 5 μ M SKI-178 [Figure 3c and d], consistent with our previous observations.²⁵

We previously demonstrated that CDK1 mediated phosphorylation at Ser70 and inhibition of anti-apoptotic Bcl-2 proteins underlies SKI-178 induced apoptotic cell death. Similar to

SKI-178, vincristine is also known to induce the inhibitory phosphorylation of anti-apoptotic Bcl-2 proteins which has likewise been linked to prolonged CDK1 activity.^{58–60} Therefore it is possible that SphK1 inhibition with PF-543 could act cooperatively with vincristine to enhance these effects. In Figure 3e, we show that in HL-60 cells, treatment with a non-cytotoxic dose of vincristine (3 nM) induces Bcl-2 phosphorylation. However, when combined with 6.25 μ M PF-543, which on its own also has minimal effect, levels of Bcl-2 phosphorylation are dramatically enhanced. While caspase 7-cleavage was not examined, it's clear from the results presented in Figures 3A and 3B that the combination of vincristine and PF-543 significantly induces apoptotic cell death.

To confirm that these effects were not specific to vincristine, we employed combretastatin A4, another MTA that induces apoptotic cell death through disruption of microtubule polymerization like vincristine.⁶¹ We again treated HL-60 cells with non-cytotoxic doses of either combretastatin A4 or PF-543 alone or in combination. The percentage of total apoptotic cells was quantified by Annexin V positive staining. While 1.25 nM combretastatin A4 alone led to an approximately 2.5-fold increase in apoptotic cell death, the combination with a non-cytotoxic dose of PF-543 (5 μ M) almost doubled this effect [Figure 3f and g]. These results support the hypothesis that SphK1 inhibition can sensitize cells to the effects of various MTAs. SKI-178 exerts both of these effects as a single agent and is therefore a novel and promising chemotherapeutic approach and suggests that further development of SKI-178 may still be warranted if efficacy could be demonstrated in a mouse model of cancer.

Determination of the toxicity profile of SKI-178 in naïve mice

We recognize that any investigational drug can potentially have dose-limiting toxicity, thus, to initially evaluate the toxicity profile of SKI-178, prior to disease efficacy determination, we determined the maximum tolerated dosage (MTD) of SKI-178 in naïve Swiss-Webster mice. SKI-178 was delivered via tail-vein injection dissolved in 45% w/v β -hydroxy-propyl-cyclodextrin (β -HPCD) in 1 $\times$ PBS at a dose of 80 mg/Kg ($n = 3$). At this dose all animals displayed no signs of overt toxicity and survived 24 h. We escalated the dosage of SKI-178 to 100 mg/Kg and observed transient lethargy (15 min) after which the animals recovered and survived 24 h. Further escalation of the dosage to 200 mg/Kg resulted in severe lethargy and the animals were euthanized. Given the transient lethargy at 100 mg/Kg, we established 80 mg/Kg as an approximate MTD for SKI-178. Repeated dosage at 80 mg/Kg every day for 3 days was well tolerated with no body weight loss (data not shown). To further examine the potential toxicological and hematopoietic effects of SKI-178, we performed a repeated dose study of SKI-178 in naïve Swiss-Webster mice. SKI-178 (in 45% β -HPCD) was injected daily, via tail-vein, for 14 days at $\frac{1}{2}$ and $\frac{1}{4}$ MTD (40 mg/Kg and 20 mg/Kg respectively). As shown in Figure 4, SKI-178 was well tolerated at both doses over the 14 day injection period, with no apparent body weight loss at either dose. Complete blood counts (CBC) and serum clinical chemistry demonstrated that SKI-178 at 40 mg/Kg appears to be well tolerated [Supplementary Tables 1 and 2].

SKI-178 induces complete remission in the MLL-AF9 mouse model system

Investigators have actively pursued the development of mouse models that accurately reflect the clinical pathology of the cancers that they wish to study. Orthotopic implantation models are generally considered to be a more accurate reflection of the proper tumor microenvironment relative to subcutaneous xenograft models. Similarly, immune-competent models with intact immune systems and bearing syngeneic tumor models have a decided advantage over immuno-compromised mouse models. On the other hand, the immune-compromised models allow engraftment of human cancer cell lines. Clearly, no single mouse model can directly predict the therapeutic efficacy of a developmental compound such as SKI-178. Thus, performing proof-of-concept animal studies in multiple models of both hematological and solid tumor models may provide a more accurate estimation of the therapeutic potential of a multi-targeted agent such as SKI-178.

To this end, one model system we have chosen to employ in our assessment of SKI-178 is an immuno-competent murine AML model induced by the MLL-AF9 (MLL/MLLT3) fusion oncogene that allows growth of cells with features consistent with a Leukemic Stem Cell (LSC) phenotype. MLL-AF9 arises as a reciprocal translocation of the AF9 gene on chromosome 9p22 and the aforementioned MLL gene on chromosome 11q23.⁶² In patients, MLL-AF9-expressing AML cells are consistent with myelomonocytic differentiation and are associated with an aggressive disease and poor prognosis.⁶² Knock-in and retroviral gene transfer mouse models of MLL-AF9 closely parallel human patient AML making these models an appropriate system with which to examine new therapeutic strategies for treatment of AML.⁶² As shown in Figure 5A, we generated MLL-AF9 transduced Kit⁺Sca1⁺Lin⁻ HSCs and transplanted them into irradiated recipients to generate AML mice. Donor and recipient cells were differentiated using CD45.1 and CD45.2 alleles as indicated and GFP was used to distinguish MLL-AF9 expressing cells. Mice confirmed to have white blood cell (WBC) counts greater than 10⁴ cells/ μ L after BM transplant were considered to have developed AML.

To determine the *in vivo* efficacy of SKI-178 in the MLL-AF9 mouse model system, leukemic mice were divided into vehicle and SKI-178 treatment groups ($n = 5$ /group). SKI-178 (20 mg/Kg) was administered to mice every other day in 45% β -HPCD vehicle. As shown in Figure 5B, PB samples were obtained from vehicle treated and SKI-178 treated groups after 1 week and 3 weeks of treatment administration. Among the vehicle treated controls, WBC counts increased with time from their initial $\sim 10^4$ cells/ μ L levels to greater than 7×10^4 cells/ μ L after 3 weeks of treatment. In contrast, after 1 week of treatment with SKI-178, WBC counts decreased from their initial 10^4 cells/ μ L levels and continued to decline after 3 weeks of treatment until they reached normal levels ($\sim 4 \times 10^3$ cells/ μ L). Two vehicle treated mice died within 1 week of initiation of treatment and after 4 weeks of treatment, and the remaining 3 vehicle treated mice were found to be moribund and were sacrificed [Figure 5c]. In contrast, all 5 SKI-178 treated mice were healthy, active and continued to receive SKI-178 treatment *Qod* for 19 weeks without weight loss or any overt signs of toxicity.

SKI-178 treatment results in a dose dependent increase in survival

To confirm and extend these findings, we next determined whether SKI-178 acted in a dose dependent manner by initiating treatments at three different dosages (20, 10 and 5 mg/Kg). Again, MLL-AF9⁺ mice displaying signs of leukemia were divided into vehicle and treatment cohorts ($n = 5/\text{group}$) and retro-orbitally injected with SKI-178 at the different dosages *Qod*. After 1 week of treatment, WBC counts were determined for all treatment groups and compared to pretreatment WBC counts. As shown in Figure 6a, after 1 week of treatment WBC counts increased significantly in vehicle treated mice consistent with the previous study. Also consistent with the previous study, we observed a significant reduction in WBC counts in the 20 mg/Kg SKI-178 treatment groups with WBC counts returning to the normal range. The SKI-178 mediated decrease in WBC counts was also observed in the 10 mg/Kg treatment group. The 5 mg/Kg SKI-178 treatment group displayed essentially no change in WBC counts after 1 week of treatment.

As shown in Figure 6b, after 2 weeks of treatment all 5 vehicle treated mice succumbed to AML. Similarly, all 5 of the 5 mg/Kg SKI-178 treatment group died within 3 weeks of the initiation of treatment. In contrast, 4 of 5 of the 10 mg/Kg SKI-178 treated group and all 5 of the 20 mg/Kg SKI-178 treated group survived the entire course of the treatment period (10 weeks). After 10 weeks of treatment, the surviving 10 mg/Kg and 20 mg/Kg SKI-178 treated mice were euthanized and peripheral blood WBC counts and the presence of MLL-AF9⁺ cells in the bone marrow and spleen were analyzed by flow cytometry. Representative samples of bone marrow and spleen from both 10 mg/Kg and 20 mg/Kg SKI-178 treated mice demonstrated that CD45.2⁺ cells were not detected in either the bone marrow or spleen samples from the 20 mg/Kg SKI-178 treated mice. Similarly, few to no CD45.2⁺ WBCs were detected in the 10 mg/Kg SKI-178 treated bone marrow and spleen samples [Figure 6c]. The WBC counts returned to normal values (data not shown). Eighty percent of the mice treated with 10 mg/Kg SKI-178 survived and at 10 weeks post treatment appeared to be leukemia free. But the kinetics of recovery were different in this group compared to the 20 mg/Kg treatment group. At 5 weeks of treatment the 10 mg/Kg treatment group still exhibited MLL-AF9⁺ cells in the peripheral blood while the 20 mg/Kg treatment group had no detectable MLL-AF9 cells (data not shown). Taken together with the survival data, the absence of MLL-AF9⁺ AML cells from both the bone marrow and spleen of both the 10 mg/Kg and 20 mg/Kg SKI-178 treated mice strongly suggests that SKI-178 is capable of inducing complete remission of MLL-AF9 leukemia in this mouse model.

A major impediment to long term treatment for AML is the inability to completely eliminate leukemia stem cells, which results in relapse of disease post treatment. To ensure that LSCs capable of inducing AML did not survive SKI-178 treatment, bone marrow from both the 10 mg/Kg and 20 mg/Kg SKI-178 treated mice was transplanted into secondary recipient mice. These mice were monitored for development of disease for 6 weeks in the absence of treatment. None of these recipient mice developed disease and WBC counts at 6 weeks post-transplant for mice that received bone marrow from either the 10 mg/Kg or 20 mg/Kg SKI-178 treated donors were within the normal range indicating that, indeed, SKI-178 induced complete remission of MLL-AF9 leukemia [Figure 6d].

SKI-178 effects on NSG mice engrafted with the human cell line MOLM-13 (MLL-AF9⁺)

The murine MLL-AF9 model of AML recapitulates many aspects of human AML, but human AML is a heterogeneous disease and cells often exhibit complex cytogenetics. Leukemia cells with multiple mutations often develop aggressive phenotypes. While the data presented above demonstrate the effectiveness of SKI-178 *in vivo*, the ability of SKI-178 to reduce the AML burden in mice bearing human AML cells has not been established. To this end, we utilized a second AML model system by engrafting the human AML cell line, MOLM-13, into immunocompromised NOD.Cg-Prkdc^{scid}Il2rg^{tm1/Wjl/SzJ} (NSG) mouse strain. MOLM-13 is an adult MLL-AF9 cell line carrying a FLT3-ITD mutated allele that induces very aggressive disease in immunocompromised mice.^{63,64} The FLT3-ITD mutation induces constitutive activation of JAK/STAT and Erk1/2 signaling leading to rapid proliferation contributing to the aggressiveness of MOLM-13 cell growth *in vivo*. Activation of STAT5 dependent signaling is known to promote leukemia stem cell self-renewal. Interestingly, we have recently shown that SKI-178 inhibits JAK/STAT signaling in natural killer-large granular lymphocyte leukemia (NK-LGL-leukemia).⁶⁵ Thus, it was of interest to determine whether SKI-178 affects JAK/STAT signaling in the MOLM-13 cell line. Indeed as shown in Figure 7a, STAT5 is constitutively active (as judged by phosphorylation of Tyr694) in MOLM-13 cells. SKI-178 treatment of these cells abrogated phosphorylation/activation of Tyr694 of STAT5 as well as phosphorylation/activation of Erk1/2 (Thr202/Tyr204), suggesting that SKI-178 may have *in vivo* efficacy in a MOLM-13 xenograft model.

We utilized MOLM-13 cells stably expressing luciferase (MOLM-13/Luc2) for non-invasive monitoring of disease burden. These cells were injected into NSG mice and the development of AML was monitored by *in vivo* imaging. As expected, SKI-178 treatment significantly reduced leukemia burden as determined by luciferase activity [Figure 7b and c] and extended survival [Figure 7d] as compared to vehicle treated controls.

DISCUSSION

We originally developed SKI-178 as a novel, SphK1- selective inhibitor and demonstrated that it was cytotoxic to a broad range of cancer cell lines including multi-drug resistant types.²⁴ Herein, we have employed the state-of-the-art CETSA technique to re-examine the isoform selectivity of SKI-178 in intact cells. This re-analysis has revealed that SKI-178 directly engages both SphK1 and SphK2 in cells, at concentrations consistent with the cytotoxic IC₅₀ of SKI-178, in multiple cancer cell lines.

The cytotoxicity of SKI-178 is at odds with a recent report claiming that inhibition of either SphK1 or SphK2 alone is not sufficient to induce apoptotic cell death.¹⁹ Thus, we considered the possibility that SKI-178 may have additional targets that contribute to its cytotoxic effects. A detailed examination of the apoptotic mechanism-of-action of SKI-178 revealed that, SKI-178 induced apoptotic cell death resulted from sustained cyclin-dependent kinase 1(CDK1) activation during prolonged mitotic arrest.²⁵ While SphK1 and SphK2 have been associated with mitotic arrest, we noted a striking similarity between the MOA of SKI-178 and various microtubule targeting agents. Thus, we examined the possibility that SKI-178, besides being a SphK inhibitor, also affects microtubule dynamics.

To this end, we have presented evidence that SKI-178 does indeed function as anMTA, preventing the polymerization of the microtubule network both *in vitro* and in intact cells. Interestingly, we separately demonstrate that simultaneous inhibition of the SphKs and disruption of the microtubule network results in synergistic induction of apoptosis suggesting that the two activities of SKI-178 interact in a synergistic manner. We further demonstrate that SKI-178 is well tolerated in normal healthy mice, with little to no overt toxicity. Most importantly, we have also demonstrated that SKI-178 has therapeutic efficacy in several mouse models of AML. Together, these results established SKI-178 as a multi-targeted inhibitor with promising chemotherapeutic potential.

Another interesting observation was the SKI SKI-178 induced attenuation of STAT5 signaling in the FLT3-ITD AML cell line MOLM-13. FLT3 has been shown to induce production of IL-6 via activation of NF- κ B signaling in AML cells.⁶⁶ Recently, an autocrine feed forward loop between IL-6, SphK1/S1P and STAT3 has been proposed in colorectal cancer.⁶⁷ The effects of SKI-178 on STAT3/5 signaling suggest that SphK1 may play a similar role in amplification of FLT3 induced STAT signaling in FLT3-ITD AMLs. This intriguing observation suggests that the ability of SKI-178 to inhibit JAK/STAT signaling would allow for the potential inclusion of SKI-178 in targeted therapeutic regimens for FLT3-ITD AMLs. Furthermore, inhibition of SphK1 activity using SKI-178 could represent an alternative strategy to block FLT3 signaling, especially in patients that have developed resistance to FLT3 inhibitors.

Personalized medicine and rational drug design have offered the hope of so-called “magic bullet” therapies that are highly potent, and highly selective/specific for single molecular targets. In cancer therapy, this approach was based primarily on the concept that driver mutations of proto-oncogenic proteins were directly linked to the development of cancer and that pharmacological inhibition of the deregulated target would, at a minimum, restore normal function or, preferentially, induce apoptosis. Unfortunately, despite a few modest success stories (i.e. Imatinib), acquired drug resistance, the presence of multiple molecular alterations and the intrinsic clonal heterogeneity of primary tumors have all conspired to dampen the enthusiasm for “magic bullet” therapies for cancer.

As kinome profiling and direct target engagement assays have matured, it has become clear that the vast majority of “targeted therapies” modulate the activities of multiple cellular targets. However, it was soon realized that these multi-targeted or “poly-pharmacological” agents had distinct advantages that made them attractive therapeutic strategies for anti-cancer therapy.

While there are numerous multi-targeted agents in the literature, we believe that SKI-178 is unique and we have directly demonstrated that the two separate functional activities of SKI-178 (i.e. SphK Inhibition and Microtubule Disruption) synergize to induce apoptotic cell death in cancer cells. With regard to the further development of SKI-178, there are several paths forward in its development. First and foremost, SKI-178 represents a novel chemotype of MTA and could be further developed as such. However, the fact that SphK inhibition synergizes with disruption of the microtubule network indicates that there is a strong rationale for maintenance the multi-targeted nature of SKI-178. Combined with our

data demonstrating the therapeutic efficacy of SKI-178 in multiple mouse models of cancer, we believe that SKI-178 represents a unique class of multi-targeted therapeutic agents that can and should be further optimized for therapeutic efficacy, potency, deliverability and stability rather than for increased target selectivity or specificity.

A separate issue concerns the future of selective SphK inhibitor development. Based upon the recent studies suggesting that targeting SphK1 and/or SphK2 alone is insufficient to affect cell viability, further efforts to discover and develop selective SphK1 and/or SphK2 inhibitors may not yield therapeutically active anti-cancer agents outright.¹⁹ However, it is possible to envision other avenues for continued SKI development. One such avenue would be as adjuvants to currently approved chemotherapies, especially in patients with high SphK expression in their tumors (i.e. development of the SphKs as biomarkers). Indeed, there are numerous examples in the literature of SKIs interacting synergistically with both targeted and cytotoxic chemotherapies.^{39,68–70} A highly selective non-cytotoxic SphK inhibitor, such as PF-543, may be an ideal adjuvant to current chemotherapeutic regimens. In such an instance, inhibition of S1P production and/or accumulation of Cer could enhance induction of apoptosis of the standard-of-care regimens. Another avenue of use for the currently available SKIs (such as PF-543) would be as chemical probes for study of SphK1 and SphK2 biological functions.⁷¹ Lastly, identification of non-cancerous diseases, where SphK inhibition, in the absence of induction of cellular cytotoxicity, would be preferable (such as sickle-cell anemia) could support further development of more selective SphK inhibitors.⁷²

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (P01 CA171983), The Jake Gittlen Memorial Golf Tournament, and The Penn State Hershey Cancer Research Center. This project was also funded, in part, under a grant with the Pennsylvania Department of Health using Tobacco CURE Funds.

References

1. French KJ, Schrecengost RS, Lee BD, Zhuang Y, Smith SN, Eberly JL, Yun JK, Smith CD. Discovery and evaluation of inhibitors of human sphingosine kinase. *Cancer Res.* 2003; 63(18): 5962–9. [PubMed: 14522923]
2. Johnson KR, Johnson KY, Crellin HG, Ogretmen B, Boylan AM, Harley RA, Obeid LM. Immunohistochemical Distribution of Sphingosine Kinase 1 in Normal and Tumor Lung Tissue. *J Histochem Cytochem.* 2005; 53(9):1159–66. [PubMed: 15923363]
3. Bektas M, Jolly PS, Muller C, Eberle J, Spiegel S, Geilen CC. Sphingosine kinase activity counteracts ceramide-mediated cell death in human melanoma cells: role of Bcl-2 expression. *Oncogene.* 2005; 24(1):178–87. [PubMed: 15637591]
4. Kawamori T, Osta W, Johnson KR, Pettus BJ, Bielawski J, Tanaka T, Wargovich MJ, Reddy BS, Hannun YA, Obeid LM, Zhou D. Sphingosine kinase 1 is up-regulated in colon carcinogenesis. *FASEB J.* 2006; 20(2):386–8. [PubMed: 16319132]
5. Sobue S, Iwasaki T, Sugisaki C, Nagata K, Kikuchi R, Murakami M, Takagi A, Kojima T, Banno Y, Akao Y, Nozawa Y, Kannagi R, Suzuki M, Abe A, Naoe T, Murate T. Quantitative RT-PCR analysis of sphingolipid metabolic enzymes in acute leukemia and myelodysplastic syndromes. *Leukemia.* 2006; 20(11):2042–6. [PubMed: 16990773]

6. Van Brocklyn JR, Jackson CA, Pearl DK, Kotur MS, Snyder PJ, Prior TW. Sphingosine kinase-1 expression correlates with poor survival of patients with glioblastoma multiforme: roles of sphingosine kinase isoforms in growth of glioblastoma cell lines. *J Neuropathol Exp Neurol*. 2005; 64(8):695–705. [PubMed: 16106218]
7. Le Scolan E, Pchejetski D, Banno Y, Denis N, Mayeux P, Vainchenker W, Levade T, Moreau-Gachelin F. Overexpression of sphingosine kinase 1 is an oncogenic event in erythroleukemic progression. *Blood*. 2005; 106(5):1808–16. [PubMed: 15890687]
8. Zhang Y, Wang Y, Wan Z, Liu S, Cao Y, Zeng Z. Sphingosine kinase 1 and cancer: a systematic review and meta-analysis. *PLoS One*. 2014; 9(2):e90362. [PubMed: 24587339]
9. Xia P, Gamble JR, Wang L, Pitson SM, Moretti PA, Wattenberg BW, D'Andrea RJ, Vadas MA. An oncogenic role of sphingosine kinase. *Curr Biol*. 2000; 10(23):1527–30. [PubMed: 11114522]
10. Wallington-Beddoe CT, Bradstock KF, Bendall LJ. Oncogenic properties of sphingosine kinases in haematological malignancies. *Br J Haematol*. 2013; 161(5):623–38. [PubMed: 23521541]
11. Neubauer HA, Pham DH, Zebol JR, Moretti PA, Peterson AL, Leclercq TM, Chan H, Powell JA, Pitman MR, Samuel MS, Bonder CS, Creek DJ, Gliddon BL, Pitson SM. An oncogenic role for sphingosine kinase 2. *Oncotarget*. 2016; 7(40):64886–99. [PubMed: 27588496]
12. Vadas M, Xia P, McCaughan G, Gamble J. The role of sphingosine kinase 1 in cancer: oncogene or non-oncogene addiction? *Biochim Biophys Acta*. 2008; 1781(9):442–7. [PubMed: 18638570]
13. Wang Z, Min X, Xiao SH, Johnstone S, Romanow W, Meininger D, Xu H, Liu J, Dai J, An S, Thibault S, Walker N. Molecular basis of sphingosine kinase 1 substrate recognition and catalysis. *Structure*. 2013; 21(5):798–809. [PubMed: 23602659]
14. Plano D, Amin S, Sharma AK. Importance of sphingosine kinase (SphK) as a target in developing cancer therapeutics and recent developments in the synthesis of novel SphK inhibitors. *J Med Chem*. 2014; 57(13):5509–24. [PubMed: 24471412]
15. Edsall LC, Van Brocklyn JR, Cu villier O, Kleuser B, Spiegel S. N,N-Dimethylsphingosine is a potent competitive inhibitor of sphingosine kinase but not of protein kinase C: modulation of cellular levels of sphingosine 1-phosphate and ceramide. *Biochemistry*. 1998; 37(37):12892–8. [PubMed: 9737868]
16. Paugh SW, Paugh BS, Rahmani M, Kapitonov D, Almenara JA, Kordula T, Milstien S, Adams JK, Zipkin RE, Grant S, Spiegel S. A selective sphingosine kinase 1 inhibitor integrates multiple molecular therapeutic targets in human leukemia. *Blood*. 2008; 112(4):1382–91. [PubMed: 18511810]
17. French KJ, Zhuang Y, Maines LW, Gao P, Wang W, Beljanski V, Upson JJ, Green CL, Keller SN, Smith CD. Pharmacology and antitumor activity of ABC294640, a selective inhibitor of sphingosine kinase-2. *J Pharmacol Exp Ther*. 2010; 333(1):129–39. [PubMed: 20061445]
18. Kennedy AJ, Mathews TP, Kharel Y, Field SD, Moyer ML, East JE, Houck JD, Lynch KR, Macdonald TL. Development of amidine-based sphingosine kinase 1 nanomolar inhibitors and reduction of sphingosine 1-phosphate in human leukemia cells. *J Med Chem*. 2011; 54(10):3524–48. [PubMed: 21495716]
19. Rex K, Jeffries S, Brown ML, Carlson T, Coxon A, Fajardo F, Frank B, Gustin D, Kamb A, Kassner PD, Li S, Li Y, Morgenstern K, Plant M, Quon K, Ruefli-Brasse A, Schmidt J, Swearingen E, Walker N, Wang Z, Watson JE, Wickramasinghe D, Wong M, Xu G, Wesche H. Sphingosine kinase activity is not required for tumor cell viability. *PLoS One*. 2013; 8(7):e68328. [PubMed: 23861887]
20. Schnute ME, McReynolds MD, Kasten T, Yates M, Jerome G, Rains JW, Hall T, Chrencik J, Kraus M, Cronin CN, Saabye M, Highkin MK, Broadus R, Ogawa S, Cukyne K, Zawadzke LE, Peterkin V, Iyanar K, Scholten JA, Wendling J, Fujiwara H, Nemirovskiy O, Wittwer AJ, Nagiec MM. Modulation of cellular S1P levels with a novel, potent and specific inhibitor of sphingosine kinase-1. *Biochem J*. 2012; 444(1):79–88. [PubMed: 22397330]
21. Gustin DJ, Li Y, Brown ML, Min X, Schmitt MJ, Wanska M, Wang X, Connors R, Johnstone S, Cardozo M, Cheng AC, Jeffries S, Franks B, Li S, Shen S, Wong M, Wesche H, Xu G, Carlson TJ, Plant M, Morgenstern K, Rex K, Schmitt J, Coxon A, Walker N, Kayser F, Wang Z. Structure guided design of a series of sphingosine kinase (SphK) inhibitors. *Bioorg Med Chem Lett*. 2013; 23(16):4608–16. [PubMed: 23845219]

22. Xiang Y, Asmussen G, Booker M, Hirth B, Kane JL Jr, Liao J, Noson KD, Yee C. Discovery of novel sphingosine kinase 1 inhibitors. *Bioorg Med Chem Lett*. 2009; 19(21):6119–21. [PubMed: 19800228]
23. Xiang Y, Hirth B, Kane JL Jr, Liao J, Noson KD, Yee C, Asmussen G, Fitzgerald M, Klaus C, Booker M. Discovery of novel sphingosine kinase-1 inhibitors. Part 2. *Bioorg Med Chem Lett*. 2010; 20(15):4550–4. [PubMed: 20598880]
24. Hengst JA, Wang X, Sk UH, Sharma AK, Amin S, Yun JK. Development of a sphingosine kinase 1 specific small-molecule inhibitor. *Bioorg Med Chem Lett*. 2010; 20(24):7498–502. [PubMed: 21050755]
25. Dick TE, Hengst JA, Fox TE, Colledge AL, Kale VP, Sung SS, Sharma A, Amin S, Loughran TP Jr, Kester M, Wang HG, Yun JK. The Apoptotic Mechanism of Action of the Sphingosine Kinase 1 Selective Inhibitor SKI-178 in Human Acute Myeloid Leukemia Cell Lines. *J Pharmacol Exp Ther*. 2015; 352(3):494–508. [PubMed: 25563902]
26. Gillies L, Lee SC, Long JS, Ktistakis N, Pyne NJ, Pyne S. The sphingosine 1-phosphate receptor 5 and sphingosine kinases 1 and 2 are localised in centrosomes: possible role in regulating cell division. *Cell Signal*. 2009; 21(5):675–84. [PubMed: 19211033]
27. Kotelevets N, Fabbro D, Huwiler A, Zangemeister-Wittke U. Targeting sphingosine kinase 1 in carcinoma cells decreases proliferation and survival by compromising PKC activity and cytokinesis. *PLoS One*. 2012; 7(6):e39209. [PubMed: 22761740]
28. Hengst JA, Guilford JM, Fox TE, Wang X, Conroy EJ, Yun JK. Sphingosine kinase 1 localized to the plasma membrane lipid raft microdomain overcomes serum deprivation induced growth inhibition. *Arch Biochem Biophys*. 2009; 492(1–2):62–73. [PubMed: 19782042]
29. Morrison KC, Hergenrother PJ. Whole cell microtubule analysis by flow cytometry. *Anal Biochem*. 2012; 420(1):26–32. [PubMed: 21893022]
30. Jafari R, Almqvist H, Axelsson H, Ignatushchenko M, Lundback T, Nordlund P, Martinez Molina D. The cellular thermal shift assay for evaluating drug target interactions in cells. *Nat Protoc*. 2014; 9(9):2100–22. [PubMed: 25101824]
31. Finkelstein LD, Ney PA, Liu QP, Paulson RF, Correll PH. Sf-Stk kinase activity and the Grb2 binding site are required for Epo-independent growth of primary erythroblasts infected with Friend virus. *Oncogene*. 2002; 21(22):3562–70. [PubMed: 12032858]
32. Thiel AT, Blessington P, Zou T, Feather D, Wu X, Yan J, Zhang H, Liu Z, Ernst P, Koretzky GA, Hua X. MLL-AF9-induced leukemogenesis requires coexpression of the wild-type Mll allele. *Cancer Cell*. 2010; 17(2):148–59. [PubMed: 20159607]
33. Evans MS, Chaurette JP, Adams ST Jr, Reddy GR, Paley MA, Aronin N, Prescher JA, Miller SC. A synthetic luciferin improves bioluminescence imaging in live mice. *Nat Methods*. 2014; 11(4):393–5. [PubMed: 24509630]
34. Liu H, Sugiura M, Nava VE, Edsall LC, Kono K, Poulton S, Milstien S, Kohama T, Spiegel S. Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J Biol Chem*. 2000; 275(26):19513–20. [PubMed: 10751414]
35. Patel AG, De Lorenzo SB, Flatten KS, Poirier GG, Kaufmann SH. Failure of iniparib to inhibit poly(ADP-Ribose) polymerase in vitro. *Clin Cancer Res*. 2012; 18(6):1655–62. [PubMed: 22291137]
36. Martinez Molina D, Jafari R, Ignatushchenko M, Seki T, Larsson EA, Dan C, Sreekumar L, Cao Y, Nordlund P. Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. *Science*. 2013; 341(6141):84–7. [PubMed: 23828940]
37. Pitson SM, Moretti PA, Zebol JR, Lynn HE, Xia P, Vadas MA, Wattenberg BW. Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. *EMBO J*. 2003; 22(20):5491–500. [PubMed: 14532121]
38. Sakurikar N, Eichhorn JM, Alford SE, Chambers TC. Identification of a mitotic death signature in cancer cell lines. *Cancer Lett*. 2014; 343(2):232–8. [PubMed: 24099917]
39. Noack J, Choi J, Richter K, Kopp-Schneider A, Regnier-Vigouroux A. A sphingosine kinase inhibitor combined with temozolomide induces glioblastoma cell death through accumulation of dihydrosphingosine and dihydroceramide, endoplasmic reticulum stress and autophagy. *Cell Death Dis*. 2014; 5:e1425. [PubMed: 25255218]

40. Dijkhuis AJ, Klappe K, Jacobs S, Kroesen BJ, Kamps W, Sietsma H, Kok JW. PDMP sensitizes neuroblastoma to paclitaxel by inducing aberrant cell cycle progression leading to hyperploidy. *Mol Cancer Ther.* 2006; 5(3):593–601. [PubMed: 16546973]
41. Qiu L, Zhou C, Sun Y, Di W, Scheffler E, Healey S, Wanebo H, Kouttab N, Chu W, Wan Y. Paclitaxel and ceramide synergistically induce cell death with transient activation of EGFR and ERK pathway in pancreatic cancer cells. *Oncol Rep.* 2006; 16(4):907–13. [PubMed: 16969513]
42. Sietsma H, Veldman RJ, Kolk D, Ausema B, Nijhof W, Kamps W, Vellenga E, Kok JW. 1-phenyl-2-decanoylamino-3-morpholino-1-propanol chemosensitizes neuroblastoma cells for taxol and vincristine. *Clin Cancer Res.* 2000; 6(3):942–8. [PubMed: 10741719]
43. Myrick D, Blackinton D, Klostergaard J, Kouttab N, Maizel A, Wanebo H, Mehta S. Paclitaxel-induced apoptosis in Jurkat, a leukemic T cell line, is enhanced by ceramide. *Leuk Res.* 1999; 23(6):569–78. [PubMed: 10374850]
44. Best C, Calianese D, Szulak K, Cammarata G, Brum G, Carbone T, Still E, Higgins K, Ji F, Di W, Wanebo H, Wan Y. Paclitaxel disrupts polarized entry of membrane-permeable C6 ceramide into ovarian cancer cells resulting in synchronous induction of cell death. *Oncol Lett.* 2013; 5(6):1854–8. [PubMed: 23833655]
45. Lee AJ, Roylance R, Sander J, Gorman P, Endesfelder D, Kschischo M, Jones NP, East P, Nicke B, Spassieva S, Obeid LM, Birkbak NJ, Szallasi Z, McKnight NC, Rowan AJ, Speirs V, Hanby AM, Downward J, Tooze SA, Swanton C. CERT depletion predicts chemotherapy benefit and mediates cytotoxic and polyploid-specific cancer cell death through autophagy induction. *J Pathol.* 2012; 226(3):482–94. [PubMed: 21953249]
46. Chen JY, Hwang CC, Chen WY, Lee JC, Fu TF, Fang K, Chu YC, Huang YL, Lin JC, Tsai WH, Chang HW, Chen BH, Chiu CC. Additive effects of C(2)-ceramide on paclitaxel-induced premature senescence of human lung cancer cells. *Life Sci.* 2010; 87(11–12):350–7. [PubMed: 20624405]
47. van Vlerken LE, Duan Z, Little SR, Seiden MV, Amiji MM. Augmentation of therapeutic efficacy in drug-resistant tumor models using ceramide coadministration in temporal-controlled polymer-blend nanoparticle delivery systems. *AAPS J.* 2010; 12(2):171–80. [PubMed: 20143195]
48. Desai A, Vyas T, Amiji M. Cytotoxicity and apoptosis enhancement in brain tumor cells upon coadministration of paclitaxel and ceramide in nanoemulsion formulations. *J Pharm Sci.* 2008; 97(7):2745–56. [PubMed: 17854074]
49. Mehta S, Blackinton D, Omar I, Kouttab N, Myrick D, Klostergaard J, Wanebo H. Combined cytotoxic action of paclitaxel and ceramide against the human Tu138 head and neck squamous carcinoma cell line. *Cancer Chemother Pharmacol.* 2000; 46(2):85–92. [PubMed: 10972477]
50. Swanton C, Marani M, Pardo O, Warne PH, Kelly G, Sahai E, Elustondo F, Chang J, Temple J, Ahmed AA, Brenton JD, Downward J, Nicke B. Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. *Cancer Cell.* 2007; 11(6):498–512. [PubMed: 17560332]
51. Chipuk JE, McStay GP, Bharti A, Kuwana T, Clarke CJ, Siskind LJ, Obeid LM, Green DR. Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis. *Cell.* 2012; 148(5):988–1000. [PubMed: 22385963]
52. Siskind LJ, Feinstein L, Yu T, Davis JS, Jones D, Choi J, Zuckerman JE, Tan W, Hill RB, Hardwick JM, Colombini M. Anti-apoptotic Bcl-2 Family Proteins Disassemble Ceramide Channels. *J Biol Chem.* 2008; 283(11):6622–30. [PubMed: 18171672]
53. Siskind LJ, Kolesnick RN, Colombini M. Ceramide forms channels in mitochondrial outer membranes at physiologically relevant concentrations. *Mitochondrion.* 2006; 6(3):118–25. [PubMed: 16713754]
54. Siskind LJ. Mitochondrial ceramide and the induction of apoptosis. *J Bioenerg Biomembr.* 2005; 37(3):143–53. [PubMed: 16167171]
55. Siskind LJ, Kolesnick RN, Colombini M. Ceramide channels increase the permeability of the mitochondrial outer membrane to small proteins. *J Biol Chem.* 2002; 277(30):26796–803. [PubMed: 12006562]
56. Siskind LJ, Colombini M. The lipids C2- and C16-ceramide form large stable channels. Implications for apoptosis. *J Biol Chem.* 2000; 275(49):38640–4. [PubMed: 11027675]

57. Truman JP, Garcia-Barros M, Obeid LM, Hannun YA. Evolving concepts in cancer therapy through targeting sphingolipid metabolism. *Biochim Biophys Acta*. 2014; 1841(8):1174–88. [PubMed: 24384461]
58. Fan M, Goodwin M, Vu T, Brantley-Finley C, Gaarde WA, Chambers TC. Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. *J Biol Chem*. 2000; 275(39):29980–5. [PubMed: 10913135]
59. Chu R, Terrano DT, Chambers TC. Cdk1/cyclin B plays a key role in mitotic arrest-induced apoptosis by phosphorylation of Mcl-1, promoting its degradation and freeing Bak from sequestration. *Biochem Pharmacol*. 2012; 83(2):199–206. [PubMed: 22024133]
60. Terrano DT, Upreti M, Chambers TC. Cyclin-dependent kinase 1-mediated Bcl-xL/Bcl-2 phosphorylation acts as a functional link coupling mitotic arrest and apoptosis. *Mol Cell Biol*. 2010; 30(3):640–56. [PubMed: 19917720]
61. Skoufias DA, Wilson L. Mechanism of inhibition of microtubule polymerization by colchicine: inhibitory potencies of unliganded colchicine and tubulin-colchicine complexes. *Biochemistry*. 1992; 31(3):738–46. [PubMed: 1731931]
62. Wei J, Wunderlich M, Fox C, Alvarez S, Cigudosa JC, Wilhelm JS, Zheng Y, Cancelas JA, Gu Y, Jansen M, Dimartino JF, Mulloy JC. Microenvironment determines lineage fate in a human model of MLL-AF9 leukemia. *Cancer Cell*. 2008; 13(6):483–95. [PubMed: 18538732]
63. Pan R, Hogdal LJ, Benito JM, Bucci D, Han L, Borthakur G, Cortes J, DeAngelo DJ, Debose L, Mu H, Dohner H, Gaidzik VI, Galinsky I, Golfman LS, Haferlach T, Harutyunyan KG, Hu J, Levenson JD, Marcucci G, Muschen M, Newman R, Park E, Ruvalo PP, Ruvalo V, Ryan J, Schindela S, Zweidler-McKay P, Stone RM, Kantarjian H, Andreeff M, Konopleva M, Letai AG. Selective BCL-2 inhibition by ABT-199 causes on-target cell death in acute myeloid leukemia. *Cancer Discov*. 2014; 4(3):362–75. [PubMed: 24346116]
64. Fazzina R, Lombardini L, Mezzanotte L, Roda A, Hrelia P, Pession A, Tonelli R. Generation and characterization of bioluminescent xenograft mouse models of MLL-related acute leukemias and in vivo evaluation of luciferase-targeting siRNA nanoparticles. *Int J Oncol*. 2012; 41(2):621–8. [PubMed: 22665135]
65. LeBlanc FR, Liu X, Hengst J, Fox T, Calvert V, Petricoin EF 3rd, Yun J, Feith DJ, Loughran TP Jr. Sphingosine kinase inhibitors decrease viability and induce cell death in natural killer-large granular lymphocyte leukemia. *Cancer Biol Ther*. 2015; 16(12):1830–40. [PubMed: 26252351]
66. Takahashi S, Harigae H, Ishii KK, Inomata M, Fujiwara T, Yokoyama H, Ishizawa K, Kameoka J, Licht JD, Sasaki T, Kaku M. Over-expression of Flt3 induces NF-kappaB pathway and increases the expression of IL-6. *Leuk Res*. 2005; 29(8):893–9. [PubMed: 15978940]
67. Nguyen AV, Wu YY, Lin EY. STAT3 and sphingosine-1-phosphate in inflammation-associated colorectal cancer. *World J Gastroenterol*. 2014; 20(30):10279–87. [PubMed: 25132744]
68. Ding X, Chaiteerakij R, Moser CD, Shaleh H, Boakye J, Chen G, Ndzengue A, Li Y, Zhou Y, Huang S, Sinicrope FA, Zou X, Thomas MB, Smith CD, Roberts LR. Antitumor effect of the novel sphingosine kinase 2 inhibitor ABC294640 is enhanced by inhibition of autophagy and by sorafenib in human cholangiocarcinoma cells. *Oncotarget*. 2016; 7(15):20080–92. [PubMed: 26956050]
69. Yang J, Yang C, Zhang S, Mei Z, Shi M, Sun S, Shi L, Wang Z, Wang Y, Li Z, Xie C. ABC294640, a sphingosine kinase 2 inhibitor, enhances the antitumor effects of TRAIL in non-small cell lung cancer. *Cancer Biol Ther*. 2015; 16(8):1194–204. [PubMed: 26054751]
70. Casson L, Howell L, Mathews LA, Ferrer M, Southall N, Guha R, Keller JM, Thomas C, Siskind LJ, Beverly LJ. Inhibition of ceramide metabolism sensitizes human leukemia cells to inhibition of BCL2-like proteins. *PLoS One*. 2013; 8(1):e54525. [PubMed: 23342165]
71. Arrowsmith CH, Audia JE, Austin C, Baell J, Bennett J, Blagg J, Bountra C, Brennan PE, Brown PJ, Bunnage ME, Buser-Doepner C, Campbell RM, Carter AJ, Cohen P, Copeland RA, Cravatt B, Dahlin JL, Dhanak D, Edwards AM, Frederiksen M, Frye SV, Gray N, Grimshaw CE, Hepworth D, Howe T, Huber KV, Jin J, Knapp S, Kotz JD, Kruger RG, Lowe D, Mader MM, Marsden B, Mueller-Fahrnow A, Muller S, O'Hagan RC, Overington JP, Owen DR, Rosenberg SH, Roth B, Ross R, Schapira M, Schreiber SL, Shoichet B, Sundstrom M, Superti-Furga G, Taunton J, Toledo-Sherman L, Walpole C, Walters MA, Willson TM, Workman P, Young RN, Zuercher WJ. The promise and peril of chemical probes. *Nat Chem Biol*. 2015; 11(8):536–41. [PubMed: 26196764]

72. Zhang Y, Berka V, Song A, Sun K, Wang W, Zhang W, Ning C, Li C, Zhang Q, Bogdanov M, Alexander DC, Milburn MV, Ahmed MH, Lin H, Idowu M, Zhang J, Kato GJ, Abdulmalik OY, Dowhan W, Kellems RE, Zhang P, Jin J, Safo M, Tsai AL, Juneja HS, Xia Y. Elevated sphingosine-1-phosphate promotes sickling and sickle cell disease progression. *J Clin Invest*. 2014; 124(6):2750–61. [PubMed: 24837436]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. SKI-178 Directly Engages SphK1 and SphK2 Protein in Cells

(a) CETSA was performed on His_{6X}-SphK1 cells as described in materials and methods. The stabilization effect of SKI-178 on SphK1 and active SphK1 at various temperatures was evaluated by Western blot using indicated antibodies. Actin was used as a loading control. (b) Similarly, the stabilization effect of SKI-178 on SphK2 was determined by performing CETSA on His_{6X}-SphK2 cells. (c) CETSA was used as described to generate an isothermal dose-response fingerprint demonstrating a dose dependent stabilization effect of SKI-178 on SphK1 and SphK2 at different concentrations. GAPDH was used as a loading control.

Figure 2. SKI-178 Directly Impairs Microtubule Assembly

(a) GFP-tubulin tagged U251 cell lines treated with either vehicle control, SKI-178, or PF-543. Disruption of normal microtubule dynamics was visualized with fluorescence microscopy. (b) Whole cell analysis of tubulin polymerization was performed on HL-60 cells after 18h treatment with vincristine (200 nM), paclitaxel (100 nM), SKI-178 (5 μM), or PF-543 (10 μM) for 18 h. Error bars represent standard deviation of the mean ($n = 3$). $*P < 0.05$ relative to DMSO control based on a two-tailed Student's t test. (c) Tubulin subunits were allowed to polymerize in the presence of vehicle, PF-543 (20 μM), SKI-178 (2.5 μM),

SKI-178 (5 μM), and vincristine (3 μM) for 60 min. Tubulin polymerization (i.e. lengthening of microtubules) was assayed in terms of absorbance at 340 nM. The assay indicates a decreased rate of tubulin polymerization in the presence of SKI-178 and vincristine, but not with PF-543 compared to control.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. SphK1 inhibition sensitizes cells to the effects of microtubule disruption

(a) Cytograms of HL-60 cells treated with PF-543 (5 μ M) and vincristine (6 nM) alone and in combination for 48 h. Apoptosis was defined as Annexin V-positive staining. (b) Quantification of the percentage of total apoptosis from (a). (c) Cytograms of HL-60 cells treated with PF-543 (5 μ M) or SKI-178 (5 μ M). Apoptosis was defined as Annexin V-positive staining. (d) Quantification of the percentage of total apoptosis from (c). * P < 0.05 relative to DMSO control based on a two-tailed Student's t test. (e) HL-60 cells treated for 16h with PF-543 (6.25 μ M) and vincristine (3 nM) alone and in combination. Western blot

analysis was performed on whole cell lysates using indicated antibodies. **(f)** Cytograms of HL-60 cells treated with PF-543 (5 μ M) and combretastatin A4 (1.25 nM) alone and in combination for 48h. Apoptosis was defined as Annexin V-positive staining. **(g)** Quantification of the percentage of total apoptosis from **(f)** * $P < 0.05$ relative to DMSO control based on a two-tailed Student's t test.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Body weights of Swiss-Webster mice

Body weight in grams of Swiss-Webster mice treated with SKI-178 at 20 mg/Kg and 40 mg/Kg every day for 14 consecutive days. Compared to untreated and Vehicle (45% β -HPCD) controls.

Figure 5. SKI-178 inhibits leukemic progression in the MLL-AF9 model

(a) Schematic representation of the development of the MLL-AF9 AML mouse model. (b) Leukemic mice induced by MLL-AF9 were treated with either vehicle control, untreated or SKI-178 (20 mg/Kg), three times per week. After 1 and 3 weeks of treatment, peripheral blood samples were collected and counted for total WBC. (c) SKI-178 treatment promotes survival of leukemic mice. Eight weeks after transplant of bone marrow cells infected with MLL-AF9, groups of 5 mice were treated with either vehicle control or SKI-178 (20 mg/Kg), three times per week.

Figure 6. SKI-178 induces complete remission in the MLL-AF9 model

Leukemic mice were treated with SKI-178 every other day at doses of 20, 10 and 5 mg/Kg for 10 weeks, ($n = 5$). (a) After 1 week of treatment, PB was collected and WBC counts were determined. (b) Survival curve for SKI-178. (c) Flow cytometric analysis of BM and spleen upon termination of experiment. (d) WBC counts after secondary transplant (6 weeks post transplantation).

Figure 7. Evaluation of the efficacy of SKI-178 in male MOLM-13 xenotransplanted NSG mice (a) Western blot analysis of the effects of SKI-178 on STAT5 and Erk1/2 signaling in MOLM-13 cells *in vitro*, treated with 5 μ M SKI-178 for 16 h. (b) IVIS analysis of luciferase signal in vehicle (PTD) and SKI-178 treated male mice on Days 6 and 12. (c) Quantitation of luciferase signal on days indicated. * $P < 0.05$ relative to DMSO control based on a two-tailed Student's *t* test. (d) Survival curve for SKI-178 treated mice.