The Apoptotic Mechanism of Action of the Sphingosine Kinase 1 Selective Inhibitor SKI-178 in Human Acute Myeloid Leukemia Cell Lines

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ABSTRACT

We previously developed SKI-178 (N‘-[(1E)-1-(3,4-dimethoxyphenyl)ethylidene]-3-[(4-methoxyphenyl)-1H-pyrazole-5-carbohydrazide] as a novel sphingosine kinase-1 (SphK1) selective inhibitor and, herein, sought to determine the mechanism-of-action of SKI-178-induced cell death. Using human acute myeloid leukemia (AML) cell lines as a model, we present evidence that SKI-178 induces prolonged mitosis followed by apoptotic cell death through the intrinsic apoptotic cascade. Further examination of the mechanism of action of SKI-178 implicated c-Jun NH2-terminal kinase (JNK) and cyclin-dependent protein kinase 1 (CDK1) as critical factors required for SKI-178-induced apoptosis. In cell cycle synchronized human AML cell lines, we demonstrate that entry into mitosis is required for apoptotic induction by SKI-178 and that CDK1, not JNK, is required for SKI-178–induced apoptosis. We further demonstrate that the sustained activation of CDK1 during prolonged mitosis, mediated by SKI-178, leads to the simultaneous phosphorylation of the prosurvival Bcl-2 family members, Bcl-2 and Bcl-xl, as well as the phosphorylation and subsequent degradation of Mcl-1. Moreover, multidrug resistance mediated by multidrug-resistant protein1 and/or prosurvival Bcl-2 family member overexpression did not affect the sensitivity of AML cells to SKI-178. Taken together, these findings highlight the therapeutic potential of SKI-178 targeting SphK1 as a novel therapeutic agent for the treatment of AML, including multidrug-resistant/recurrent AML subtypes.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by a multitude of genetic mutations and chromosomal abnormalities (Kumar, 2011). Although extensive efforts are being made to develop new therapeutics, the standard of care for AML has remained relatively unchanged for more than four decades, and long-term survival remains poor (Sweet and Lancet, 2014). Bioactive sphingolipids have emerged as important regulators of numerous biologic processes, including cell survival, cell proliferation, inflammation, and carcinogenesis (Adan-Gokbulut et al., 2013; Heffernan-Stroud and Obeid, 2013). Among these sphingolipids, ceramide, sphingosine, and sphingosine-1-phosphate (S1P) play key roles in determining cell fate by promoting opposing effects on cell survival and proliferation. Ceramide and sphingosine exert antiproliferative, proapoptotic effects (Hannun and Luberto, 2000; Taha et al., 2006), whereas S1P promotes proliferation, cell survival, and inhibition of apoptosis (Goetzl et al., 1999; Kwon et al., 2001; Spiegel and Milstien, 2003). Sphingosine kinase-1 (SphK1), an

ABBREVIATIONS: 7AAD, 7-amino-actinomycin; AML, acute myeloid leukemia; AS601245, (2S)-2-(benzodithiazol-2(3H)-ylidene)-2-[(2-[(3-pyridine-3-yl ethyl)(amino)propyl]dithio)acetonitrile; CDK1, cyclin-dependent kinase 1; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; JNK, c-Jun NH2-terminal kinase; KD, knockdown; LCC, long-chain ceramide; MAPK, mitogen-activated protein kinase; MDR, multidrug-resistant protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-pyrazole-4-carboxylate; PK, propidium iodide; PI9K, phosphoinositide 3-kinase; R03360, (5Z)-5-quinolin-6-ylmethylene-2-[[thiophen-2-ylmethyl]amino]thiazol-4-one; SKI-178, sphingosine-1-phosphate; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole; siRNA, small interfering RNA; SKI-178, N‘-[(1E)-1-(3,4-dimethoxyphenyl)ethylidene]-3-[(4-methoxyphenyl)-1H-pyrazole-5-carbohydrazide; SP600125, 1,9-pyrazoloanthrone; SphK1, sphingosine kinase-1; VLCC, very long-chain ceramide.

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oncogenic lipid kinase responsible for converting sphingosine to S1P, plays a fundamental role in this process as it regulates the levels of all three above-mentioned bioactive sphingolipids (Spiegel and Milstien, 2003).

Elevated expression of SphK1 is associated with numerous cancer types (Li et al., 2007; Sobue et al., 2008; Paugh et al., 2009; Knapp et al., 2010; Malavaud et al., 2010; Guan et al., 2011), and several lines of evidence link SphK1 deregulation with the progression of various hematologic malignancies (Van Brocklyn et al., 2005; Bonhoure et al., 2006; Bayerl et al., 2008; Paugh et al., 2008; Pitson et al., 2011). For example, overexpression of SphK1 has been shown to be an oncogenic event in acute erythroid leukemia progression (Le Scolan et al., 2005). Furthermore, SphK1 is also shown to mediate chemotherapy sensitivity in AML cells. In fact, a study by Sobue et al. (2008) identified the cellular ceramide:S1P ratio as a critical biosensor for predicting AML cell sensitivity to daunorubicin. Specifically, they demonstrated that in AML cells, a low ceramide:S1P ratio, associated with high SphK1 activity, is correlated with a robust intrinsic chemoresistance to daunorubicin. They further demonstrated that increasing the ceramide:S1P ratio (using SphK inhibitors (SKI-II) or SphK1 small interfering RNAs (siRNAs) to reduce SphK1 activity and upregulate intracellular ceramide levels) sensitizes AML cells to daunorubicin. Another study demonstrated synergism between SKI-II treatment (resulting in increased ceramide accumulation) and imatinib mesylate in K562 chronic myelogenous leukemia cells, which results in increased apoptosis (Ricci et al., 2009). Additionally, Bonhoure et al. (2006) demonstrated that targeting SphK1 induces apoptosis of multidrug-resistant HL-60 cells (HL-60/VCR). Together, these findings implicate SphK1 as a novel chemotherapeutic target to overcome chemoresistance in hematologic malignancies.

We developed SKI-178 ([N’-(1E)-1-(3,4-dimethoxyphenyl)ethylidene]-3-(4-methoxyxyphenyl)-1H-pyrazole-5-carboxydrazide) as a novel small-molecule, isotype-specific, nonlipid substrate inhibitor of SphK1 that is cytotoxic toward a broad panel of cancer types, including AML (Hengst et al., 2010). Given the association of SphK1 with development and progression of leukemias in general, we believe that SKI-178 could represent a novel, effective therapeutic strategy for the treatment of AML. As a first step in this process we sought to elucidate the molecular mechanism by which SKI-178 induces apoptosis in human AML cell lines. Herein, we present evidence that SKI-178 induces prolonged mitosis, leading to induction of apoptotic cell death in HL-60 cells. Detailed analysis of this mitosis-dependent mechanism revealed that SKI-178-induced sustained activation of cyclin-dependent protein kinase 1 (CDK1) activity is responsible for the apoptotic effect. To ensure that these findings were not limited to HL-60 cells, SKI-178–induced apoptotic cell death was further extended to multiple AML cell lines, including multidrug resistance types. Taken together, we provide evidence that SKI-178 induces apoptosis in a CDK1-dependent manner and is not a substrate for multidrug-resistant protein 1 (MDR1), making it a promising chemotherapeutic candidate for the treatment of AML, including those known to be drug resistant.

Materials and Methods

Reagents. Reagents were purchased as follows: SKI-178 (ChemBridge Corporation, San Diego, CA), vincristine (Thermo Fisher Scientific, Waltham, MA), colchicine (Enzo Life Sciences, Farmingdale, NY), RO3306 ([5Z]-5-quinoxin-6-ylmethylene-2-[thiophen-2-ylmethyl]-amino)-thiazol-4-one; Sigma-Aldrich, St. Louis, MO), antibodies against total Bcl-2, pBcl-2 (Ser70), McI-1, caspase-3, caspase-9, cleaved caspase-3 (active), cleaved caspase-9 (active), cleaved caspase-7 (active), pHistone H3 (Ser10), pCDK1 (Tyr15), p-e-Jun N-terminal kinase (JNK) (Thr183/Tyr185), poly(ADP ribose) polymerase, and actin (Cell Signaling Technology, Danvers, MA), Sphk1 (Abgent, San Diego, CA), pBcl-xl (Ser62) (Abcam, Cambridge, MA), and glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Lines and Culture Conditions. Human AML-derived cell lines, HL-60 (CCL-240), U937 (CRL-1593.2), and THP-1 (TIB-202), as well as the human pancreatic cell line MIA PaCa-2 (CRL-1420), were obtained from the American Type Culture Collection (Manassas, VA). HL-60/VCR cells were a gift from H.-G. W. (Penn State College of Medicine, Hershey, PA). HL-60 and HL-60/VCR were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Denville Scientific, South Plainfield, NJ). U937 and THP-1 cell lines were maintained in RPMI (Thermo Fisher Scientific) supplemented with 10% FBS. All cell lines were maintained at 37°C with 5% CO2 in a humidified incubator.

Cell Viability Assays. Cells were treated with increasing concentrations of SKI-178, vincristine, or colchicine in 96-well plates for 48 hours. Cell viability was measured using the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell metabolism assay (American Type Culture Collection) according to manufacturer recommendations. Briefly, following treatment, 10 µl of MTT was added to each well and incubated for 4 hours at 37°C with 5% CO2 in a humidified incubator. After the 4-hour incubation, stop solution was added, kept overnight at room temperature, and absorbance was recorded at 570 nM. The results were normalized to vehicle [dimethyl sulfoxide (DMSO)] control. IC50 values based on cell viability were determined using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA) based on three separate experiments (n = 3).

Sphingosine Kinase Activity Assay and Thin-Layer Chromatography. Briefly, whole cell lysates were prepared from 1.0 × 106 cells in buffers selective for SKI-1 (20 mM Tris pH 7.4, 1 mM β-mercaptoethanol, 1 mM EDTA, 1.0% Triton X-100, 1 mM Na3VO4, 15 mM NaF, 0.5 mM 4-deoxypyridoxine) or SphK2 (20 mM Tris pH 7.4, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 M KCl, 1 mM Na3VO4, 15 mM NaF, 0.5 mM 4-deoxypyridoxine). Fifty micrograms of total protein lysates was combined with 50 µM D-erythro-sphingosine, 200 µM ATP, and 2 µCi [γ-32P]ATP in a 100 µl final reaction volume and incubated for 1 hour at 37°C with shaking. Kinase reactions were terminated by the addition of 10 µl 6N HCl, and the radiolabeled lipids were extracted by the addition of 400 µl of chloroform/MeOH (100:200 v/v) and 125 µl chloroform and 125 µl 1 M KCl. The organic phase containing lipids was dried down under nitrogen stream. Samples, along with separate SIP standards generated by sphingosine kinase activity assay using purified recombinant SphK1 as an enzyme source (Sigma-Aldrich), were then resuspended in 30 µl chloroform and applied to a Silica Gel thin-layer chromatography plate (Whatman, Florham Park, NJ) and the lipids were separated using a butanol:water:acetic acid (3:1:1 v/v/v) solvent system. The plates were analyzed by X-ray film exposure, and the region of the thin-layer chromatography plate corresponding to the Rf value (0.32) of SIP was examined.

Sphingolipid Analyses. Sphingolipids from treated HL-60 cells were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry essentially as described previously (Fox et al., 2011) with minor modifications described herein. Total lipids were extracted from HL-60 cell lysates equivalent to 500 µg of total protein. Extracted lipids were then separated on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) according to chromatography conditions described previously (Fox et al., 2011), but with 0.1% formic acid in the mobile phase at a flow rate of 0.4 milliliter per minute on a Phenomenex Kinetex C8 (2.6 µm) 2 mm internal diameter × 5 cm column maintained at 60°C (Phenomenex, Torrance, CA). The eluate was analyzed with an inline AB Sciex 4000 Q Trap mass spectrometer equipped with a turbo ion spray source (Applied Biosystems, Framingham, MA). The peak areas for the different sphingolipid subspecies were compared with that of the
internal standards. All data reported are based on monoisotopic mass and are represented as picoemols per milligram total cellular protein.

**Western Blot Analysis.** This was performed as previously described (Francy et al., 2007) with some modifications. Briefly, whole cell lysates were harvested in 100 µl 1× radioimmunoprecipitation assay buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂ EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 30 mM sodium fluoride, and complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged (≥10,000g) for 15 minutes at 4°C to remove cell debris. Total protein concentrations were quantified using the BCA assay from Pierce (Thermo Fisher Scientific). Equal amounts of denatured total protein were resolved by NuPAGE 12.5% Bis-Tris gel electrophoresis (Life Technologies, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Life Technologies). Membranes were blocked for 1 hour at room temperature in 5% milk/Tris-buffered saline/Tween 20, incubated overnight at 4°C with primary antibodies (1:1,000), and immunodetection was done with corresponding secondary IgG horse-radish peroxidase–linked antibodies (1:5,000) using the ECL chemiluminescence reagents (Thermo Fisher Scientific).

**Analysis of Apoptosis.** HL-60 cells were treated with either vehicle (DMSO) for 48 hours or SKI-178 (5 µM) for 16, 24, or 48 hours. 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) according to manufacturer's recommendations. Final analysis of the data were done using FlowJo 3.2 software (Tree Star, Inc., San Carlos, CA). Histograms are representative of three independent experiments (n = 3).

**Cell Cycle Synchronization.** Cells were synchronized according to the previously established double-thymidine block-and-release protocol (Bostock et al., 1971). Briefly, cells were synchronized at the G1/S phase border by culturing cells in DMEM + 10% FBS containing 2 mM thymidine (Sigma-Aldrich) for 19 hours. Cells were then released from the G1/S phase block by washing twice with phosphate-buffered saline (PBS) and resuspending them in thymidine-free culture medium for 9 hours. Cells were again treated with 2 mM thymidine in DMEM + 10% FBS for an additional 16 hours. After the second block, cells were washed twice with PBS and resuspended in thymidine-free culture medium containing appropriate treatment or control.

**Cell Cycle Analysis.** The cell cycle distribution of HL-60 cells after SKI-178 or DMSO treatment was determined by flow cytometry of propidium iodide (PI)–stained cells. Briefly, cells were treated with SKI-178 (5 µM) or DMSO (vehicle) for 4, 8, or 16 hours. Cells were washed with PBS, fixed in cold 70% ethanol, and incubated at −20°C for at least 30 minutes. Once all time points were collected, fixed cells were washed twice with PBS and resuspended in 1 ml PBS containing 0.1% (v/v) Triton X-100, 100 µg/ml PI (Invitrogen, Carlsbad, CA), and 200 µg/ml ribonuclease A (Qiagen, Venlo, The Netherlands). Cells were stained with PI for at least 30 minutes and analyzed using the FACScan analyzer (Becton Dickinson, Franklin Lakes, NJ). Data were processed and analyzed using FlowJo V10 software (Tree Star, Inc.).

**siRNA Transfection.** Duplexed Stealth siRNA (Life Technologies) was used to knockdown SphK1 expression in human MIA PaCa-2 cell line. siRNA sequences:

- siSphK1 #1: 5′-CCUACUUGGUAUAUGGGCCGUGU-3′.
- siSphK1 #2: 5′-GAGGCGAAGACUCUCAUGCGU-3′.

Transfections of MIA PaCa-2 cells were carried out by reverse-transfecting 0.5 × 10^6 cells with RNAiMAX (Life Technologies) containing 12.5 or 25 pmol of respective siRNAs according to manufacturer’s recommendations. Lysates for Western blotting or SphK activity assays were collected after a 72-hour incubation.

**Results**

**SKI-178 Induces Cytotoxicity in a Range of AML Cell Lines, Including Multidrug-Resistant HL-60/VCR.** We previously demonstrated that SKI-178 is cytotoxic toward a broad panel of cancer cell lines (Hengst et al., 2010). The efficacy of SKI-178 toward the various leukemia cell lines tested in this previous study highlighted the potential utility of SKI-178 as a novel AML therapeutic strategy. To further evaluate the therapeutic potential and determine the mechanism of action of SKI-178, we chose to restrict the focus of our studies to human AML cell lines. Numerous studies have employed HL-60, HL-60/VCR, and U937 cell lines in the evaluation of SphK inhibitors and determination of the role of SphK1 in leukemia pathogenesis, indicating that these human AML cell lines are appropriate models for the determination of the mechanism of action of SKI-178 (Bonhoure et al., 2006; Paugh et al., 2008; Kennedy et al., 2011). As shown in Fig. 1A, we confirmed that SphK1 protein is expressed in each of these cell lines. Interestingly, we observed that THP-1 cells expressed the highest levels of SphK1 protein among the cell lines examined. Consistent with our previous studies (Hengst et al., 2010), we determined that SKI-178 is cytotoxic toward all AML cell lines examined, with IC₅₀ values ranging from ~500 nM to ~1 µM (Table 1). Although not statistically significant, the range of IC₅₀ values appears to correlate with the relative levels of SphK1 catalytic activity (Fig. 1B) as determined by thin-layer chromatography. For example, HL-60 cells have the lowest level of SphK1 expression, as determined by Western blot analysis, of the four cell lines examined. They also have lower levels of SphK1 activity and lower IC₅₀ values than HL-60/VCR, U937, and THP-1 cells.

A major obstacle for treatment of AML patients is the fact that many patients have leukemic cells that are either inherently refractory to certain chemotherapeutics or develop multidrug resistance in relapsed AML (Cortes et al., 2004; Funato et al., 2004). We therefore included in our panel the HL-60 AML cell line and its vincristine-resistant variant, HL-60/VCR, to determine whether SKI-178 would be effective against multidrug-resistant cells. HL-60/VCR cells are known to be highly resistant to numerous chemotherapeutics (Baran et al., 2006; Zhao et al., 2010). Figure 1C confirms that HL-60/VCR express high levels of MDR1 compared with all other AML cell lines tested. Vincristine and colchicine are chemotherapeutics known to be effluxed by multidrug-resistant proteins (Yang et al., 1999; Licht et al., 2000). We therefore confirmed drug resistance in HL-60/VCR cells by comparing IC₅₀ values for vincristine and colchicine to the parental HL-60 cell line. As outlined in Table 1, the IC₅₀ values of vincristine and colchicine were significantly higher in HL-60/VCR compared with HL-60 cells, verifying that HL-60/VCR cells are indeed multidrug resistant. This observation is consistent with previous findings that MDR1 overexpression in HL-60/VCR underlies its multidrug resistance (Marquardt et al., 1990; Baran et al., 2006). Interestingly, as shown in Fig. 1D and summarized in Table 1, the IC₅₀ value for SKI-178 is only about 2-fold higher in HL-60/VCR compared with HL-60 cells. This is considerably lower than the 1000- and 60-fold differences for vincristine and colchicine, respectively, indicating that SKI-178 is not a substrate of drug efflux pump proteins (i.e., MDR1, MDR2).

**SKI-178 Induces Apoptosis through the Intrinsic Apoptotic Signaling Pathway.** To confirm SKI-178–induced cytotoxicity is a result of apoptotic cell death, HL-60 and HL-60/VCR cells were treated with SKI-178 for indicated time periods, and the stages of apoptosis were quantified using Annexin V-PE as a marker for apoptosis and 7-aminomycin
D (7AAD) as a marker of necrosis. In this assay, cells in the early stages of apoptosis stain Annexin V-negative and 7AAD-negative (lower right quadrant, Q3), whereas cells in the later stages of apoptosis stain Annexin V-positive and 7AAD-positive (upper right quadrant, Q2). As shown in Fig. 2A, the majority of vehicle treated cells was viable and stain negative for both Annexin V and 7AAD (lower left quadrant, Q4). After 16 hours of SKI-178 treatment, we observed an increase in the number of early apoptotic cells (Q3). By 24 hours of SKI-178 treatment, almost 60% of HL-60 and almost 50% of HL-60/VCR cells were apoptotic (Q2 + Q3) (Fig. 2B) with an increasing percentage of these in the later stages of apoptosis (Q2) (Fig. 2A). By 48 hours, greater than 90% of HL-60 and almost 70% of HL-60/VCR cells were in either the early or late stages of apoptosis (Fig. 2B). The fact that cells progress through early apoptosis in a time-dependent manner before staining positive for 7AAD suggests that positive 7AAD staining is indicative of cells dying as a result of apoptosis and not necrosis.

Mitochondrion-centered intrinsic apoptosis is mediated by mitochondrial outer membrane permeabilization, resulting in apoptosisosome formation, activation of caspase-9, and subsequent activation of effector caspases-3 and -7. To confirm intrinsic apoptotic cell death, HL-60 cells were treated with 5 μM SKI-178 for indicated time points. The activation of proapoptotic signaling [i.e., poly(ADP ribose) polymerase cleavage] correlated with the cleavage and activation of caspases-9 and -3 (Fig. 2C), indicating an activation of the intrinsic apoptotic pathway. In contrast, we were unable to detect any changes in signaling pathways indicative of the extrinsic apoptotic pathway, such as caspase-8 activation (data not shown).

**SKI-178 Alters Bioactive Sphingolipid Levels.** In previous studies we demonstrated selective SphK1 inhibition with SKI-178 in vitro as well as in whole cells using A549 lung cancer cells (Hengst et al., 2010). Hence, we used an established liquid chromatography–tandem mass spectrometry method (Sullards and Merrill, 2001; Seefelder et al., 2002; French et al., 2006) to examine the changes in intracellular levels of sphingolipid metabolites in HL-60 cells in response to SKI-178 treatment. Levels of S1P were significantly decreased after 24-hour SKI-178 (5 μM) treatment relative to the control vehicle (DMSO)-treated HL-60 cells (Table 2). Sphingosine levels were also decreased after 24 hours, most likely attributed to the reacylation of sphingosine to ceramide by the action of ceramide synthase enzymes. Interestingly, SKI-178 had a differential effect on the levels of long-chain ceramide species (LCC: C16–C18) compared with very long-chain ceramide (VLCC: C22–C24) species. The LCC species are known to exert proapoptotic effects, whereas VLCC species were recently suggested to promote cell growth (Koybasi et al., 2004; Grösch et al., 2012; Hartmann et al., 2012). According, we observed a significant increase in the levels of LCC species and a simultaneous decrease in the levels of VLCC species in cells treated with SKI-178 for 24 hours relative to

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**TABLE 1**

Cytotoxicity (IC50) of SKI-178 versus vincristine and colchicine

<table>
<thead>
<tr>
<th>AML Cell Line</th>
<th>SKI-178 (nM)</th>
<th>Vincristine</th>
<th>Colchicine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>472 ± 2.95</td>
<td>2.3 ± 0.016</td>
<td>9.5 ± 0.034</td>
</tr>
<tr>
<td>HL-60/VCR</td>
<td>1057 ± 3.06</td>
<td>2280 ± 9.3</td>
<td>583 ± 2.6</td>
</tr>
<tr>
<td>THP-1</td>
<td>686.7 ± 1.98</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>U-937</td>
<td>885.4 ± 1.98</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not available.
vehicle control. Together, these results demonstrate that SKI-178 treatment alters levels of bioactive sphingolipids that favor antiproliferation and/or apoptosis.

**SKI-178-Induced Apoptotic Cell Death Correlates with Prolonged Bcl-2 Phosphorylation.** The overall balance between ceramide and S1P is known to play an important role in determining cell fate through differential effects on members of the mitogen-activated protein kinase (MAPK) family of proteins (Cuvillier et al., 1996). S1P is known to exert some of its mitogenic effects through the activation of both extracellular signal-regulated kinase (ERK), a prosurvival antiapoptotic MAPK, and the phosphoinositide 3-kinase (PI3K)/AKT prosurvival pathway (Wu et al., 1995; Bonnaud et al., 2010). Sphingosine and ceramide, on the other hand, are believed to induce cell death via activation of proapoptotic MAPKs, such as JNK and p38 mitogen-activated protein kinase (p38) (Westwick et al., 1995; Verheij et al., 1996; Jarvis et al., 1997; Yoon et al., 2009). Based on these previous studies, we examined the effect

**Fig. 2.** SKI-178 induces apoptosis through the intrinsic apoptotic signaling pathway. (A) Cytometric analysis of Annexin V/7AAD-stained HL-60 and HL-60/VCR cells treated with SKI-178 (5 μM) for indicated time periods or vehicle (DMSO) control for 48 hours. Results shown are representative of three separate experiments. (B) Quantification of Annexin V–positive staining including both early (Q3) and late (Q2) stage apoptosis. Error bars indicate S.D. of triplicate counts of 5000 cells. Statistical significance was assessed by two-tailed paired Student’s t test. Asterisks indicate significance: *P ≤ 0.001; **P ≤ 0.0001. (C) HL-60 cells treated with SKI-178 (5 μM) for indicated time intervals. Western blot analysis was performed on whole cell lysates with proteins indicated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. PARP, poly(ADP-ribose) polymerase.

**TABLE 2**

<table>
<thead>
<tr>
<th>Sph</th>
<th>S1P</th>
<th>C16</th>
<th>C18</th>
<th>C22</th>
<th>C24:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>21.96 ± 1.98</td>
<td>6.98 ± 1.24</td>
<td>22.72 ± 2.07</td>
<td>0.22 ± 0.06</td>
<td>1.58 ± 0.43</td>
</tr>
<tr>
<td>SKI-178</td>
<td>15.12 ± 1.22</td>
<td>2.02 ± 0.02</td>
<td>49.84 ± 2.57</td>
<td>0.89 ± 0.08</td>
<td>0.3 ± 0.07</td>
</tr>
</tbody>
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of inhibiting MAPK and PI3K/AKT signaling on SKI-178–induced cell death. Specifically, HL-60 cells were treated with SKI-178 alone, or in combination with SP600125 (1,9-pyrazoloanthrone), SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole], PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one], or Wortmannin, inhibitors of JNK, p38, mitogen-activated protein kinase kinase 1 (ERK1/2 inhibition), and PI3K (AKT inhibition), respectively. Interestingly, inhibiting either p38, ERK1/2, or AKT had little to no effect on SKI-178–mediated apoptotic induction, indicated by caspase-7 cleavage/activation (Fig. 3A), demonstrating these MAPK are not required for SKI-178–induced apoptosis. On the other hand, JNK inhibition with SP600125 profoundly inhibited SKI-178–mediated caspase-7 cleavage. To further evaluate the role of JNK activity in the apoptotic mechanism of SKI-178, we next examined the effects of SKI-178 treatment on JNK activation and correlated its activity to the induction of apoptotic signaling. As shown in Fig. 3B, JNK activity (indicated by phosphorylation at Thr183/Tyr185) increased in a time-dependent manner starting as early as 2 hours after 5 μM SKI-178 treatment and continued to increase for at least 24 hours. Between 8 and 24 hours of SKI-178 treatment, where JNK activity was at its highest, there was a concomitant increase in apoptotic cell death indicated by the cleavage of caspase-7. As an indication of JNK activity, we also examined the phosphorylation of Bcl-2 at Ser70, a known JNK substrate (Yamamoto et al., 1999; Deng et al., 2001). Bcl-2 phosphorylation at Ser70 increased with time in response to SKI-178 treatment, reaching maximal levels at 8 hours, which was consistent with the timing of caspase-7 activation (Fig. 3B). Numerous studies have implicated JNK in the phosphorylation of Bcl-2 at Ser70 in response to agents that induce mitotic arrest and subsequent apoptotic cell death (Fan et al., 2000; Kelk et al., 2012). However, a recent study provided evidence that a known JNK inhibitor, SP600125, also inhibits CDK1 (Kim et al., 2010). Our findings in Fig. 3A, implicating JNK in the apoptotic mechanism of SKI-178, are based on the use of SP600125. To further clarify whether JNK or CDK1 was required for induction of apoptosis in response to SKI-178, we examined the effect of more selective inhibitors of JNK (AS601245 [(2Z)-2-(benzyl(2H-tiazol-2-yl)-yldene)-2-(2-(4-pyridine-3-yl)-ethyl)amino) pyridine-4-yl]acetoniitrile) and CDK1 (RO3306) on SKI-178 mediated Bcl-2 phosphorylation. Interestingly, as shown in Fig. 3C, both the JNK- and CDK-specific inhibitors abrogated SKI-178–induced Bcl-2 phosphorylation and caspase-7 cleavage similar to SP600125. Together, these results indicated that SKI-178 induces phosphorylation of Bcl-2 at Ser70 through a JNK- and/or CDK1-dependent mechanism. Thus, we next focused our efforts on deciphering the roles of these kinases in Bcl-2 phosphorylation and its role in the apoptotic mechanism of action of SKI-178.

**Prolonged G2/M Precedes Apoptosis in Response to SKI-178 Treatment.** Bcl-2 is known to undergo transient cell cycle–dependent phosphorylation at multiple sites, including Ser70, during mitosis (Scatena et al., 1998; Barboule et al., 2005). In contrast, enhanced and prolonged levels of Bcl-2 phosphorylation in mitosis are commonly associated with apoptotic cell death (Haldar et al., 1998; Eichhorn et al., 2013). Therefore, we next examined the effects of SKI-178 on cell cycle progression to determine whether SKI-178–induced apoptosis is associated with mitotic progression. HL-60 cells were treated with SKI-178 or vehicle for 4, 8, and 16 hours, and DNA content was examined using propidium iodide staining followed by flow cytometry. As shown in Fig. 4A, the DNA content of vehicle-treated cells reflected a normal distribution of cells in G1, S, or G2/M at all three time points. In contrast, we observed a substantial increase in the number of cells in G2/M after only 4 hours of SKI-178 treatment. By 8 and 16 hours of treatment, the majority of cells were in G2/M phase. Importantly, we observed a significant increase in sub-G1 DNA content, indicative of DNA condensation/fragmentation during apoptotic cell death. It is important to note that DNA fragmentation in cells arrested in mitosis results in an incremental decrease in DNA (Sakurikar et al., 2014). This incremental degradation of DNA starting in cells with G2/M DNA content (i.e., 4N DNA) results in a broad sub-G1 peak that is not easily distinguished from G1 or S phase. The quantification of the sub-G1 peak includes only cells with strictly sub-G1 DNA content (<2N DNA) and therefore may be an underrepresentation of the number of cells undergoing apoptosis. Nonetheless, these results, in combination with Annexin V staining in Fig. 2, A and B, strongly indicate that SKI-178–induced G2/M arrest precedes apoptotic cell death. To verify that these effects are not cell type specific, three additional human AML cell lines, including multidrug resistant HL-60/VCR, were treated with SKI-178 for 16 hours. Analysis of their DNA content also revealed substantial increase in cells with G2/M DNA content (Fig. 4B). These results indicate that SKI-178 induces apoptosis through a sustained/prolonged cell cycle at mitosis.

**SKI-178 Induces Sustained Bcl-2 Phosphorylation during Mitosis.** The results presented in Fig. 4, A and B, strongly suggest SKI-178–induced apoptosis may be the result of prolonged mitosis. Because analysis of DNA content does not distinguish between G2 and M phase, we employed a cell synchronization method to further examine the relationship between cell cycle and apoptosis in response to SKI-178. To this end, HL-60 cells were synchronized at the G1/S phase transition using a double thymidine block method (Bostock et al., 1971) and released into either 5 μM SKI-178 or vehicle DMSO control for a period of up to 30 hours. Whole cell lysates were prepared from cells collected every 2 hours and subjected to Western blot analysis for pBcl-2 (Ser70) as well as pHistone H3 (Ser10), which is a known marker for mitosis (Hendzel et al., 1997). Consistent with previous studies, HL-60 cells released from G1/S blockade into vehicle containing medium have a slight but transient increase in the phosphorylation of Bcl-2, because the cells progress through mitosis, indicated by histone H3 phosphorylation 10–14 hours after release (Yamamoto et al., 1999). Cells released into 5 μM SKI-178 containing medium also entered mitosis about 10 hours after release, but unlike vehicle-treated cells, histone H3 remains phosphorylated for up to 20 hours (Fig. 5). Furthermore, histone H3 phosphorylation in SKI-178–treated cells is paralleled by a robust and sustained phosphorylation of Bcl-2 at Ser70. Importantly, although no caspase-7 cleavage was detected in vehicle-treated cells, SKI-178 treatment showed substantial cleavage/activation of caspase-7 beginning about 4 hours after the onset of mitotic arrest (14 hours postrelease). These results indicate that SKI-178–induced apoptotic cell death is closely associated with prolonged mitosis.

**CDK1 Inhibitor Completely Abrogates SKI-178–Induced Bcl-2 Phosphorylation and Caspase-7 Activation.** The results presented thus far clearly indicate that SKI-178 induces prolonged mitosis indicated by prolonged Bcl-2 and histone H3 phosphorylation. Additionally, our pharmacologic inhibitor studies indicate that JNK and/or CDK1 are required for SKI-178–induced apoptosis. However, elucidation of the role of
JNK and CDK1 is complicated by the fact that inhibition of either kinase was previously shown to block cell cycle progression from G2 into mitosis. Hence, we next sought to clarify the roles of JNK and CDK1 in the apoptotic mechanism of SKI-178 associated with prolonged mitosis.

CDK1, in complex with cyclin B1, functions as a mitosis promoting factor initiating the transition into early prophase (Morgan, 1995). Inhibition of CDK1 activity before entry into mitosis blocks cell cycle progression at the G2/M boundary (Vassilev, 2006). Similarly, there are data to suggest that JNK is involved in progression from G2 into mitosis by regulating expression of Aurora B (Oktay et al., 2008). Thus it is possible that simultaneous addition of JNK/CDK1 inhibitors with SKI-178 in unsynchronized cells or in synchronized cells before entry into mitosis would abrogate SKI-178–induced apoptosis. However, this effect could be an artifact of the blockage of cell cycle before mitosis and would not accurately reflect the roles of these kinases in SKI-178–induced mitosis-dependent apoptotic cell death.

To overcome these limitations, HL-60 cells were synchronized in G1/S phase using a double thymidine block, released into fresh medium containing SKI-178 and then treated with JNK (AS601245) or CDK1 (RO3306) inhibitors at different time points as cells progress through the cell cycle. Whole cell lysates were prepared from cells collected at the indicated time points and subjected to Western blot analysis for pBcl-2 (Ser70) and cleaved active caspase-7. GAPDH serves as a loading control. Results shown are representative of at least three independent experiments.
(AS601245) at the time of release from G1/S blockade, we observed abrogation of pBcl-2 (Ser70) and pHistone H3 (Ser10) (Fig. 6C) and no caspase-7 cleavage (Active) (Fig. 6F). Conversely, when synchronized HL-60 cells were released from the G1/S blockade into medium containing SKI-178, allowed to enter into mitosis [\sim 10\text{ hours indicated by pHistone H3 (Ser10)}], and subsequently treated with AS601245 (addition at 14 hours), JNK inhibition did not have an effect on SKI-178–induced Bcl-2 or histone H3 phosphorylation (Fig. 6D). Similarly, AS601245 addition at 14 hours after release did not have an effect on caspase-7 cleavage (activation) (Fig. 6F). Together, these results indicate that JNK activity plays a critical role in cell cycle progression from G2 into mitosis and confirms that entry into mitosis is required for SKI-178–mediated apoptotic cell death.

Fig. 4. Prolonged mitosis precedes SKI-178–induced apoptotic cell death. (A) Flow cytometry analysis of cell cycle distribution in HL-60 cells treated with SKI-178 or vehicle (DMSO) control for indicated time intervals. Histograms are representative of three separate experiments. (B) Percentage of AML cell lines in G2/M after 16 hours of treatment with SKI-178 (5 \mu M) or vehicle (DMSO). Error bars indicate S.D. of triplicate counts of 25,000 cells. Statistical significance was assessed by two-tailed paired student’s t test. Asterisks indicate significance: *P \leq 0.01.
The same experimental design was employed to evaluate the role of CDK1 on SKI-178–induced apoptosis. Given the established role of CDK1/cyclin B1 as the mitosis promoting factor (Vassilev, 2006), we did not evaluate the role of CDK1 inhibition before to entry into mitosis. As shown in Fig. 6E, inhibition of CDK1 with RO3306 after synchronized cells entered mitosis (14 hours after release) completely abrogated SKI-178–induced Bcl-2 phosphorylation, indicating that CDK1 is responsible for Bcl-2 phosphorylation. Similarly, RO3306 treatment also abrogated histone H3 phosphorylation. This is likely due to the fact that CDK1 regulates the activity of Aurora kinase A, the kinase responsible for histone H3 phosphorylation at Ser10 (Van Horn et al., 2010; Ding et al., 2011). In addition, RO3306 inhibition of CDK1 dramatically reduced SKI-178–induced apoptosis indicated by abrogation of caspase-7 cleavage (activation) (Fig. 6F). Together, these results indicate that CDK1, not JNK, is the kinase responsible for SKI-178–induced apoptotic cell death.

**SKI-178 Induces Prolonged CDK1 Activation.** The results presented above clearly indicate that SKI-178 induces prolonged mitosis where CDK1 is active. Furthermore, inhibition of CDK1 during mitosis completely abrogates SKI-178–induced enhanced/sustained Bcl-2 phosphorylation and caspase-7 cleavage. These results suggest that SKI-178 induces sustained activation of CDK1 once cells enter into mitosis. Before mitosis, CDK1 is held inactive by phosphorylation at two residues, Thr14 and Tyr15 (Chow et al., 2011). Tyr15 phosphorylation, in particular, is an essential checkpoint preventing entry into and progression through mitosis (Fletcher et al., 2002; Welburn et al., 2007). To confirm sustained CDK1 activity, HL-60 cells were synchronized at G1/S and released into SKI-178 or vehicle control for a period of 24 hours. CDK1 activity was examined by the presence or absence of the inhibitory phosphorylation of CDK1 at Tyr15. HL-60 cells released into both SKI-178 and vehicle control showed sustained CDK1 inactivation indicated by phosphorylation at pCDK1 (Tyr15) up to 8 hours after release (Fig. 7). Between 8 and 12 hours, the inactivating phosphorylation of CDK1 (pCDK1 (Tyr15)) is rapidly reduced in both vehicle- and SKI-178–treated cells as cells enter mitosis. Vehicle-treated cells start to exit mitosis around 16 hours, indicated by the reappearance of CDK1 inhibitory phosphorylation. On the other hand, CDK1 remained active in SKI-178–treated cells (indicated by the lack of phosphorylation at Tyr15) once cells have entered into mitosis. These results are consistent with our previous finding that SKI-178 induces prolonged mitotic arrest and further indicate that sustained activation of CDK1 in response to SKI-178 is responsible for the phosphorylation and inactivation of antiapoptotic Bcl-2.

**SKI-178–Induced CDK1 Activation Results in Mcl-1 Degradation.** Although our data suggest a strong correlation between Bcl-2 phosphorylation at Ser70 and induction of apoptotic cell death, the functional role of this phosphorylation is not yet clear. Although many studies have implicated this Bcl-2 phosphorylation in making cells more susceptible to apoptosis (Haldar et al., 1998; Eichhorn et al., 2013), there are recent studies that suggest it actually enhances its cytoprotective effects (Dai et al., 2013; Zhou et al., 2014). Nonetheless, Bcl-2 is not the only CDK1 substrate that displays extensive/prolonged phosphorylation during prolonged mitotic arrest. CDK1 has been shown to phosphorylate and inactivate other antiapoptotic Bcl-2 family members including Bcl-xl and Mcl-1. Bcl-xl phosphorylation during mitotic arrest inhibits its antiapoptotic activity by disrupting its interaction with Bax, leading to mitochondrial outer membrane permeabilization and cytochrome c release (Bah et al., 2014). Unlike Bcl-2 and Bcl-xl, Mcl-1 phosphorylation at Thr92 by CDK1 quickly targets it for proteasomal degradation (Harley et al., 2010).

As demonstrated in Fig. 8A, all four AML cell lines, to varying degrees, express Bcl-2, Mcl-1, and Bcl-xl. Relative to HL-60 cells, HL-60/VCR cells express higher levels of all three antiapoptotic Bcl-2 family members. Interestingly, THP-1 cells express extensively higher levels of Bcl-2 relative to all other cell lines examined. Given that CDK1-dependent phosphorylation of Mcl-1 targets it for degradation, it is hypothesized that CDK1 inhibition would prevent Mcl-1 degradation in response to SKI-178. To test this hypothesis, HL-60 and HL-60/VCR cells were treated with SKI-178 alone or in combination with RO3306 for a 24-hour period, and the expression levels of pBcl-2 (Ser70), pBcl-xl (Ser62), and total Mcl-1 were examined by Western blot analysis. As expected, SKI-178 treatment led to a dramatic increase in Bcl-2 phosphorylation, Mcl-1 degradation, and caspase-7 cleavage (activation) in both HL-60 and HL-60/VCR cells (Fig. 8B). SKI-178 also induced phosphorylation of Bcl-xl in HL-60/VCR cells, whereas Bcl-xl phosphorylation in HL-60 was not detected (data not shown), likely due to antibody limitations because HL-60 express considerably lower levels of total Bcl-xl relative to HL-60/VCR cells (Fig. 8A).
Fig. 6. Inhibition of CDK1 completely abrogates SKI-178–induced Bcl-2 phosphorylation and caspase-7 activation after mitotic arrest. HL-60 cells were synchronized at G1/S phase transition using a double thymidine block and released into either vehicle (A) or SKI-178 (5 μM) (B). Cells released into SKI-178 were subdivided into four additional treatments: (C) HL-60 cells released into SKI-178 and cotreated with AS601245 at the time of release; (D) HL-60 cells released into SKI-178 and cotreated with AS601245 14 hours after release; (E) HL-60 cells released into SKI-178 and cotreated with RO3306 14 hours after release. Whole cell lysates were collected at indicated time points and blot analysis was performed using antibodies for pBcl-2 (Ser70) or pHistone H3 (Ser10). (F) To directly compare pBcl-2 (Ser70), pHistone H3 (Ser10), and caspase cleavage between the various treatments, Western blot analysis was performed using indicated antibodies on the 26-hour time points from (A–E). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serves as a loading control. Results shown are representative of at least three independent experiments.
As discussed previously with regard to Bcl-2 phosphorylation, inhibition of Mcl-1 degradation by RO3306 could occur indirectly by inhibiting cell cycle entry into mitosis where Mcl-1 phosphorylation/degradation occurs. To clarify this point, HL-60/VCR cells were synchronized as previously described, released into media containing SKI-178, and treated with RO3306 after cells had entered into mitosis (~14 hours after release). HL-60/VCR were chosen based on their high expression of Mcl-1 relative to other cell lines (Fig. 8A) and to extend the cell cycle profiling seen in HL-60 to a multidrug-resistant cell line. The results seen here with HL-60/VCR (Fig. 8C) mimicked those previously observed in HL-60 cells. Specifically, cells released into either vehicle or SKI-178 alone entered into mitosis, as indicated by the presence histone H3 phosphorylation (Ser10), approximately 10–12 hours after release from G1/S blockade. Vehicle-treated cells showed a slight but transient increase in Bcl-2 phosphorylation as cells progress through mitosis 10–16 hours after release. Cells treated with SKI-178 alone showed sustained Bcl-2 phosphorylation, prolonged mitosis, and subsequent caspase-7 cleavage (active) starting around 6–8 hours after the onset of mitosis. As expected, SKI-178 also lead to almost complete Mcl-1 degradation beginning shortly after entry into mitosis but before the appearance of caspase-7 cleavage. As shown previously in HL-60 cells, inhibition of CDK1 in HL-60/VCR cells during mitosis completely blocked Bcl-2 phosphorylation and caspase-7 activation. Furthermore, inhibition of CDK1 prevented the complete degradation of Mcl-1 observed in cells treated with SKI-178 alone. Together, these results indicate that SKI-178 induces apoptotic cell death during prolonged apoptosis by activating intrinsic apoptosis signaling pathways (i.e., Bcl-2 phosphorylation and Mcl-1 degradation).

**siRNA Knockdown of SphK1 Recapitulates the Effects of SKI-178.** To further demonstrate that the mechanism of action we observed with SKI-178 was attributable to its ability to inhibit SphK1 activity, we treated MIA PaCa-2 cells with siRNAs directed to SphK1. MIA PaCa-2 cells were used to overcome the difficulty of transfection of suspension cell lines, such as HL-60 cells, and to extend our observations to non-AML cell lines. In vitro SphK activity assays using isotype specific buffer conditions confirm the ability of SphK1 siRNAs to block SphK1 expression without affecting SphK2 activity (Supplemental Fig. 1A). Western blot analysis of siRNA-treated cells revealed that blockage of SphK1 expression activated JNK and induced phosphorylation of Bcl-2 (Ser70) relative to scrambled controls (Supplemental Fig. 1B), as was observed previously with SKI-178 treatment. Furthermore, we also treated MIA PaCa-2 cells that have been transfected with SphK1 siRNAs with low doses of SKI-178 (500 nM). As shown in Supplemental Fig. 1C, SphK1 knockdown (KD) increases the phosphorylation of JNK and Bcl-2 to the same extent as scrambled siRNA control cells treated with 500 nM SKI-178. Treatment of SphK1 KD cells with 500 nM SKI-178 further enhanced the activation of JNK, phosphorylation of Bcl-2, and induced substantial activation of caspase-7. Together, the fact that SphK1 siRNAs recapitulate the effects of SKI-178 and that SphK1 KD enhances the effects of SKI-178 strongly supports the mechanism of action of SKI-178 described herein is due to the target-specific effects of SKI-178 on SphK1 activity rather than “off-target” effects.

**Discussion**

In this study we use multiple human AML cell lines to elucidate the molecular mechanism of action of SKI-178, a potent, selective inhibitor of SphK1. Elevated expression of SphK1 has consistently been observed in various cancer types, including leukemia (Heffernan-Stroud and Obeid, 2013), and has become a promising target for novel therapeutic strategies (Orr Gandy and Obeid, 2013). We previously established SKI-178 as a potent selective inhibitor of SphK1 through in vitro activity assays with purified SphK1 protein (Hengst et al., 2010). Our results presented here support these findings by demonstrating that SKI-178 treatment of HL-60 cells induces alterations in intracellular levels of bioactive sphingolipid metabolites that are consistent with SphK1 inhibition. After 24 hours of treatment, levels of S1P are significantly decreased compared with control, whereas levels of proapoptotic long-chain ceramide species (C_{16} and C_{18}) are increased. Interestingly, SKI-178 treatment concomitantly decreased levels of very long chain ceramide species, which recent studies suggest have antiapoptotic properties (Koybasi et al., 2004; Grösch et al., 2012; Hartmann et al., 2012). Ceramide is produced by the addition of a fatty acyl chain to a molecule of sphingosine by the activity of six different ceramide synthase enzymes (CerS1–6), each with different substrate specificities (Park et al., 2014). The majority of very long chain ceramide species are produced specifically by ceramide synthase 2 (CerS2) (Petrache et al., 2013). The decrease in very long chain ceramide species implies that CerS2 might be inhibited by SKI-178. Considering both SphK1 and ceramide synthase enzymes use sphingosine as a substrate and SKI-178 acts as a sphingosine competitive inhibitor, this is a distinct possibility. Another potential explanation is that CerS2 activity is regulated by sphingolipid levels. Indeed, Laviad et al. (2008) demonstrated that S1P inhibits CerS2 activity. Because SKI-178 is a SphK1-selective inhibitor and does not inhibit SphK2, it is possible that S1P levels remain high enough to restrict CerS2 activity such that very long chain ceramide levels remain unchanged or decrease over time through further metabolic conversion in response to SKI-178. Conversely, Beverly et al. (2013) recently demonstrated that induction of apoptotic signaling stimulates the formation of long-chain ceramide species. Thus SKI-178 may induce the formation of long-chain ceramides from the excess pool of sphingosine that is liberated by the inhibition of S1P formation. Regardless of how SKI-178 induces these opposing effects, simultaneous induction and inhibition of pro- and antiapoptotic ceramide species, respectively, presents a unique
Fig. 8. SKI-178–induced CDK1 activation results in MCL-1 degradation. (A) Whole cell lysates from the indicated AML cell lines were subjected to Western blot analysis to assess expression of various antiapoptotic family members (Bcl-2, Bcl-xl, and Mcl-1). (B) HL-60 and HL-60/VCR cells treated for 24 hours with SKI-178, RO3306, or a combination of SKI-178 and RO3306. Western blot analysis was performed on whole cell lysates using indicated antibodies. (C) HL-60/VCR cells were synchronized at the G1/S phase transition using a double thymidine block and released into either vehicle or SKI-178. Cells released into SKI-178 were either maintained in SKI-178 alone or cotreated with RO3306 14 hours after release. Whole cell lysates were collected at indicated time points, and Western blot analysis was performed using indicated antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serves as a loading control.
characteristic of SKI-178, making it a promising tool in the field of sphingolipid biology.

We next verified SKI-178–induced cytotoxicity is the result of mitochondria-dependent apoptosis based on Annexin V staining and caspases-9, -3, and -7 activation. Numerous MAPKs, such as ERK, p38, JNK, and Akt, have been identified as important regulators of this intrinsic apoptotic pathway (Xia et al., 1995; Kennedy et al., 1997; Cagnol and Chambard, 2010). Furthermore, activation of these MAPKs plays an important role in ceramide-induced apoptotic cell death (Oh et al., 2006; Kim et al., 2008). Therefore, as SKI-178 treatment induces accumulation of proapoptotic ceramide species, we proposed that activation of various MAPKs may be critical to the apoptotic mechanism of SKI-178. Of all the MAPKs tested, only JNK activation appeared to be essential for the apoptotic mechanism for SKI-178. SKI-178 treatment in combination with JNK inhibition completely abrogated SKI-178–induced caspase cleavage, whereas inhibition of p38, ERK, or Akt had little to no effect. In addition to JNK activation, SKI-178 induced a time-dependent increase in the phosphorylation of antiapoptotic Bcl-2 at Ser70. Although JNK has been linked to the phosphorylation of Bcl-2 in mitotically arrested cells (Yamamoto et al., 1999; Fan et al., 2000; Du et al., 2004), there is conflicting evidence in the literature implicating JNK activation, SKI-178–induced prolonged mitosis, and inactivation of JNK before mitosis induces the phosphorylation of Aurora kinase B and phosphorylation of histone H3 at Ser10 (Du et al., 2004; Oktay et al., 2008).

Our findings further suggest a link between SphK1 inhibition with SKI-178 and prolonged activation of CDK1 leading to apoptotic cell death. Although it is possible that SKI-178 induces prolonged mitosis as a polypharmacologic agent (i.e., “off-target” effects), several studies provide evidence to suggest that SphK1 and/or SphK2 plays an important role in cell cycle progression. For example, SphK1, SphK2, and the S1P5 receptor have been shown to colocalize to the centrosome of mammalian cells, where the authors proposed that the S1P5 receptor acts as a guanine nucleotide exchange factor stimulated by localized S1P production (Gillies et al., 2009). Thus, SphK1 inhibition by SKI-178 or other SphK inhibitors could alter the activity of the centrosome. In support of this, SphK inhibition and RNAi-mediated SphK1 downregulation has been shown to block mitotic exit, leading to cytokinesis failure in breast carcinoma cell lines (Kotelevets et al., 2012), and nonselective small molecule inhibitors of SphK1 and SphK2 induce substantial G2/M arrest in melanoma cell lines (Madhunapanthula et al., 2012). Although outside the scope of this investigation, it is interesting to suggest that sphingolipid metabolism plays a role in regulation of the cell cycle.

Of all the stages of the cell cycle, mitosis is considered the most critical. During mitosis, condensed chromosomes are no longer protected by the nuclear membrane and DNA damage is not easily repairable (Heijink et al., 2013). This leaves cells extremely vulnerable and potentially more susceptible to apoptotic stimuli. Mitosis is tightly controlled by the spindle-assembly checkpoint that ensures proper chromosome replication and segregation. A failure to satisfy the spindle-assembly checkpoint often results in prolonged mitotic arrest and the induction of an intrinsic proapoptotic pathway responsible for clearing cells that fail to exit mitosis in a timely fashion (Topham and Taylor, 2013). This intrinsic phenomenon, normally used as a quality control mechanism to ensure proper chromosome segregation, has been widely exploited by many current chemotherapeutics to induce apoptotic cell death in rapidly growing cancer cells (Chan et al., 2012).

Several lines of evidence indicate that extensive phosphorylation of Bcl-2 at Ser70 and other sites by CDK1 inactivates its antiapoptotic function and is a key feature of apoptotic cell death in response to agents that induce prolonged mitotic arrest (Haldar et al., 1998; Barboule et al., 2005; Eichhorn et al., 2013). However, Dai et al. (2013) recently demonstrated that phosphorylation of Bcl-2 at Ser70 enhances the antiapoptotic function of Bcl-2 by promoting Bim and Bak binding. Interestingly, CDK1-mediated phosphorylation of Bcl-xl at Ser62 during mitotic arrest has more consistently been shown to play a proapoptotic role, at least in part by disrupting its ability to bind and inhibit proapoptotic Bax (Upreti et al., 2008; Bah et al., 2014). Furthermore, CDK1-mediated phosphorylation of Mcl-1 at Thr92 and its subsequent proteosomal degradation during prolonged mitotic arrest have also been shown to play a key role in linking mitotic arrest to the induction of apoptotic cell death (Harley et al., 2010). Our results clearly show that during SKI-178–induced prolonged mitosis, these antiapoptotic Bcl-2 family members undergo these posttranslational modifications, and that they are strictly dependent upon sustained CDK1 activity. The functional role of Bcl-2 phosphorylation at Ser70 remains controversial, but irrespective of whether it enhances its antiapoptotic effects or induces apoptosis, pBcl-2 (Ser70) is a clear maker of CDK1 activity that consistently correlates with induction of apoptotic cell death.

It is important to note that different cell lines depend more heavily on different antiapoptotic Bcl-2 family members for survival. Although many leukemic cells are highly dependent on Bcl-2 (Totova et al., 2002), the other antiapoptotic Bcl-2 family members, Mcl-1 and Bcl-xl, have overlapping functions and can compensate for Bcl-2 inhibition (van Delft et al., 2006; Lin et al., 2007; Wei et al., 2012). Furthermore, alterations in the expression of Bcl-2 family members play a potential role in the mechanism of resistance to various chemotherapeutic agents. Resistance to ABT-737, a small molecule inhibitor of Bcl-2 currently undergoing clinical trials, is attributed to transcriptional upregulation of Mcl-1 (Yecies et al., 2010). Similarly, high levels of Bcl-xl expression confers resistance to Mcl-1 inhibitors (Wei et al., 2012). All four AML cell lines expressed Bcl-2, Bcl-xl, or Mcl-1 to varying degrees. THP-1, in particular, expresses extensively high levels of Bcl-2 relative to all other cell lines but remain sensitive to the cytotoxic effects of SKI-178.

Although our findings here focus more heavily on Bcl-2, it is well established in the literature that CDK1, in response to inducers of mitotic arrest, simultaneously phosphorylates Bcl-2, Bcl-xl, and Mcl-1 (Harley et al., 2010; Terrano et al., 2010; Chu et al., 2012). We demonstrate that in addition to Bcl-2, SKI-178–induced CDK1 activity also leads to the inhibitory phosphorylation of Bcl-xl (Ser62) and Mcl-1 phosphorylation, leading to subsequent degradation. The fact that SKI-178 leads to the prolonged and simultaneous inhibition of multiple antiapoptotic Bcl-2 proteins makes it an attractive potential...
therapeutic agent for the treatment of AML. In fact, many of the most effective chemotherapeutic agents currently in clinical use are inducers of mitotic arrest (Mukhtar et al., 2014). Unfortunately, a major problem in the treatment of leukemic cancers is the development of resistance to such chemotherapeutic agents (Holohan et al., 2013). Much of this resistance has been attributed to the overexpression of MDR1 (Broxterman and Schuurhuis, 1997; Cianfriglia, 2013). Taken together, our results demonstrate that SKI-717 induces apoptotic cell death in multiple leukemic cell lines, including multidrug-resistant cell lines. These results highlight the promising potential of developing SKI-717 as a therapeutic strategy, not only for AML but also for an array of cancer types including those with multidrug resistance. Further studies are underway to examine the in vivo efficacy of SKI-717 in animal models.

**Authorship Contributions**

**Participated in research design:** Dick, Hengst, Fox, Sung, Sharma, Amin, Loughran, Kester, Wang, Yun.

**Conducted experiments:** Dick, Hengst, Fox, College, Kule.

**Contributed reagents or analytic tools:** Fox.

**Performed data analysis:** Dick, Hengst, Yun.

**Wrote or contributed to the writing of the manuscript:** Dick, Hengst, College, Kule.

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The Apoptotic Mechanism of Action of the Sphingosine Kinase 1 Selective Inhibitor, SKI-178, in Human AML Cell Lines.


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Supplementary Figure 1: siRNA knockdown of SphK1 recapitulates the effects of SKI-178: (A) MIA PaCa-2 cells were transfected with 25pmol siRNA for 72h. Scramble siRNA serves as a negative control. Whole cell lysate were prepared in buffers selective for SphK1 or SphK2 activity. SphK1 and SphK2 catalytic activity was subsequently determined by thin-layer chromatography. (B) MIA PaCa-2 cells were transfected with 12.5 or 25pmol siRNA for 72h. Scramble siRNA serves as a negative control. Western blot analysis was performed on whole cell lysate using indicated antibodies. (C) MIA PaCa-2 cells were transfected with 25pmol siRNA. After 48h, transfected cells were treated with 500nM SKI-178 and incubated for an additional 24h. Scramble siRNA serves as a negative control. Western blot analysis was performed on whole cell lysate using indicated antibodies. GAPDH serves as a loading control.
Supplementary
Figure 1