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A PROMISING ANTI-CANCER AGENT THAT FUNCTIONS AS A DUAL TARGET INHIBITOR OF SPHK1 AND MICROTUBULE DYNAMICS

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ABSTRACT

An extensive amount of data points to sphingosine kinase 1 (SphK1) and aberrant regulation of sphingolipids as important components of tumorigenesis. We previously developed SKI-178 as a novel small molecule, SphK1-selective inhibitor that is cytotoxic toward a broad panel of cancer cell lines. Nonetheless, the mechanism underlying SKI-178 induced apoptotic cell death had not yet been elucidated. Therefore, the overarching goal of this research study was to uncover the detailed mechanism-of-action (MOA) of SKI-178 induced apoptosis. 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. We further demonstrate that the sustained activation of cyclin-dependent kinase 1 (CDK1) during SKI-178 mediated prolonged mitosis leads to the simultaneous phosphorylation of pro-survival Bcl-2 family members, Bcl-2 and Bcl-xl, as well as the phosphorylation and subsequent degradation of Mcl-1. Based on evidence in the literature suggesting a link between SphK1 activity and mitotic arrest, we originally attributed much of the MOA to SphK1 inhibition. However, in light of recent reports claiming that SphK1 inhibition alone is not sufficient to induce apoptotic cell death; we considered the possibility that SKI-178 may have additional targets that contribute to its cytotoxicity. Based on the striking similarly between the MOA of SKI-178 and various agents that disrupt microtubules, we considered the possibility that SKI-178, in addition to being a SphK1 selective inhibitor, also affects normal microtubule dynamics. We hypothesize that SKI-178 mediated apoptosis is due to its ability to work as a microtubule targeting agent (MTA), and that its concomitant inhibition of SphK1 sensitizes cells to this

effect. Herein we present evidence that SKI-178 does indeed function as MTA, preventing the polymerization of the microtubule network. Furthermore, we demonstrate that independent of SKI-178, inhibition of SphK1 alone sensitizes cells to vincristine, a MTA known for its ability to disrupt microtubule polymerization. We also definitively confirm that in cells, SKI-178 is able to directly target engage both SphK1 and tubulin proteins at concentrations known to induce apoptotic cell death in a panel of cancer cell lines. Moreover, multi-drug resistance, mediated by multidrug resistant protein-1 and/or pro-survival Bcl-2 family member over-expression, did not affect the sensitivity of cells to SKI-178. Together, these results establish SKI-178 as a dual target inhibitor with promising chemotherapeutic potential for a wide variety of cancer types including those know to be multi-drug resistant.

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List of Abbreviations

7-AAD	7-amino-actinomycin D
ABCC	ATP-binding cassette
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC/C	Anaphase promoting complex/cyclosome
ATRA	All-trans-retinoic acid
CDK1	Cyclin-dependent kinase 1
CIB1	Calcium and integrin binding protein 1
CML	Chronic myeloid leukemia
Des1	Dihydroceramide desaturase
dhCers	Dihydroceramide
DMS	*N*,*N*-dimethylsphingosine
eEF1A1	Eukaryotic elongation factor 1a
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
HDAC	Histone deacetylase
HPLC	High performance liquid chromatography
IL-1	Interleukin 1
JNK	c-Jun NH_2-terminal kinase
KSP	Kinesin spindle protein
KSR	Kinase suppressor of Ras
MAPK	Mitogen activated protein kinase
MDR1	Multidrug resistant protein-1
MOA	Mechanism-of-action

MOMP	Mitochondrial outer membrane permeabilization
MTA	Microtubule targeting agents
NSCLC	Non-small-cell lung cancer
p38	p38 mitogen-activated protein kinase
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
Pgp	P-glycoprotein
PI3K	The phosphoinositide 3-kinase
PKC	Protein kinase C
PKCα	Protein kinase C alpha
PKCζ	Protein kinase C zeta
PLK	Polo-like kinase
PP2A	Protein phosphatase 2A
PTI-1	Prostate tumor inducer-a
ROMe	(R)-FTY720-Ome
S1P	Sphingosine-1-phosphate
S1PR	S1P receptors
SAC	Spindle-assembly-checkpoint
SphK	Sphingosine kinase
Spns2	Spinster homolog 2
TNF	Tumor necrosis factor
TRAF2	(TNF) receptor-associated factor 2
VCR	Vincristine resistant

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Chapter 1

Review of the Literature

Sphingosine Kinase as an Anticancer Chemotherapeutic Target

Overview of Sphingolipid Metabolism

Sphingolipids are a complex heterogeneous family of lipids characterized by the presence of a sphingosine base (Figure 1.1) and were initially thought to function solely as critical structural components of cellular membranes. Relative to phospholipids, sphingolipids tend to associate more tightly with each other in cell membranes and thereby modulate the fluidity of the plasma membranes. Together with cholesterol, they form the basis for densely packed regions of the membrane referred to as lipid rafts [1]. Although sphingolipids serve as critical biophysical components of eukaryotic plasma membranes [2, 3], their more fascinating functions stem from their role as potent bioactive signaling molecules. Bioactive sphingolipids, such as ceramide, sphingosine, sphingosine-1-phosphate (S1P), and other sphingolipid metabolites have been shown to play an important role in various intracellular signaling pathways that regulate cell fate. They constitute a class of second messengers that exert a powerful influence over a variety of cellular processes such as cellular differentiation, proliferation, senescence, apoptosis, and even chemoresistance. While the exact mechanisms are still being elucidated, some general dogmas about sphingolipid signal transduction have emerged. Ceramide and sphingosine exert anti-proliferative, pro-apoptotic effects while S1P promotes proliferation, cell survival, and inhibits apoptosis [4-8]. More specifically, sphingosine plays a role in regulating apoptosis and cell cycle progression partially due to its effects on lipid activated kinases, such as protein kinase C (PKC) [9]. While sphingosine was one of the first bioactive lipids identified,

more emphasis has been placed on investigating the roles of ceramide and S1P on cell fate [10]. Ceramide's role in mediating numerous cellular responses to stress, including apoptosis [11] and senescence [12], has been well documented. S1P, on the other hand, plays a crucial role in cell survival [13]. Only a few enzymatic reactions are involved in the inter-conversion between these opposing sphingolipids (Figure 1-1), suggesting a “sphingolipid rheostat” between the levels of ceramide, sphingosine, and S1P that plays an important role in determining cell fate (Figure 1-2) [10, 14]. Therefore, enzymes such as sphingosine kinase 1 (SphK1) that regulate levels of these bioactive sphingolipids are of particular interest to lipid biologists and cancer researchers.

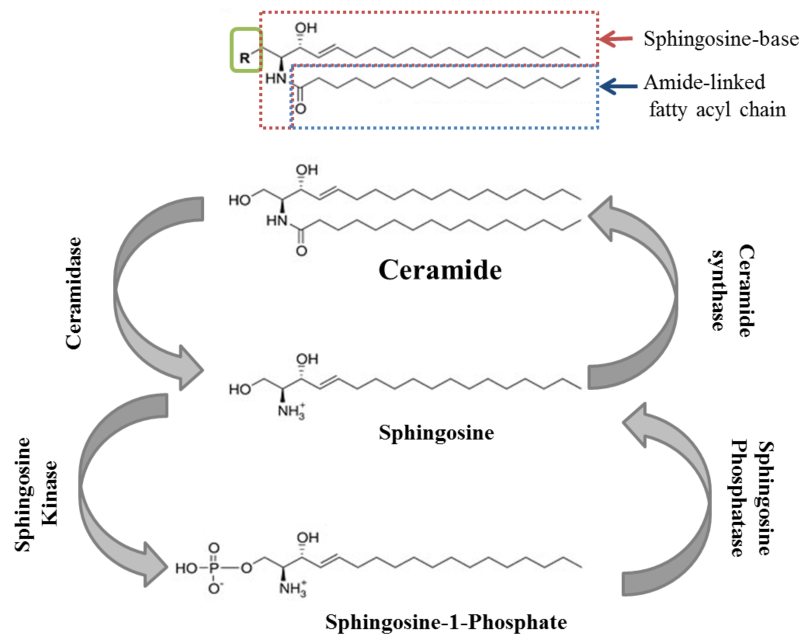


Figure 1-1. The structure and synthesis of ceramide, sphingosine, and sphingosine-1-phosphate

The backbone of sphingolipids is composed of an 18-carbon sphingosine-base. The amino-group of this sphingosine base is linked to the acyl group of an amide-linked fatty acyl chain through an amide bond. These two groups are referred to as the ceramide unit. Most sphingolipids contain a ceramide unit and are differentiated by a wide array of head groups (R) that attach to the sphingosine backbone. Ceramide has an (R) group composed of a

hydroxyl group (–OH) and is a central molecule of sphingolipid metabolism that can be metabolized into various other sphingolipids. Ceramide can also be metabolized by ceramidase, which cleaves the fatty acyl chain from ceramide, producing sphingosine, which in turn gets phosphorylated by sphingosine kinase to form sphingosine-1-phosphate.

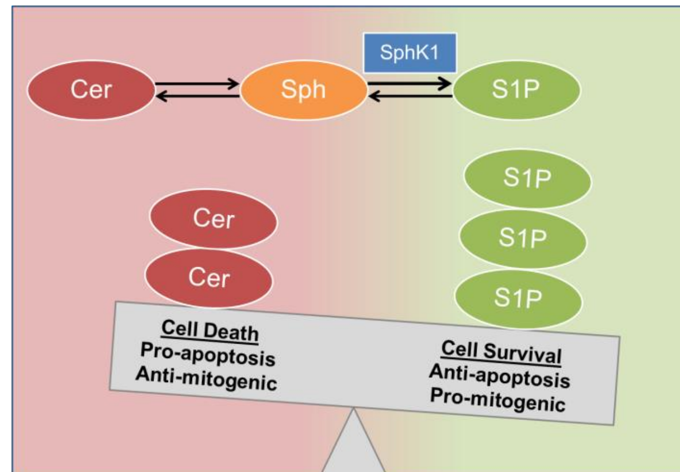


Figure 1-2. The “sphingolipid rheostat” concept

The delicate balance between levels of ceramide, sphingosine, and S1P plays an important role in determining cell fate. Both sphingosine (Sph) and ceramide (Cer) in particular exert anti-proliferative pro-apoptotic effects, whereas sphingosine-1-phosphate (S1P) promotes proliferation, cell survival and inhibition of apoptosis. SphK1 therefore acts as an oncogenic lipid kinase responsible for converting Sph to S1P, and plays a fundamental role in regulating the levels of all three of these bioactive sphingolipids.

Ceramide Signaling:

Ceramide is a potent bioactive sphingolipid that can be synthesized through multiple different cellular pathways. It’s composed of a sphingosine backbone attached to a fatty acyl chain which can vary in length from $C_{14} – C_{26}$ [15, 16]. Ceramide is considered a powerful tumor suppressor, promoting both intrinsic and extrinsic apoptosis, cell growth inhibition, and even autophagy [17, 18]. Therefore, it’s not surprising that cancers are often found to have defects in many enzymes involved in regulating ceramide [19]. In fact,

disruption of ceramide signaling is a key tumorigenic strategy used by cancer cells to escape apoptotic stimuli [20]. On the other hand, ceramide formation can be induced by a variety of death-inducing stimuli such as ϒ-radiation [21], tumor necrosis factor (TNF) activation [22], CD95 clustering [23], and oxidative stress [24]. Furthermore, many chemotherapeutic agents, including anti-mitotic drugs, DNA-damaging agents, kinase inhibitors, histone deacetylase (HDAC) inhibitors, and even certain herbal medicines are known to induce apoptosis through the accumulation of ceramide [25-29]. For example, daunorubicin induced apoptosis has been shown to be dependent upon ceramide generation through activation of ceramide synthase enzymes [26]. In fact, cell-permeable analogs of ceramide were able to mimic daunorubicin induced apoptosis [26]. The combination of sorafenib and vorinostat, a kinase and HDAC inhibitor respectively, is known to increase CD95 activation through a mechanism dependent upon elevating ceramide levels [28]. Ceramide has also proven to be a key regulator of cisplatin induced cell death via activation of ceramide generating pathways [30, 31].

Ceramide often promotes cell death and growth inhibition by acting as an intracellular second messenger, particularly in signaling pathways associated with stress response [18, 32] . For example, ceramide activates various stress- and mitogen- activated protein kinases including protein kinase C zeta (PKCζ) [33], p38 mitogen-activated protein kinase (p38) [34, 35], c-Jun NH_2-terminal kinase (JNK) [36, 37], and the kinase suppressor of Ras (KSR) [38]. Conversely, ceramide inhibits the activities of various pro-survival anti-apoptotic kinases such as extracellular signal-regulated kinase (ERK1/2) [36], the phosphoinositide 3-kinase (PI3K)/Akt pathway [39], and protein kinase C alpha (PKCα) [40]. The mechanism by which ceramide regulates the activity and inhibition of these protein

kinases is believed to involve the activation of protein phosphatases, including the PP2A family [41, 42] as well as PP1 [43]. Ceramide induced activation of protein phosphatases not only induces growth inhibition and death by regulating various protein kinases, but also promotes the de-phosphorylation and inhibition of anti-apoptotic Bcl-2, a protein directly involved in mitochondrial mediated intrinsic apoptosis [44, 45]

While ceramide often promotes cell death and growth inhibition by acting as an important second messenger, it's interesting to note that ceramide is not only an intracellular singling molecule. As a lipid, ceramide is extremely hydrophobic and can alter the biophysical properties of cell membranes. For example, ceramide generation at the plasma membrane can form distinct regions, often termed ceramide-enriched membrane platforms, which co-localize with various proteins promoting their death-inducing downstream signaling [46-48]. Furthermore, several intracellular organelles are involved in the diverse apoptotic signaling induced by ceramide. For example, long chain ceramides species (C_{16} and C_{18}) can directly promote apoptotic signaling by forming channels within the mitochondrial membrane [49].

Taken together, these studies clearly implicate ceramide as a pro-apoptotic, anti-proliferative signaling lipid. However, more recent findings suggest that this simple picture of ceramide signaling doesn't always hold true. In reality, ceramide's role in cell survival is substantially more diverse than originally thought. For one, several studies suggest that many of the observed roles of ceramide may be chain length specific [50-52]. Furthermore, ceramides with different fatty-acid chain lengths have significantly diverse effects on cell growth and survival [53]. For example, long chain ceramides induce growth inhibition and apoptosis in human breast cancer cell lines but very long chain ceramides actually promote

their proliferation. Therefore, these findings support the continued search for chemotherapeutic strategies aimed at increasing intracellular levels of ceramide species with favorable chain lengths.

S1P Signaling:

Unlike the pro-apoptotic anti-proliferative effects of ceramide, S1P is known to promote cell survival, proliferation, migration, angiogenesis [54], and even neovascularization [10, 15]. S1P functions either as an intracellular second messenger or extracellularly through five G-protein-coupled transmembrane receptors S1PR(1-5) [8, 55]. S1P is solely produced by the phosphorylation of sphingosine which is catalyzed by the activity of SphK enzymes. Levels of S1P are also regulated by degradative enzymes such as S1P phosphatase and S1P lyase [56]. Once produced inside the cell, S1P is unable to diffuse across the plasma membrane due to the charge of its polar head group, but can be exported by various transporter proteins including ATP-binding cassette (ABCC) transporter family members, ABCC1 and ABCC2 [57, 58], or spinster homolog 2 (Spns2) [59]. Once excreted, S1P is free to activate the five S1PRs that, through a variety of interacting G-proteins, regulate an array of intracellular signaling pathways. S1PRs vary greatly in their expression patterns. For example, S1PR1, 2 and 3 are expressed in a wide variety of mammalian tissues, whereas S1PR4 is predominantly expressed in certain hematopoietic cells, and the S1PR5 is mostly confined to the central nervous system [60, 61]. These expression patterns, in combination with the wide variety of G-proteins that couple to the receptors, contribute to the diverse array of downstream signaling pathways activated by exogenous S1P [62]. The role

of S1P as a potent antigenic growth factor is one of its more widely studied functions. For example, various *in vitro* studies found that S1P is able to stimulate migration and the formation of capillary-like tubes through binding and stimulating various cell surface S1P receptors like S1PR1 and S1PR3 [63-66]. In further support of these findings, *in vivo* studies have confirmed the pro-angiogenic effects of S1P using ischemic hind limbs of mice [67], where they showed that pharmacological inhibition or siRNA induced downregulation of S1P receptors suppressed tumor angiogenesis and growth [67, 68]. Extracellular S1P also enhances cell proliferation and survival through various mechanisms including activating phosphatidylinositol 3-kinase (PI3K) and Akt signaling [69], significantly suppressing expression levels of pro-apoptotic Bax [6], and enhancing the production of nitric-oxide [7].

In addition to these extracellular actions of S1P, numerous studies have implicated S1P as an intracellular second messenger that promotes cell proliferation, survival, and suppression of apoptosis [62]. A wide variety of growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and TNF-α are known to activate sphingosine kinase [70, 71], the sole enzyme responsible for S1P generation. Unlike the extensively studied extracellular signaling of S1P, the exact mechanisms by which S1P functions intracellularly are not as well characterized. Recent studies have demonstrated S1P's ability to regulate gene expression by binding and inhibiting the enzymatic activity of histone deacetylases HDAC1 and HDAC2 [72]. S1P, produced by SphK1 bound to TNF receptor-associated factor 2 (TRAF2), plays a critical role in NF-κB signaling induced by TNF-α [73], leading to the activation of downstream pathways that promote cell survival. Furthermore, intracellular S1P has also been shown to activate the pro-survival ERK pathway [74] while also inhibiting the pro-apoptotic JNK pathway [75], opposing the effects

of ceramide on these pathways. Many other studies have confirmed a role for S1P in inhibiting ceramide induced apoptosis [75-77]. Together, these findings summarize the importance of S1P as a critical signaling molecule in cancer biology.

Sphingosine Kinase-1 and Cancer

Isoforms of Sphingosine Kinase:

Two human isoforms of sphingosine kinase (SphK), SphK1 and SphK2, are responsible for maintaining the balance between pro-apoptotic ceramide and anti-apoptotic S1P by catalyzing the conversion of sphingosine to S1P. SphK1 and SphK2 are located on separate chromosomes (17q25.2 and 19q13.2 respectively) [78], have individual splice variants [78], and differ slightly in their size (43kDa and 65kDa respectively), structure [79], cellular localization [80], and physiological function [78]. In adult mice, SphK1 and SphK2 have distinct differences in the tissue distribution of their mRNA. SphK2 appears to be ubiquitously expressed in most tissues including the liver, heart, kidney, testis, and brain, with the highest levels detected in the liver and heart [78]. The skeletal muscle and spleen, however, had little to no detectable levels of SphK2 [78]. SphK1 on the other hand had the highest expression in the lung, spleen, and liver [78]. While SphK1 is predominantly cytosolic, post translational modifications, such as phosphorylation, induce its translocation to the inner leaflet of the plasma membrane [81]. In contrast, SphK2 is predominantly located on or in organelles such as the ER [82] and the nucleus [83, 84]. SphK1 or SphK2 knockout

mice develop normally whereas SphK1/SphK2 double knockout is embryonically lethal [85]. Therefore, despite differences, SphK1 and SphK2 appear to have some overlapping physiological functions, particularly during embryonic development. In regards to cancer, however, SphK1 and SphK2 appear to have conflicting roles. While substantial evidence supports the role of SphK1 in promoting cell growth, inhibiting apoptosis, and promoting cancer progression (discussed in more detail in subsequent sections), the role of Sphk2 is much more controversial.

Unlike SphK1, overexpression of SphK2 has been shown to inhibit cell growth and even induce apoptotic cell death [79, 82, 83]. As SphK2 is known to localize to the nucleus, SphK2 may suppress cell growth and induce apoptosis by inhibiting DNA synthesis and disrupting cell cycle progression via regulating the expression of p21, a known cyclin dependent kinase inhibitor [84]. Furthermore, Liu et al. identified a 9-amino acid motif in the SphK2 sequence that is analogous to that found in pro-apoptotic BH3-only Bcl-2 family proteins. They further showed that SphK2 is able to interact with Bcl-xl, a known anti-apoptotic Bcl-2 family protein, likely inhibiting its anti-apoptotic function [86]. While SphK1 gets translocated to the plasma membrane upon activation, SphK2 predominantly remains located at intracellular organelles [82, 83]. Therefore, due to its strict intracellular location, S1P produced by SphK2 is likely not exported across the plasma membrane to engage the pro-survival signaling induced by S1PRs. Nevertheless, there are numerous conflicting reports that suggest SphK2 may in fact have some pro-survival functions. For example, pharmacological inhibition or siRNA mediated downregulation of SphK2 has been shown to induce cell death [87, 88]. Furthermore, downregulation of SphK2 has been shown

to synergize with doxorubicin to induce apoptosis in breast and colon cancers [84], as well as sensitize colon cancer cells to the effects of all-trans-retinoic acid (ATRA) [89].

Sphingosine Kinase 1 as an Oncogene:

The oncogenic potential of SphK1 in cancer cells has been well established. In fact, overexpression of human SphK1 in NIH3T3 fibroblasts induces a transformed phenotype and promotes their ability to form tumors in mice [90]. An extensive amount of data points to SphK1 and aberrant regulation of sphingolipids as important components of tumorigenesis. For example, normal/tumor matched thyroid and lung cancer patient samples showed significantly more SphK1 positive immunostaining in tumor samples compared to the matched normal tissue [91]. Not only is transcriptional upregulation of Sphk1 associated with numerous cancer types, but SphK1 expression also correlates with poor prognosis and numerous adverse clinical parameters of tumor development (Table 1.1). Moreover, increased activity of Sphk1 in various cancer types is associated with metastasis, drug resistance, and reduced overall patient survival [92-96]. Table1.2 summarizes the role of elevated SphK1 expression in promoting drug resistance in a panel of cancer types. Accordingly, pharmacological inhibition or downregulation of SphK1 is shown to sensitize cells to the effects of various chemotherapeutic agents. For example, inhibition of SphK1 activity sensitizes chemo-resistant HL-60 AML cell lines to doxorubicin and etoposide [97] and downregulation of SphK1 reduced expression of Mcl-1 in chronic myeloid leukemia (CML) cell lines, sensitizing them to the cytotoxic effects of Bcr-Abl kinase inhibitors [98]. Since SphK1 is not subject to any known direct mutations, the concept of non-oncogene

addiction has been used to describe the reliance of numerous cancer types on its signaling [99]. While it's unclear what signal(s) lead to the over-dependence on SphK1 for cell survival, numerous mechanisms for its transcriptional upregulation have been proposed. In glioblastoma cells, interleukin 1 (IL-1) has been shown to upregulate SphK1 mRNA and protein levels via a novel AP-1 element found within the first intron of the SphK1 gene [100]. An alternative pathway for SphK1 upregulation was shown in rat pheochromocytoma cells where they demonstrated that nerve growth factor upregulates SphK1 through a pathway involving transcription factor specificity protein-1 [101].

Sphingosine kinase is known to have increased intrinsic activity as a consequence of overexpression in many cancers [102]. However, post-translational activation of SphK1 activity also seems to be important for its oncogenic signaling [103]. This regulation of SphK1 activity can occur through various mechanisms including protein-protein interactions [104] or post translational modifications [81]. For example, the oncogenic truncated isoform form of eukaryotic elongation factor 1a (eEF1A1), also called prostate tumor inducer-a (PTI-1), can directly activate SphK1, which is essential for PTI-1 induced neoplastic cell transformation [105]. Furthermore, calcium and integrin binding protein 1 (CIB1) directly interacts with SphK1 in a Ca^{2+} -dependent manner, which is required for SphK1 translocation to the plasma membrane [106]. ERK1/2 mediated phosphorylation of SphK1 at Ser225 not only increases its catalytic activity but also targets SphK1 to the plasma membrane [81]. The translocation of SphK1 to the plasma membrane has been shown to be integral for its oncogenic activity [103, 107] and transformation capacity [81]. This phosphorylation, which can occur even in the absence of external stimuli, is critical for SphK1 induced proliferation, survival, and transformation, as these effects are blocked upon mutation of the

phosphorylation site [103]. Further studies suggest that the mechanism by which phosphorylation at Ser225 promotes translocation is through modulating the interactions of other SphK1 residues, Thr^{54} and Asn^{89}, with phosphatidylserine at the plasma membrane [108]. Therefore, Ser225 not only induces the translocation of SphK1 to the plasma membrane, but also promotes retention at the membrane. Additional factors that promote SphK1 translocation to the plasma membrane include protein kinase C, which appears to promote the phosphorylation of SphK1 [80].

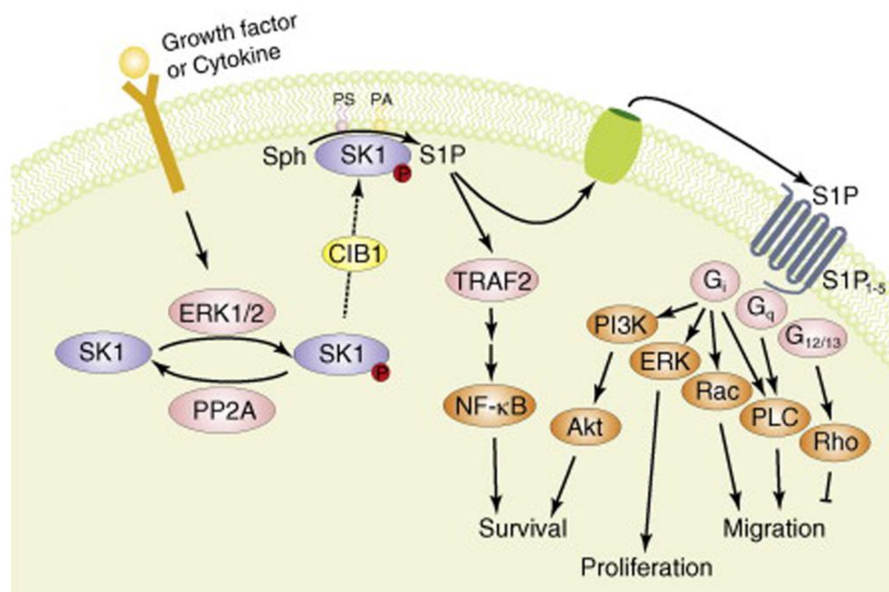


Figure 1-3. SphK1 regulation and signaling

SphK1 is predominantly a cytoplasmic protein but upon its phosphorylation by Extracellular signal-related kinase (ERK1/2) gets translocated to the plasma membrane. This relocation is supported by its interaction with calcium and integrin binding protein 1 (CIB1). Retention of SphK1 at the plasma membrane is facilitated by its interaction with phosphatidylserine (PS) or phosphatidic acid (PA). S1P generated by SphK1 at the plasma membrane can either be exported from the cell to engage the five cell surface S1P receptors or act on certain intracellular targets such as TRAF2. Taken from Piston et al. [109]

Table 1-1. The biological correlation and significance of SphK1 overexpression in numerous human cancer types

Cancer Type	Biological Correlation and Significance	
Breast	Poor prognosis and shorter recurrence time in all subtypes of breast cancer studied. Associated with the development of Tamoxifen resistance in ER+ patients.	[92, 110]
Gastric	Shorter overall survival times	[111]
Glioblastoma	Increased expression of SphK1 correlates with reduced patient survival	[95]
Glioblastoma and Astrocytoma	SphK1 mRNA and protein levels are significantly upregulated relative to adjacent noncancerous tissue. Correlated with Histological grade and shorter patient survival time	[95, 112]
Head and Neck Small Cell Lung Cancer	Overexpressed in HNSCC compared to normal tissue. Correlates with advanced tumor state, nodal involvement, recurrence, shorter patient survival time and loss of p21 expression	[113] [96, 114]
Lung	Correlate with tumor state	[115]
Non-Hodgkin Lymphoma	SphK1 expression correlates with an increase in clinical grade	[116]
Non-small cell lung	Tumor progression, reduced survival	[94]
Prostate	Higher levels of SphK1 enzymatic activity correlates with numerous clinical parameters including positive resection margins, surgical failure	[117]
Salivary Gland	Differentiation and reduced overall patient survival time	[118]
Thyroid	Correlates with the degree of thyroid malignancy and reduced survival time	[91]

Table 1-2. Elevated SphK1 confers chemotherapy resistance to a panel of cancer cell lines

Cancer Type	Chemotherapy	Mechanism of resistance	
Chronic myeloid leukemia	Imatinib Nilotinib	Increased S1P: ceramide ratio Regulating Bcr-Abl1 stability	[93] [119]
Prostate	Camptothecin	CPT induced upregulation of SphK1 enzyme and S1PR3 signaling	[120]
Lung	Cisplatin	Upregulation of p38 and JNK kinase	[121]
Leukemia	Daunorubicin	Increased S1P:ceramide ratio	[122]
Ovarian	N-(4-hydroxyphenyl) retinamide	Increased S1P: ceramide ratio	[123]
Colon	Oxaliplatin	Ceramide accumulation and Akt activation	[124]
Breast	Doxorubicin Docetaxel Tamoxifen	Stimulation of ERK1/2 activity ERK1/2 activation and EGFR transactivation	[125] [126] [127]

Sphingosine Kinase Inhibitors

As described above, SphK is an oncogenic lipid kinase that catalyzes the formation of S1P, a pro-survival signaling lipid, at the expense of the more pro-apoptotic ceramide lipid. Therefore, SphK is an attractive target for cancer therapy and extensive efforts have been made to develop inhibitors targeting SphK1, SphK2, or both. Table 1.3 lists some of the more commonly used SphK inhibitors. Their characteristics with regards to selectivity, potency, and chemotherapeutic potentials are described in more detail below.

Pan SphK1 and SphK2 inhibitors

Some of the first SphK inhibitors identified were sphingosine analogs that typically inhibited both SphK1 and SphK2. Two prime examples include *N*,*N*-dimethylsphingosine (DMS) and L-*threo*-dihydrosphingosine (safingol). Both are micromolar inhibitors of SphK enzymes. In 2003, a chemical library screen yielded four non-lipid based small molecule SphK inhibitors identified as SKI-I, SKI-II, SKI-III, and SKI-IV [128]. Among these, SKI-II is the most orally bioavailable [129] and has since been extensively studied and used as a tool for SphK inhibition. Modification of SKI-I actually lead to the development of SKI-178, the first non-lipid based small molecule SphK1 selective inhibitor [130]. The molecular mechanism of action of SKI-178 is the focus of this dissertation and will be extensively characterized in subsequent chapters.

N,*N*-dimethylsphingosine (DMS) is an *N*-methyl natural derivative of sphingosine. It was originally found to inhibit SphK activity in human platelets [131]. DMS is a competitive

inhibitor of SphK1 with a K_i around 5 μM and a non-competitive inhibitor of SphK2 with a K_i around 12 μM [132]. Interestingly, it was recently demonstrated that in addition to inhibiting SphK1 kinase activity, DMS also led to ubiquitin-mediated SphK1 proteasomal degradation [133]. DMS has been shown to increase ceramide and decrease S1P levels in a panel of cancer cell lines [134], which seems to correlate with its ability to induce apoptotic cell death in a panel of solid tumors [135] and leukemic cell lines [136]. DMS also has been reported to be phosphorylated to DMS-1-phosphate, but little has been studied on this compound since [136]. Unfortunately, DMS has been shown to have numerous off-targets that limit its use as a tool for studying SphK selective inhibition. For example, DMS is known to have inhibitory effects on numerous cellular kinases such as protein kinase C (PKC) [137], ceramide kinase [138], src tyrosine kinases[139], and mitogen activated protein kinase (MAPK) [135].

L-*threo*-dihydrosphingosine (safingol), a synthetic sphingosine analogue, was originally described as a PKC inhibitor. However, being a sphingosine analogue, it was also found to be a potent inhibitor of SphK activity [131]. Since then, safingol has been shown to induce apoptosis in a panel of cancer cell lines, including oral squamous cell carcinoma (SCC) cells, where it was also shown that Bcl-2 family proteins, such as Bim and Bcl-xL, were involved in safingol induced apoptosis [140]. Unfortunately, like DMS, various other protein kinases, such as MAPK, are reported to be directly inhibited by safingol [141]. Nonetheless, safingol is a proven chemotherapeutic agent and has been successfully used in clinical trials as monotherapy or in combination with other chemotherapeutic agents such as doxorubicin and cisplatin [142, 143]. However, due to the lack of selectivity, safingol is not an appropriate agent for studying the effects of SphK inhibition.

SKI-II (or SKi) is another commonly used inhibitor of both SphK1 and SphK2. It has been shown to inhibit proliferation and induce apoptotic cell death in a panel of cancer cell lines [128, 144]. While SKI-II is known to inhibit the kinase activity of both SphK enzymes [145], it has also been shown to induce ubiquitin-proteasomal degradation of SphK1, which has proven to be critical for its apoptotic effects [133]. In podocytes, SKI-II also induces degradation of SphK1. However, in podocytes, this was mediated by a lysosomal-dependent pathway [146]. Interestingly, this was not reproducible in prostate cancer cell lines, suggesting that this route of SphK1 degradation may not operate in these or other cancer cell lines [133]. As for its chemotherapeutic potential, SKI-II has been shown to decrease proliferation and viability of multidrug-resistant breast cancer cells [145] and induce apoptotic cell death in human prostate adenocarcinoma cell lines [147]. Furthermore, SKI-II has proven to inhibit *in vitro* growth of human acute myeloid leukemia cells and has been found to be generally safe to normal peripheral blood mononuclear cells [148]. Unlike DMS and safingol, SKI-II does not appear to inhibit kinases such as PKC, MAPK, and phosphoinositide 3-kinase (PI3K) [128]. Nonetheless, there is evidence to suggest that SKI-II exerts some of its effects through targets other than SphK. For example, SKI-II is known to reduce the transcriptional activity of estrogen-stimulated estrogen response element in a dose dependent manner through a SphK-independent mechanism [149]. Furthermore, a recent study by Cingolani et al. demonstrated that SKI-II is also a noncompetitive inhibitor of Dihydroceramide desaturase (Des1), the last enzyme in the *de novo* pathway of ceramide synthesis [150]. Des1 functions to regulate the balance between ceramide and dihydroceramide (dhCer), an important bioactive dihydrosphingolipid shown to induce autophagic cell death [151].

SphK1 selective inhibitors

FTY720 or fingolimod (commercially available as Gilenya^{TM}) is a sphingosine analog. It was originally found to exert immunosuppressive effects through binding and modulating signaling of S1PRs [152]. However, it was later shown that FTY720 inhibited SphK1 catalytic activity but did not inhibit SphK2, despite the fact that it was phosphorylated by Sphk2 before exerting its effects on S1PRs [153]. Interestingly, while FTY720 is known to modulate the activity of SphK1, it has also been shown to induce its proteasomal degradation, which likely contributes to its SphK1 inhibitory effects [144, 154]. Numerous *in vitro* and *in vivo* studies have demonstrated the chemotherapeutic potential of FTY720 in a panel of cancer cell lines including prostate [154, 155], breast [154, 156], lung [157], pancreatic [158], and bladder[159] cancers. As both S1PR and SphK1 signaling contribute to cancer growth and progression, it's possible that FTY720 induced cell death involves both these targets. Furthermore, various off-target effects of FTY720 may also contribute to its anti-cancer properties. For example, FTY720 directly activates protein phosphatase 2A (PP2A), a serine/threonine phosphatase known for its tumor suppressor functions [160]. Additionally, FTY720 may exert some of its anti-cancer properties through disruption of the PI3K/Akt pathway [40] via mechanisms involving increasing PTEN expression [161] or activation of PP2A [160].

SK1-I (BML-258) was originally developed as one of the first potent SphK1 isoenzyme selective inhibitors that induced apoptotic cell death in a panel of leukemic cancer cell lines [162]. It was subsequently shown to not only induce apoptosis, but also inhibit invasion of various human glioblastoma cell lines [163]. Furthermore, it reduced growth and

promoted apoptosis in glioblastoma xenograft models [163]. These studies found that the effects of SK1-I appeared to involve inhibition of Akt signaling with a concomitant activation of the JNK pathway. SK1-I is not only effective at killing cancer cells *in vitro*, but also in decreases hemangiogenesis and lymphangiogensis, two very important micro-environmental factors required for cancer progression and metastatic development [54]. While off-target effects can't be completely ruled out, SK1-I does not appear to inhibit SphK2 or other protein kinases such as PKC [162], which are often inhibited by some of the pan-SphK inhibitors described above [139].

Owing to the effectiveness of SK1-I and other SphK inhibitors, Mathews et al. sought to add to the pool by developing a series of amidine-based SphK inhibitors with varying degrees of SphK isoform selectivity [164]. One of the more notable was VPC45129 that inhibited the activity of both SphK1 and SphK2. These amidine-based inhibitors were selective for SphK enzymes and weren't found to inhibit other lipid or protein kinases. Furthermore, unlike SK1-I, they worked at sub-micromolar concentrations [165]. These inhibitors were used as the starting point for optimization studies in search of more specific SphK1 selective inhibitors. They developed an array of SphK1 subtype-selective inhibitors that were found to significantly lower endogenous S1P levels in human leukemia cell lines at low nM concentrations but were not shown to have any effect on cell growth or survival [166, 167]. Around the same time, Schnute et al. developed PF-543, another inhibitor that is 100-fold more selective for SphK1 over SphK2 with a K_i in the low nM range [168]. Despite being the most potent SphK1 selective inhibitor described to date, PF-543 also had little to no effect on cell viability both *in vitro* and *in vivo*.

SphK2 selective inhibitors

Although numerous studies have described the pro-apoptotic, anti-proliferative roles of SphK2 [83, 169], others studies have contradicted these findings by demonstrating that SphK2 may instead promote cancer cell survival [84, 95]. ABC294640 was developed as a sphingosine competitive, SphK2 selective inhibitor with a K_i around 9.8 µM [87]. While ABC294640 shows no direct inhibitory effects against recombinant SphK1 even at concentrations as high as 100 µM [87], recent findings have shown that ABC294640 can induce proteasomal dependent degradation of SphK1 at concentrations around 10-25 µM [170]. Although the potential for off-target effects still exist, ABC294640 was tested against a range of serine/threonine and tyrosine kinases and none were shown to be significantly inhibited. As for its chemotherapeutic potential, ABC294640 was found to inhibit proliferation and cell migration in numerous cancer cell lines [87, 171] as well as induce autophagy in prostate, breast, and kidney cancer cell lines [171]. In multiple myeloma cell lines, ABC294640 induced apoptotic cell death that correlated with a downregulation of pS6 expression (inhibiting the mTOR pathway) and the degradation of anti-apoptotic Mcl-1 [172]. Furthermore, ABC294640 prolonged survival in mouse xenograft models of hematological malignancies [173]. The success of ABC294640 has confirmed the chemotherapeutic potential of inhibiting SphK2.

A second SphK2 selective inhibitor, (R)-FTY720-Ome (ROMe), was developed shortly after ABC294640. It is actually an analogue of FTY720 where one of the prochiral-hydroxyl groups is replaced with a methoxy-group [144]. This replacement blocks the site of FTY720 that gets phosphorylated by SphK2. This change yielded a sphingosine competitive

inhibitor of SphK2 that had little to no effect on SphK1 [174]. These studies showed that ROMe induced proteasomal degradation of SphK2 similar to the findings that FTY720 induced proteasomal degradation of SphK1. Unfortunately, since its discovery, little has been done to uncover the chemotherapeutic potential of ROMe.

Table 1-3. Sphingosine Kinase inhibitors, their structures, and their inhibition properties

	SphK Inhibitor	Structure	Inhibition Properties
Pan inhibitors of SphK1/2	**SKI-II / SKi** 2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole		Small molecule. Competitive and non-competitive inhibitor of both Sphingosine and ATP binding
	DMS *N*,*N*-dimethyl-sphingosine		Sphingosine Analog. Sphingosine Competitive
	Safingol (2*S*,3*S*)-2-Aminooctadecane-1,3-diol		Sphingosine Analog. Sphingosine Competitive
SphK1 selective	**FTY720 (Fingolimod)** 2-Amino-2-[2-(4-octylphenyl)]-1,3-propanediol hydrochloride		Sphingosine Analog. Sphingosine Competitive and ATP non-competitive inhibitor
	SK1-I / BML-258 (2*R*,3*S*,4*E*)-*N*-methyl-5-(4′-pentylphenyl)-2-aminopent-4-ene-1,3-diol		Sphingosine Analog. Sphingosine Competitive
	PF-543 (R)-[1-(4-[3-methyl-5-(phenylsulfonylmethyl)phenoxy]methyl)benzyl) pyrrolidin-2-yl]methanol		Sphingosine Competitive
	SKI-178 N’-[(1E)-1-(3,4-dimethoxythenyl)ethylidene]-3-(4-methoxyphenyl)-1H-pyrazole-5-carbohydrazide		Small Molecule Sphingosine Competitive
SphK2 selective	**(R)-FTY720-Ome/ ROMe** (2*R*)-2-Amino-3-(*O*-methyl)-(2-(4′-*n*-octylphenyl)ethyl)propanol		Sphingosine Analog Sphingosine Competitive
	ABC294640 [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide]		Small Molecule. Sphingosine Competitive inhibitor

Chapter 2

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

Abstract

We have previously developed SKI-178 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 NH_2-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 pro-survival Bcl-2 family members, Bcl-2 and Bcl-xl, as well as the phosphorylation and subsequent degradation of Mcl-1. Moreover, multi-drug resistance mediated by MDR-1 and/or pro-survival Bcl-2 family member over-expression 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 multi-drug resistant/recurrent AML subtypes.

Introduction

AML is a heterogeneous disease characterized by a multitude of genetic mutations and chromosomal abnormalities [215]. 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 [216]. Bioactive sphingolipids have emerged as important regulators of numerous biological processes including cell survival, cell proliferation, inflammation, and carcinogenesis [217, 218]. 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 anti-proliferative, pro-apoptotic effects [4, 5], whereas S1P promotes proliferation, cell survival, and inhibition of apoptosis [6-8]. SphK1, an 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 [8].

Elevated expression of SphK1 is associated with numerous cancer types [91, 98, 100, 117, 122, 219] and several lines of evidence link SphK1 deregulation with the progression of various hematological malignancies [95, 97, 116, 162, 220]. For example, over-expression of SphK1 has been shown to be an oncogenic event in acute erythroid leukemia progression [221]. Furthermore, SphK1 is also shown to mediate chemotherapy sensitivity in AML cells. In fact, a study by Sobue et al. identified the cellular Ceramide:S1P ratio as a critical biosensor for predicting AML cell sensitivity to daunorubicin [122]. Specifically, they demonstrated that in AML cells, a low

Ceramide:S1P ratio, associated with high SphK1 activity, is correlated with a robust intrinsic chemo-resistance to daunorubicin. They further demonstrated that increasing the Ceramide:S1P ratio (using SphK inhibitors (SKI-II) or SphK1 siRNAs to reduce SphK1 activity and up-regulate 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 [222]. Additionally, Bonhoure et al. [97] demonstrated that targeting SphK1 induces apoptosis of multi-drug resistant HL-60 cells (HL-60/VCR). Together, these findings implicate SphK1 as a novel chemotherapeutic target to overcome chemo-resistance in hematological malignancies.

We developed SKI-178 as a novel small molecule, isotype-specific, non-lipid substrate inhibitor of SphK1 that is cytotoxic toward a broad panel of cancer types, including AML [130]. Given the association of SphK1 with development and progression of leukemia 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 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 resistant types. Taken together, we provide evidence that SKI-178 induces apoptosis in a CDK1-dependent manner, and is not a

substrate for multi-drug resistant protein (MDR), 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 (N'-[(1E)-1-(3,4-dimethoxyphenyl)ethylidene]-3-(4-methoxyphenyl)-1H-pyrazole-5-carbohydrazide) (ChemBridge Corporation, San Diego, CA), vincristine (Thermo Fisher Scientific, Waltham, MA, USA), Colchicine (Enzo Life Sciences, Farmingdale, NY, USA), RO3306 (Sigma-Aldrich, St. Louis, MO), antibodies against total Bcl-2, pBcl-2(Ser70), Mcl-1, Caspase 3, Caspase 9, Cleaved caspase 3 (Active), Cleaved caspase 9 (Active), Cleaved caspase 7 (Active), pHistone H3(Ser10), pCDK1(Tyr15) pJNK(Thr183/Tyr185), PARP, and Actin (Cell Signaling Technology, Danvers, MA, USA), Sphk1 (Abgent, San Diego, CA, USA), pBcl-xl(Ser62) (Abcam, Cambridge, MA, USA), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell lines and culture conditions: Human acute myeloid leukemia (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 (ATCC, Manassas, Virginia, USA). HL-60/VCR cells were a gift from Dr. Hong-Gang Wang (Penn State College of Medicine, PA, USA). HL-60 and HL-60/VCR were maintained in DMEM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% FBS (Denville Scientific, South Plainfield,

NJ, USA). U937 and THP-1 cell lines were maintained in RPMI (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS. All cell lines were maintained at 37°C with 5% CO_2 in a humidified incubator

Cell viability assays: Cells were treated with increasing concentrations of SKI-178, vincristine, or Colchicine in 96 well plates for 48h. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell-metabolism assay (ATCC, Manassas, Virginia, USA) according to manufacturer recommendations. Briefly, following treatment, 10µl of MTT were added to each well and incubated for 4h at 37°C with 5% CO_2 in a humidified incubator. After the 4h incubation, stop solution was added, kept overnight at room temperature, and absorbance was recorded at 570 nM. The results were normalized to vehicle (DMSO) control. IC_{50} values based on cell viability were determined using GraphPad Prism version 5.0 (GraphPad Software) based on three separate experiments (n=3).

Sphingosine Kinase activity assay and thin-layer chromatography: Briefly, whole cell lysates were prepared from $1.0x10^6$ cells in buffers selective for SphK1 (20mM Tris pH 7.4, 1mM β-mercaptoethanol, 1mM EDTA, 1.0% Triton X-100, 1mM Na_3VO_4, 15mM NaF, 0.5 mM 4-deoxypyridoxine) or SphK2 (20mM Tris pH 7.4, 1mM β-mercaptoethanol, 1mM EDTA, 1M KCl, 1mM Na_3VO_4, 15mM NaF, 0.5mM 4-deoxypyridoxine). 50µg of total protein lysates were combined with 50µM D-erythro-sphingosine, 200µM ATP and 2µCi [γ-^{32}P] ATP in a 100µL final reaction volume and incubated for 1h at 37°C with shaking. Kinase reactions were terminated by the addition of 10µL 6N HCl and the radio-labeled lipids were extracted by the addition of 400µL of Chloroform/MeOH (100:200 v/v) and 125µL Chloroform and 125µL 1M KCl. The

organic phase containing lipids was dried down under nitrogen stream. Samples, along with separate S1P standards generated by sphingosine kinase activity assay using purified recombinant SphK1 as an enzyme source (Sigma-Aldrich, St. Louis, MO), were then resuspended in 30μL chloroform and applied to a Silica Gel TLC 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 TLC plate corresponding to the *Rf* value (0.32) of S1P was examined.

Sphingolipid analyses: Sphingolipids from treated HL-60 cells were analyzed by LC/ESI-MS/MS essentially as described previously [223] with minor modifications as described herein. Total lipids were extracted from HL-60 cell lysates equivalent to 500μg of total protein. Extracted lipids were then separated on an Agilent 1100 HPLC system according to chromatography conditions described previously [223], but with 0.1% formic acid in the mobile phases at a flow rate of 0.4mL/min on a Phenomenex Kinetex C8 (2.6 μm) 2mm ID × 5cm column maintained at 60°C. The eluate was analyzed with an inline AB Sciex 4000 Q Trap mass spectrometer equipped with a turbo ion spray source. 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 pmol/mg total cellular protein.

Western blot analysis: Western blot analysis was performed as previously described [224] with some modifications. Briefly, whole cell lysates were harvested in 100μL 1X RIPA buffer (20mM Tris-HCL (pH 7.5), 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM sodium orthovandate, 30mM sodium fluoride, and complete

protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany)). Lysates were centrifuged (≥10,000g) for 15min 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 1h at room temperature (RT) 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, Massachusetts, USA).

Analysis of apoptosis: HL-60 cells were treated with either vehicle (DMSO) for 48h or SKI-178 (5 μM) for 16, 24 or 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. Final analysis of the data was 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 [225]. Briefly, cells were synchronized at the G1/S phase border by culturing cells in DMEM + 10% FBS containing 2mM thymidine (Sigma-Aldrich, St. Louis, MO) for 19h. Cells were then released from the G1/S phase block by washing twice with phosphate-buffered saline (PBS) and re-suspending them in thymidine-free culture medium for 9h. Cells were again

treated with 2mM thymidine in DMEM + 10% FBS for an additional 16h. After the second block, cell were washed twice with PBS and re-suspended 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 (5µM) or DMSO (Vehicle) for 4, 8, or 16 h. Cells were washed with PBS, fixed in cold 70% ethanol, and incubated at -20°C for at least 30min. Once all time points were collected, fixed cells were washed twice with PBS and resuspended in 1ml PBS containing 0.1%(v/v) Triton X-100, 100µg/ml PI (Invitrogen, Carlsbad, CA, USA), and 200µg/ml Ribonuclease A (Qiagen, Venlo, Netherlands). Cells were stained with PI for at least 30min and analyzed using the FACScan analyzer (Becton Dickinson, Franklin Lakes, NJ, USA). Data was processed and analyzed using FlowJo V10 software (Tree Star, Inc., San Carlos, CA).

siRNA transfection: Duplexed Stealth siRNA (Life technologies Carlsbad, CA, USA) was used to knockdown SphK1 expression in human MIA PaCa-2 cell line.

siRNA sequences:

siSphK1 #1: 5'-CCUACUUGGUAUAUGUGCCCGUGGU-3'

siSphK1 #2: 5'-GAGGCUGAAAUCUCCUUCACGCUGA-3'

Transfections of MIA Paca-2 cells were carried out by reverse-transfecting 0.5 x10^6 cells with RNAiMAX (Life Technologies) containing 12.5pmol or 25pmol of respective siRNAs according to manufacturer's recommendations. Lysates for Western blotting or SphK activity assays were collected after 72h incubation.

Results

SKI-178 induces cytotoxicity in a panel of AML cell lines, including multidrug resistant HL-60/VCR

We have previously demonstrated that SKI-178 is cytotoxic toward a broad panel of cancer cell lines [130]. 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 [97, 162, 226]. As shown in Figure 2-1A, 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 [130], we determined that SKI-178 is cytotoxic toward all AML cell lines examined, with IC_{50} values ranging from ~500nM to ~1μM (Table 2-1). Although not statistically significant, the range of IC_{50} values appears to correlate with the relative levels of SphK1 catalytic activity (Figure 2-1B) 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 the lower IC_{50} values than HL-60/VCR, U937, and THP-1 cells.

A major obstacle for treatment of AML patients is the fact that many have leukemic cells that are either inherently refractory to certain chemotherapeutics or develop multidrug resistance in relapsed AML [227, 228]. 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 [229, 230]. Figure 2-1C confirms that HL-60/VCR cells express high levels of the multi-drug resistant protein (MDR1) compared to all other AML cell lines tested. Vincristine and colchicine are chemotherapeutics known to be effluxed by multi-drug resistant proteins [231, 232]. We therefore confirmed drug resistance in HL-60/VCR cells by comparing IC_{50} values, for vincristine and Colchicine, to the parental HL-60 cell line. As outlined in Table 2-1, the IC_{50} values of vincristine and Colchicine were significantly higher in HL-60/VCR compared to 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 multi-drug resistance [229, 233]. Interestingly, as shown in Figure 2-1D and summarized in Table 2-1, the IC_{50} value for SKI-178 is only about 2 fold higher in HL-60/VCR compared to HL-60 cells. This is considerably lower than the 1,000 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).

Table 2-1. Cytotoxicity (IC_{50}) of SKI-178 vs. Vincristine and Colchicine

AML cell line:	SKI-178 (nM)	Vincristine (nM)	Colchicine (nM)
HL-60	472 ± 2.95	2.3 ± 0.016	9.5 ± 0.034
HL-60/VCR	1057 ± 3.06	2280 ± 9.3	583 ± 2.6
THP-1	686.7 ± 1.98	NA	NA
U-937	885.4 ± 1.98	NA	NA

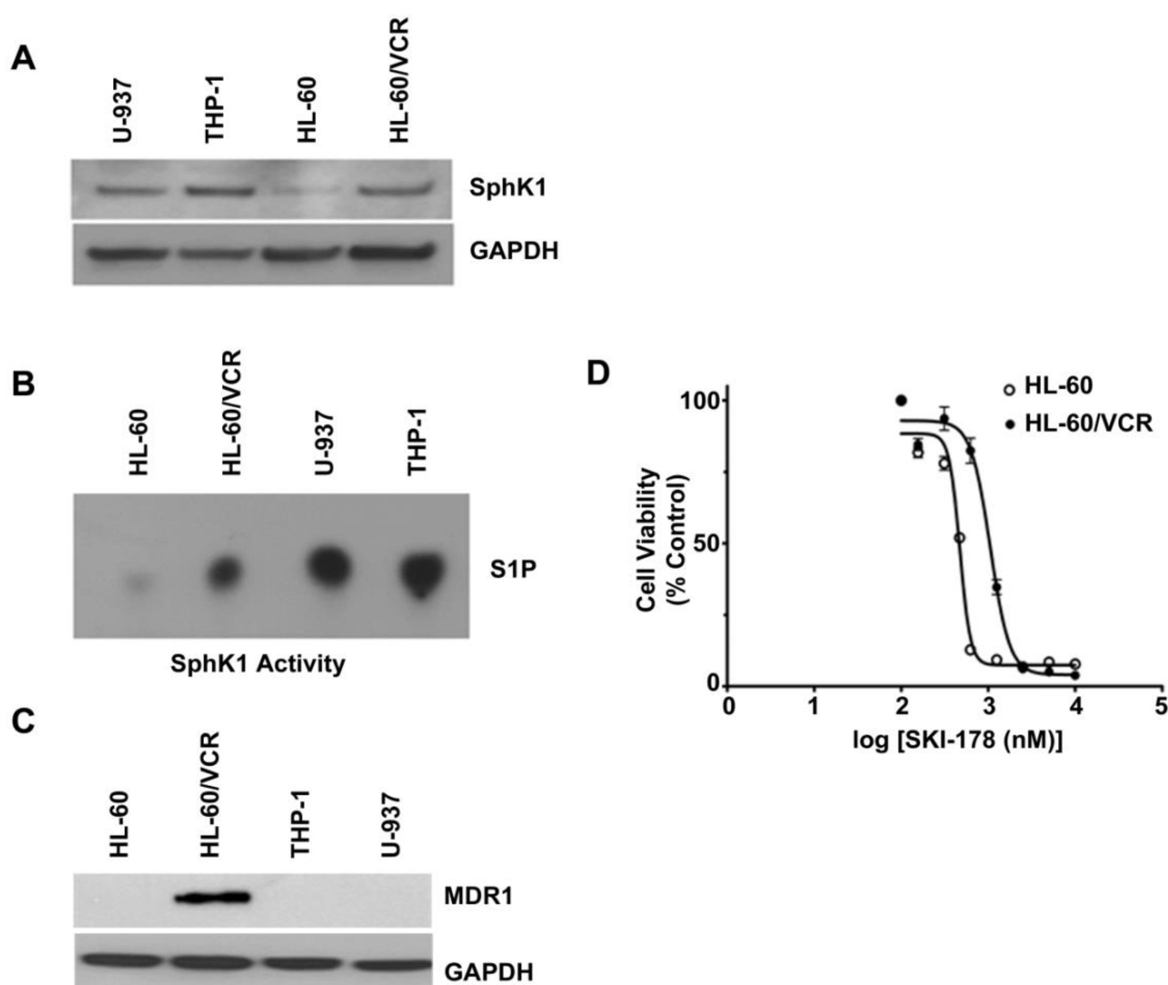


Figure 2-1. SKI-178 induces cell death in a panel of AML cell lines, including multidrug resistant HL-60/VCR.

(A and C) Whole cell lysates from the indicated AML cell lines were subjected to Western blot analysis to assess SphK1 (A) and MDR-1 (C) expression. GAPDH serves as an equal loading control. (B) Whole cell lysates from the indicated AML cell lines were prepared in buffer selective for SphK1 activity, and SphK1 catalytic activity was

subsequently determined by thin-layer chromatography. (D) HL-60 and HL-60/VCR cells were exposed to increasing concentrations of SKI-178 over a 48h period and cytotoxicity was assessed by MTT assays. Error bars indicate standard deviation (SD) of triplicate counts of 2,500 cells.

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-amino-actinomycin D (7-AAD) as a marker of necrosis. In this assay, cells in the early stages of apoptosis stain Annexin V-positive and 7-AAD-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 Figure 2-2A, the majority of vehicle treated cells were viable and stain negative for both Annexin V and 7AAD (lower left quadrant, Q4). After 16h of SKI-178 treatment, we observed an increase in the number of early apoptotic cells (Q3). By 24h of SKI-178 treatment, almost 60% of HL-60 and almost 50% of HL-60/VCR cells were apoptotic (Q2 + Q3) (Figure 2-2B) with an increasing percentage of these in the later stages of apoptosis (Q2) (Figure 2-2A). By 48h, greater than 90% of HL-60 and almost 70% of HL-60/VCR cells were in either the early or late stages of apoptosis (Figure 2-2B). The fact that cells progress through early apoptosis in a time dependent manner prior to 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 (MOMP) resulting in apoptosome 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 5µM SKI-178 for indicated time points. The activation of pro-apoptotic signaling (i.e., PARP cleavage) correlated with the cleavage and activation of Caspase 9 and Caspase 3 (Figure 2-2C) 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).

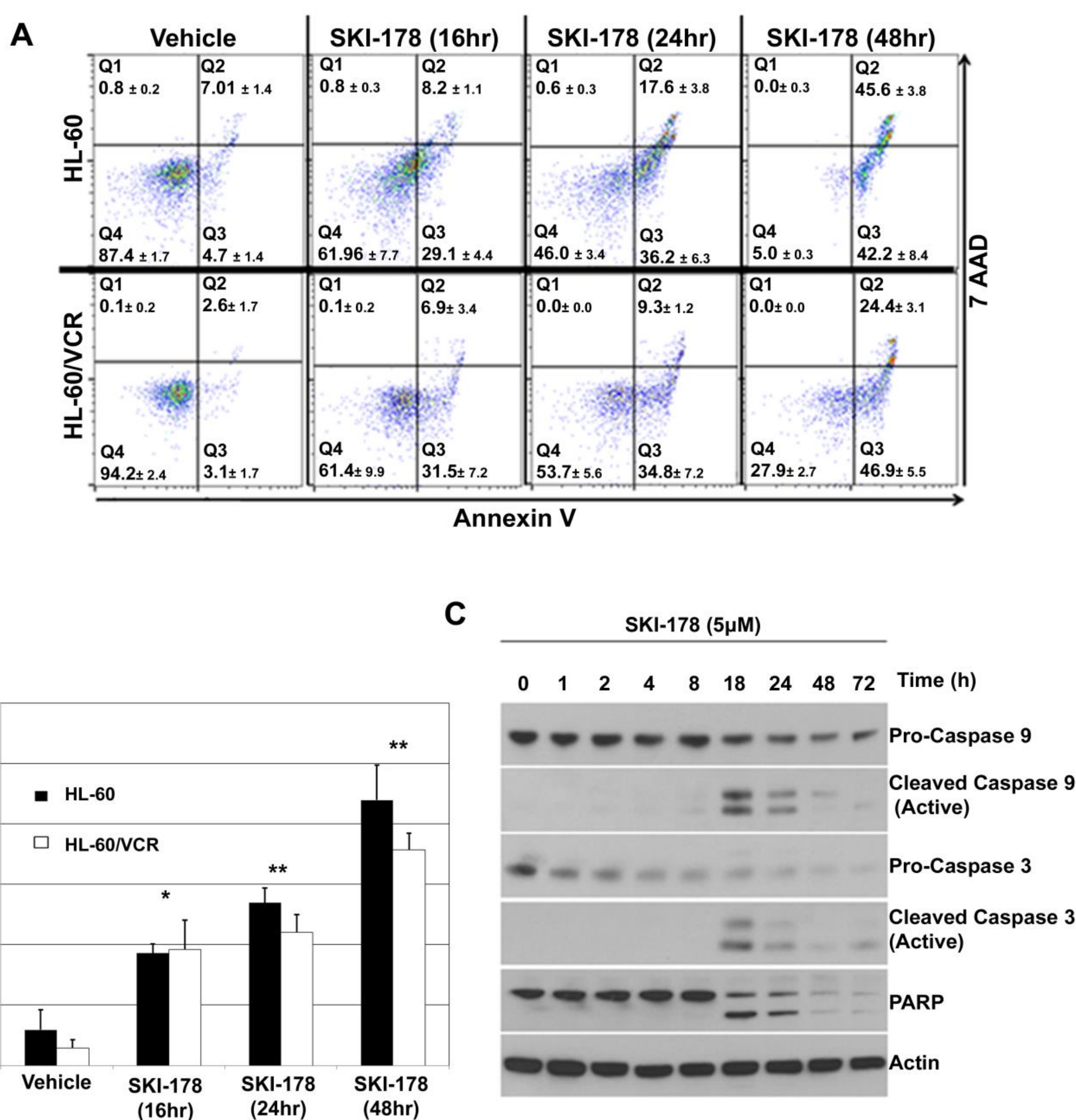


Figure 2-2. SKI-178 induces apoptosis through the intrinsic apoptotic signaling pathway:

(A) Cytometric analysis of Annexin V/7AAD stained HL-60 and HL-60/VCR cells treated with SKI-178 (5μM) for indicated time periods or vehicle (DMSO) control for 48h. 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 SD of triplicate counts of 5000 cells. Statistical significance was assessed by two-tailed paired student's t test. Asterisks indicate significance: *$p \leq 0.001$; **$p \leq 0.0001$. (C) HL-60 cells treated with SKI-178 (5μM) for indicated time intervals. Western blot analysis was performed on whole cell lysates with proteins indicated. GAPDH was used as a loading control.

SKI-178 alters bioactive sphingolipid levels

In previous studies we have demonstrated selective SphK1 inhibition with SKI-178 *in vitro* as well as in whole cells using A549 lung cancer cells [130]. Hence, we used an established LC-MS/MS method [129, 234, 235] 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 24h SKI-178 (5µM) treatment relative to the control vehicle (DMSO) treated HL-60 cells (Table 2-2). Sphingosine levels were also decreased after 24h, 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: C_{16}-C_{18}) compared to very long chain Ceramide (VLCC: C_{22}-$C_{24.1}$) species. The LCC species are known to exert pro-apoptotic effects, whereas VLCC species have recently been suggested to promote cell growth [52, 236, 237]. Accordingly, 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 24h relative to vehicle control. Together, these results demonstrate that SKI-178 treatment alters levels of bioactive sphingolipids that favor anti-proliferation and/or apoptosis

Table 2-2. Sphingolipid analysis of vehicle and SKI-178 (5 μM) treated HL-60 cells at 24h

	pmol/mg total protein					
	Sph	**S1P**	**C16**	**C18**	**C22**	**C24:1**
Vehicle	21.96 ± 1.98	6.98 ±1.24	22.72 ±2.07	0.22 ±0.06	1.58 ±0.43	4.22 ±0.42
SKI-178	15.12 ±1.22	2.02 ±0.02	49.84 ±2.57	0.89 ±0.08	0.3 ±0.07	0.86 ±0.05

SKI-178-induced apoptotic cell death correlates with prolonged Bcl-2 phosphorylation

The overall balance between levels of 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 [75]. S1P is known to exert some of its mitogenic effects through the activation of both extracellular-signal-regulated kinase (ERK), a pro-survival anti-apoptotic MAPK, and the PI3K/AKT pro-survival pathway [39, 74]. Sphingosine and Ceramide, on the other hand, are believed to induce cell death via activation of pro-apoptotic MAPKs, such as c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38) [34-36, 238]. Based on these previous studies, we examined the effect 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, SB202190, PD98059, or Wortmannin, inhibitors of JNK,

p38, MEK1 (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 (Figure 2-3A), 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 Figure 2-3B, JNK activity (indicated by phosphorylation at Thr183/Tyr185) increased in a time dependent manner starting as early as 2h after 5μM SKI-178 treatment and continued to increase for at least 24h. Between 8-24h 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 [239, 240]. Bcl-2 phosphorylation at Ser70 increased with time in response to SKI-178 treatment, reaching maximal levels at 8h which was consistent with the timing of caspase 7-activation (Figure 2-3B).

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 [241, 242]. However, a recent study provided evidence that a known JNK inhibitor, SP600125, also inhibits CDK1 [243]. Our findings in Figure 2-3A, 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) and CDK1

(RO3306) on SKI-178 mediated Bcl-2 phosphorylation. Interestingly, as shown in Figure 2-3C, both the JNK and CDK1 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.

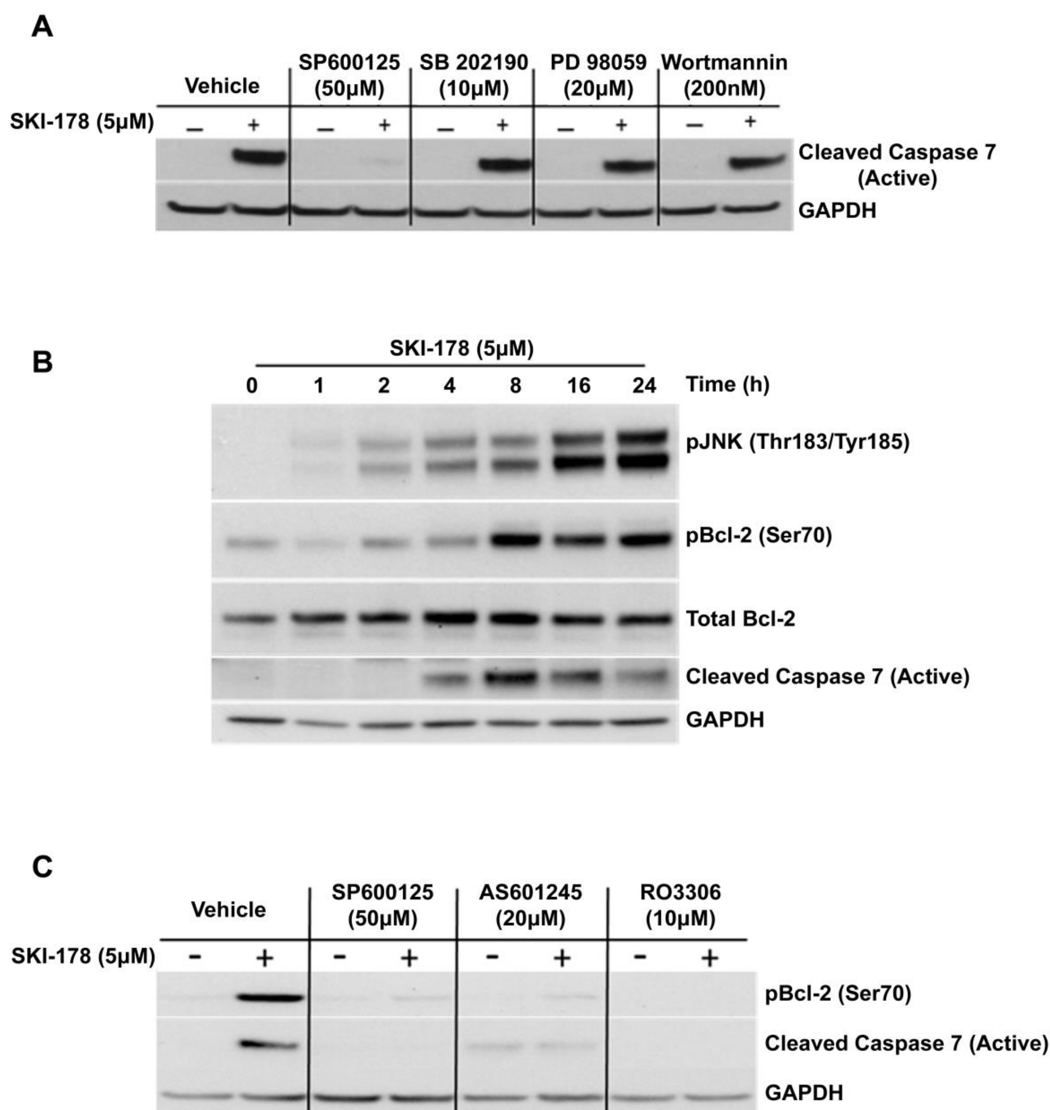


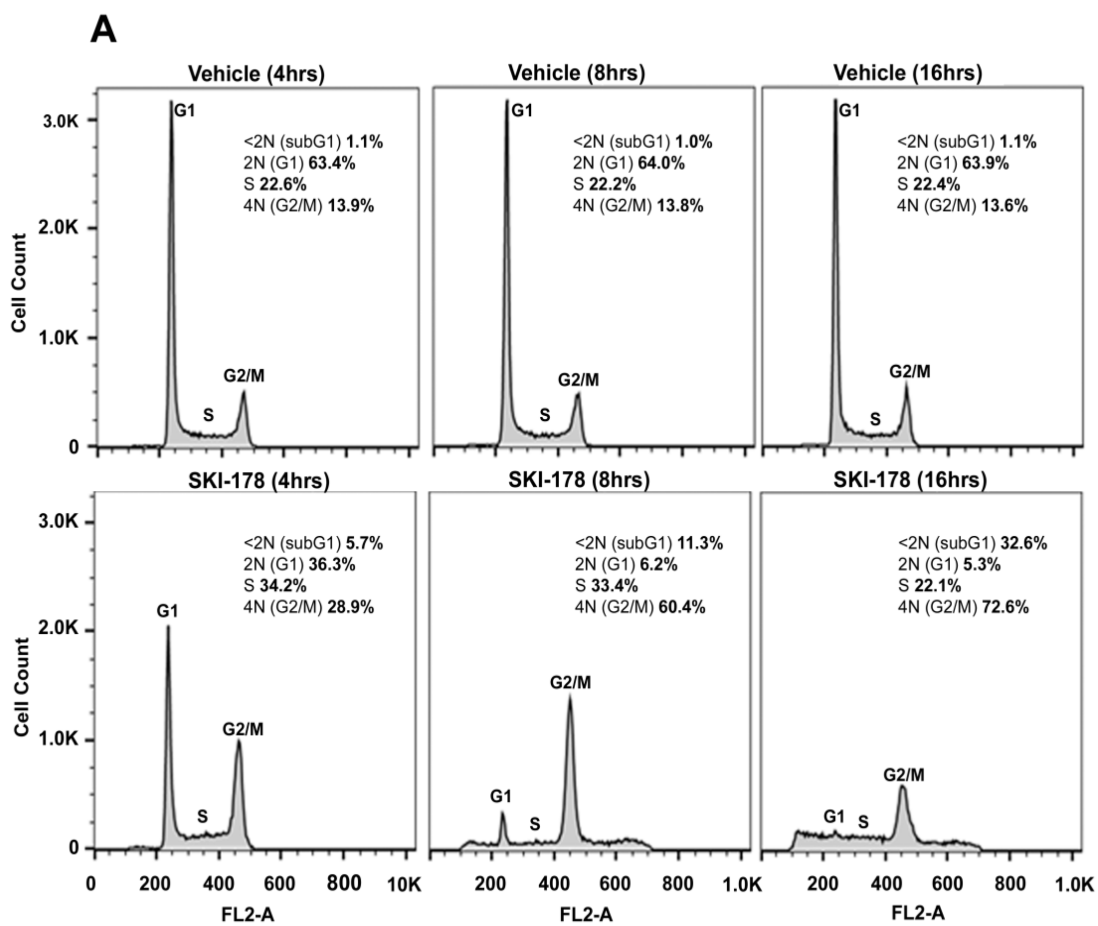
Figure 2-3. SKI-178-induced Bcl-2 phosphorylation and Caspase 7 activation are blocked by JNK and CDK1 inhibitors.

(A) HL-60 cells treated for 24h with various MAPK inhibitors or vehicle (DMSO) alone or in combination with SKI-178 (5 μM). Western blot analysis was performed on whole cell lysate using antibody for cleaved caspase 7. (B) HL-60 cells treated with SKI-178 (5 μM) for indicated time intervals. Western blot analysis was performed on whole cell lysates with pJNK (Thr183/Tyr185) antibody, or for the proteins indicated. GAPDH was used as a loading control. (C) HL-60 cells treated for 24h with vehicle (DMSO), SP600125, a JNK specific inhibitor (AS601245), or a CDK1 specific inhibitor (RO3306) alone or in combination with SKI-178. Western blot analysis was performed on whole cell lysate using antibody 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.

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 [244, 245]. In contrast, enhanced and prolonged levels of Bcl-2 phosphorylation in mitosis are commonly associated with apoptotic cell death [214, 246]. 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 16h, and DNA content was examined using propidium iodide (PI) staining followed by flow cytometry. As shown in Figure 2-4A, 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 4h of SKI-178 treatment. By 8 and 16h 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 [247]. 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 Figure 2-2A 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 16h. Analysis of their DNA content also revealed substantial increase in cells with G2/M DNA content (Figure 2-4B). These results indicate that SKI-178 induces apoptosis through a sustained/prolonged cell cycle at mitosis.


A
Vehicle (4hrs)
Vehicle (8hrs)
Vehicle (16hrs)
<2N (subG1) 1.1%
2N (G1) 63.4%
S 22.6%
4N (G2/M) 13.9%
<2N (subG1) 1.0%
2N (G1) 64.0%
S 22.2%
4N (G2/M) 13.8%
<2N (subG1) 1.1%
2N (G1) 63.9%
S 22.4%
4N (G2/M) 13.6%
SKI-178 (4hrs)
SKI-178 (8hrs)
SKI-178 (16hrs)
<2N (subG1) 5.7%
2N (G1) 36.3%
S 34.2%
4N (G2/M) 28.9%
<2N (subG1) 11.3%
2N (G1) 6.2%
S 33.4%
4N (G2/M) 60.4%
<2N (subG1) 32.6%
2N (G1) 5.3%
S 22.1%
4N (G2/M) 72.6%
G1
S
G2/M
Cell Count
FL2-A

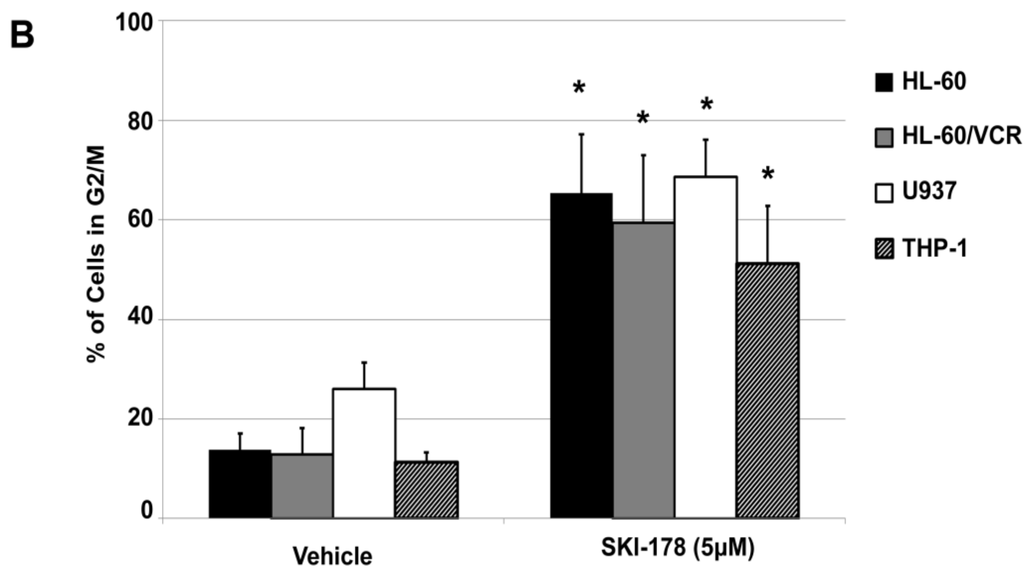

B
% of Cells in G2/M
HL-60
HL-60/VCR
U937
THP-1
Vehicle
SKI-178 (5µM)

Figure 2-4. 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 16h treatment with SKI-178 (5 μM) or vehicle (DMSO). Error bars indicate SD of triplicate counts of 25,000 cells. Statistical significance was assessed by two-tailed paired student's t test. Asterisks indicate significance: $*p \leq 0.01$.

SKI-178 induces sustained Bcl-2 phosphorylation during mitosis

The results presented in Figure 2-4A and B strongly suggest SKI-178-induced apoptosis may be the result of prolonged mitosis. As 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 [225] and released into either 5μM SKI-178 or vehicle DMSO control for a period of up to 30h. Whole cell lysates were prepared from cells collected every 2h and subjected to Western blot analysis for pBcl-2 (Ser70) as well as pHistone H3 (ser10) which is a known marker for mitosis [248]. 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 as the cells progress through mitosis, indicated by Histone H3 phosphorylation 10-14h after release [239]. Cells released into 5 μM SKI-178 containing medium also entered mitosis about 10h after release, but unlike vehicle treated cells, Histone H3 remains phosphorylated for up to 20h (Figure 2-5). Furthermore, Histone H3 phosphorylation in SKI-178 treated cells is paralleled by a robust and sustained phosphorylation of Bcl-2 at Ser70. Importantly,

while no caspase 7 cleavage was detected in vehicle treated cells, SKI-178 treatment showed substantial cleavage (activation) of caspase 7 beginning about 4h after the onset of mitotic arrest (14h post release). These results indicate that SKI-178-induced apoptotic cell death is closely associated with prolonged mitosis.

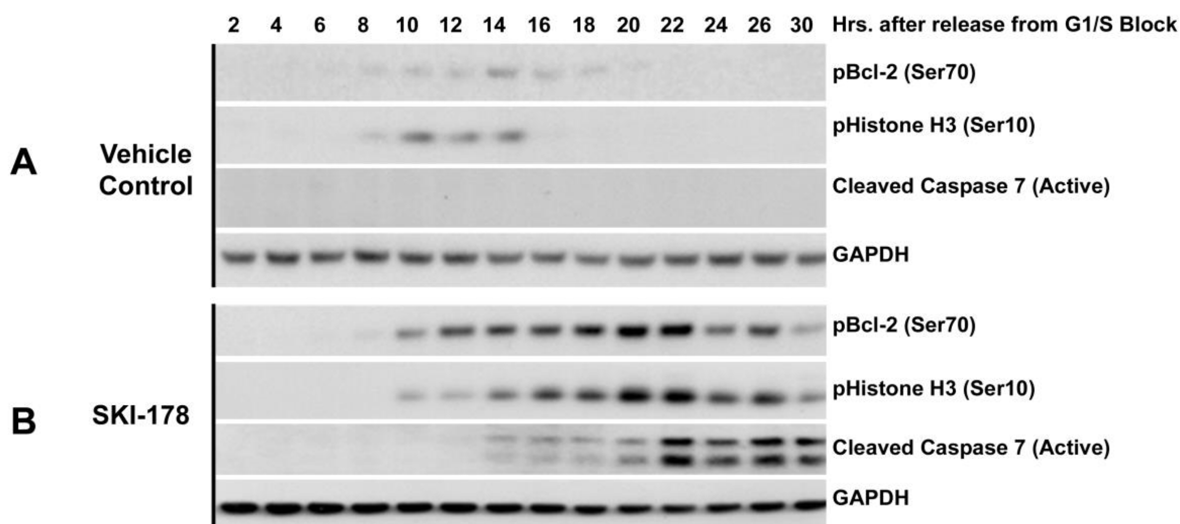


Figure 2-5. SKI-178 induces sustained Bcl-2 phosphorylation during prolonged mitosis. (A and B) Bcl-2 phosphorylation dynamics during cell cycle progression of vehicle (DMSO) treated or SKI-178 (5 µM) treated cells. HL-60 cells were synchronized at G1/S phase transition using a double thymidine block. Cells were released from the block into media containing (A) vehicle (DMSO) or (B) SKI-178 (5µM). Whole cell lysates were collected at indicated time points and Western blot analysis was performed using antibodies for pBcl-2 (Ser70), pHistone H3 (Ser10), or cleaved active caspase 7. GAPDH serves as a loading control. Results shown are representative of at least three independent experiments.

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

pharmacological 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 has been 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 (MPF) initiating the transition into early prophase [249]. Inhibition of CDK1 activity prior to entry into mitosis blocks cell cycle progression at the G2/M boundary [250]. Similarly, there is data to suggest that JNK is involved in progression from G2 into mitosis by regulating expression of Aurora B [251]. Thus, it is possible that simultaneous addition of JNK/CDK1 inhibitors with SKI-178 in un-synchronized cells or in synchronized cells prior to entry into mitosis would abrogate SKI-178-induced apoptosis. However, this effect could be an artifact of the blockage of cell cycle prior to 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 pHistone H3 (Ser10). Our results show that cells released into either vehicle or SKI-178 alone (Figures 2-6A and 2-6B respectively) responded as previously observed

(Figure 2-5) and entered into mitosis, as indicated by the presence Histone H3 phosphorylation (Ser10), approximately 10h after release from G1/S blockade. Additionally, vehicle treated control cells showed a slight but transient increase in Bcl-2 phosphorylation as the cell cycle progresses through mitosis 10-14h after release. In contrast, SKI-178 treated cells showed enhanced and prolonged Bcl-2 phosphorylation, prolonged mitosis indicated by sustained Histone H3 phosphorylation beginning 10h after release (Figure 2-6B), and apoptotic cell death indicated by caspase 7 cleavage (activation) at 26h (Figure 2-6F).

We next evaluated the role of JNK in SKI-178-induced apoptosis by treating synchronized HL-60 cells with AS601245 (selective JNK inhibitor) either at the time of release from G1/S blockade or after cells had entered mitosis. When HL-60 cells were simultaneously treated with SKI-178 and the JNK inhibitor (AS601245) at the time of release from G1/S blockade, we observed abrogation of pBcl-2 (Ser70), pHistone H3 (Ser10) (Figure 2-6C) and no caspase 7 cleavage (Active) (Figure 2-6F). Conversely, when synchronized HL-60 cells were released from the G1/S blockade into medium containing SKI-178, allowed to enter into mitosis (~10h indicated by pHistone H3 (Ser10)) and subsequently treated with AS601245 (addition at 14h), JNK inhibition did not have an effect on SKI-178-induced Bcl-2 or Histone H3 phosphorylation (Figure 2-6D). Similarly, AS601245 addition at 14h after release did not have an effect on Caspase 7 cleavage (activation) (Figure 2-6F). 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.

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 [250], we did not evaluate the role of CDK1 inhibition prior to entry into mitosis. As shown in Figure 2-6E, inhibition of CDK1 with RO3306 after synchronized cells entered mitosis (14h 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 Aurora Kinase A, the kinase responsible for Histone H3 phosphorylation at ser10 [252, 253]. In addition, RO3306 inhibition of CDK1 dramatically reduced SKI-178-induced apoptosis indicated by abrogation of caspase 7 cleavage (activation) (Figure 2-6F). Together, these results indicate that CDK1, not JNK, is the kinase responsible for SKI-178-induced apoptotic cell death.

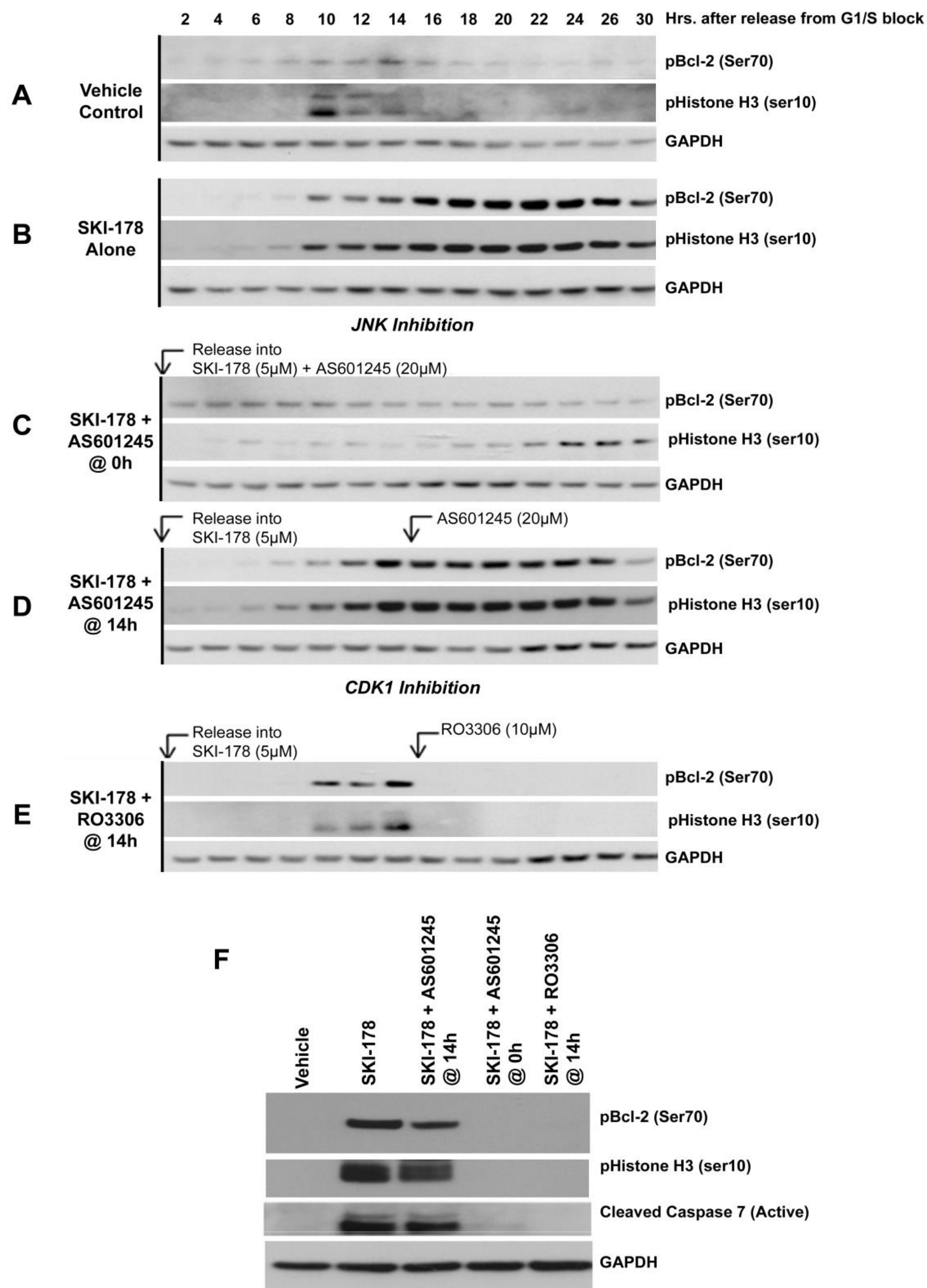

2
4
6
8
10
12
14
16
18
20
22
24
26
30
Hrs. after release from G1/S block
A
Vehicle Control
pBcl-2 (Ser70)
pHistone H3 (ser10)
GAPDH
B
SKI-178 Alone
pBcl-2 (Ser70)
pHistone H3 (ser10)
GAPDH
JNK Inhibition
Release into
SKI-178 (5μM) + AS601245 (20μM)
C
SKI-178 + AS601245 @ 0h
pBcl-2 (Ser70)
pHistone H3 (ser10)
GAPDH
Release into
SKI-178 (5μM)
AS601245 (20μM)
D
SKI-178 + AS601245 @ 14h
pBcl-2 (Ser70)
pHistone H3 (ser10)
GAPDH
CDK1 Inhibition
Release into
SKI-178 (5μM)
RO3306 (10μM)
E
SKI-178 + RO3306 @ 14h
pBcl-2 (Ser70)
pHistone H3 (ser10)
GAPDH
F
Vehicle
SKI-178
SKI-178 + AS601245 @ 14h
SKI-178 + AS601245 @ 0h
SKI-178 + RO3306 @ 14h
pBcl-2 (Ser70)
pHistone H3 (ser10)
Cleaved Caspase 7 (Active)
GAPDH

Figure 2-6. 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 (A) vehicle or (B) SKI-178 (5µM). Cells released into SKI-178 were subdivided into four additional treatments: (C) HL-60 cells released into SKI-178 and co-treated with AS601245 at the time of release, (D) HL-60 cells released into SKI-178 and co-treated with AS601245 14h after release, (E) HL-60 cells released into SKI-178 and co-treated with RO3306 14h 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 26h time points from (A-E). GAPDH serves as a loading control. Results shown are representative of at least three independent experiments.

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. Prior to mitosis, CDK1 is held inactive by phosphorylation at two residues, Thr14 and Tyr15 [254]. Tyr15 phosphorylation, in particular, is an essential checkpoint preventing entry into and progression through mitosis [255, 256]. 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 24h. 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 8h after

release (Figure 2-7). Between 8-12h, 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 16h, 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 anti-apoptotic Bcl-2.

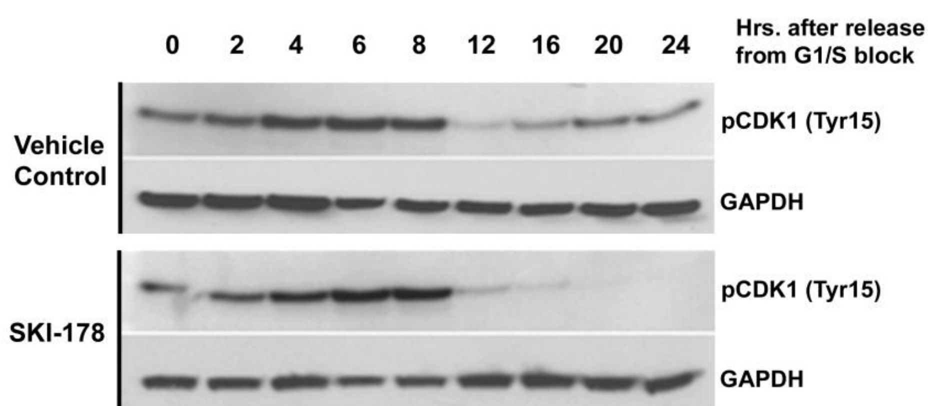


Figure 2-7. SKI-178 induces sustained CDK1 activation during mitosis.

HL-60 cells were treated with either vehicle or SKI-178 (5μM) for the indicated time points. Whole cell lysates were collected at indicated time points and Western blot analysis was performed using antibodies for pCDK1 (Tyr15). GAPDH serves as a loading control. Results shown are representative of three independent experiments.

SKI-178-induced CDK1 activation results in Mcl-1 degradation

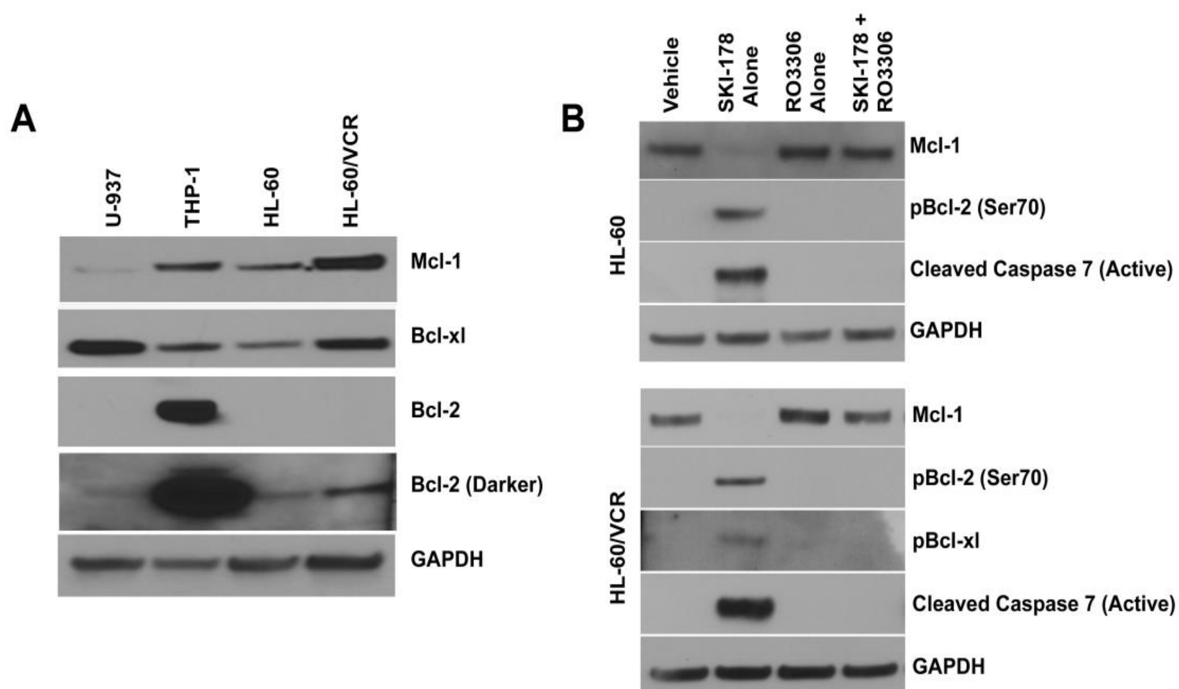
Although our data suggests a strong correlation between Bcl-2 phosphorylation at ser70 and induction of apoptotic cell death, the functional role of this phosphorylation is not yet clear. While many studies have implicated this Bcl-2 phosphorylation in making cells more susceptible to apoptosis [214, 246], there are recent studies that suggest it actually enhances its cyto-protective effects [257, 258]. 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 anti-apoptotic Bcl-2 family members including Bcl-xl and Mcl-1. Bcl-xl phosphorylation during mitotic arrest inhibits its anti-apoptotic activity by disrupting its interaction with Bax, leading to MOMP and cytochrome-c release [212]. Unlike Bcl-2 and Bcl-xl, Mcl-1 phosphorylation at Thr92, by CDK1, quickly targets it for proteasomal degradation [210].

As demonstrated in Figure 2-8A, 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 anti-apoptotic 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 24h 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 (Figure 2-8B). 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 as HL-60 express considerably lower levels of total Bcl-xl relative to HL-60/VCR cells (Figure 2-8A).

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 (~14h after release). HL-60/VCR were chosen based on their high expression of Mcl-1 relative to other cell lines (Figure 2-8A), 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 (Figure 2-8C) 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 10-12h after release from G1/S blockade. Vehicle treated cells showed a slight but transient increase in Bcl-2 phosphorylation as cells progress through mitosis 10-16h after release. Cells treated with SKI-178 alone showed sustained Bcl-2 phosphorylation, prolonged mitosis, and subsequent caspase 7 cleavage (active) starting around 6-8h after the onset of mitosis. As expected, SKI-178 also lead to almost complete Mcl-1 degradation beginning shortly after entry into mitosis but prior to 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).


A
U-937
THP-1
HL-60
HL-60/VCR
Mcl-1
Bcl-xl
Bcl-2
Bcl-2 (Darker)
GAPDH
B
Vehicle
SKI-178 Alone
RO3306 Alone
SKI-178 + RO3306
HL-60
Mcl-1
pBcl-2 (Ser70)
Cleaved Caspase 7 (Active)
GAPDH
HL-60/VCR
Mcl-1
pBcl-2 (Ser70)
pBcl-xl
Cleaved Caspase 7 (Active)
GAPDH

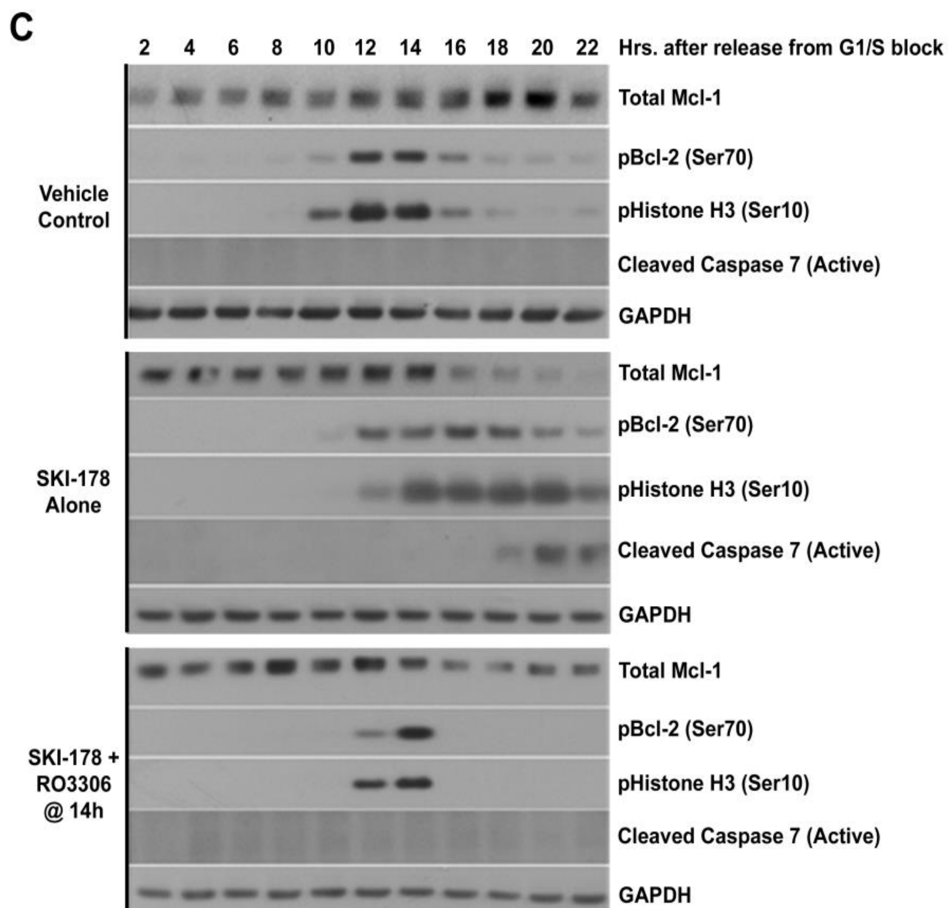

C
2 4 6 8 10 12 14 16 18 20 22
Hrs. after release from G1/S block
Vehicle Control
Total Mcl-1
pBcl-2 (Ser70)
pHistone H3 (Ser10)
Cleaved Caspase 7 (Active)
GAPDH
SKI-178 Alone
Total Mcl-1
pBcl-2 (Ser70)
pHistone H3 (Ser10)
Cleaved Caspase 7 (Active)
GAPDH
SKI-178 + RO3306 @ 14h
Total Mcl-1
pBcl-2 (Ser70)
pHistone H3 (Ser10)
Cleaved Caspase 7 (Active)
GAPDH

Figure 2-8. 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 anti-apoptotic family members (Bcl-2, Bcl-xl, and Mcl-1). (B) HL-60 and HL-60/VCR cells treated for 24h 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 co-treated with RO3306 14h after release. Whole cell lysates were collected at indicated time points and Western blot analysis was performed using indicated antibodies. GAPDH serves as a loading control.

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 utilized 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 (Figure 2-9A). 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 (Figure 2-9B), 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 (500nM). As shown in Figure 2-9C, SphK1 knockdown (KD) increases the phosphorylation of JNK and Bcl-2 to the same extent as scrambled siRNA control cells treated with 500nM SKI-178. Treatment of SphK1 KD cells with 500nM 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 support 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.

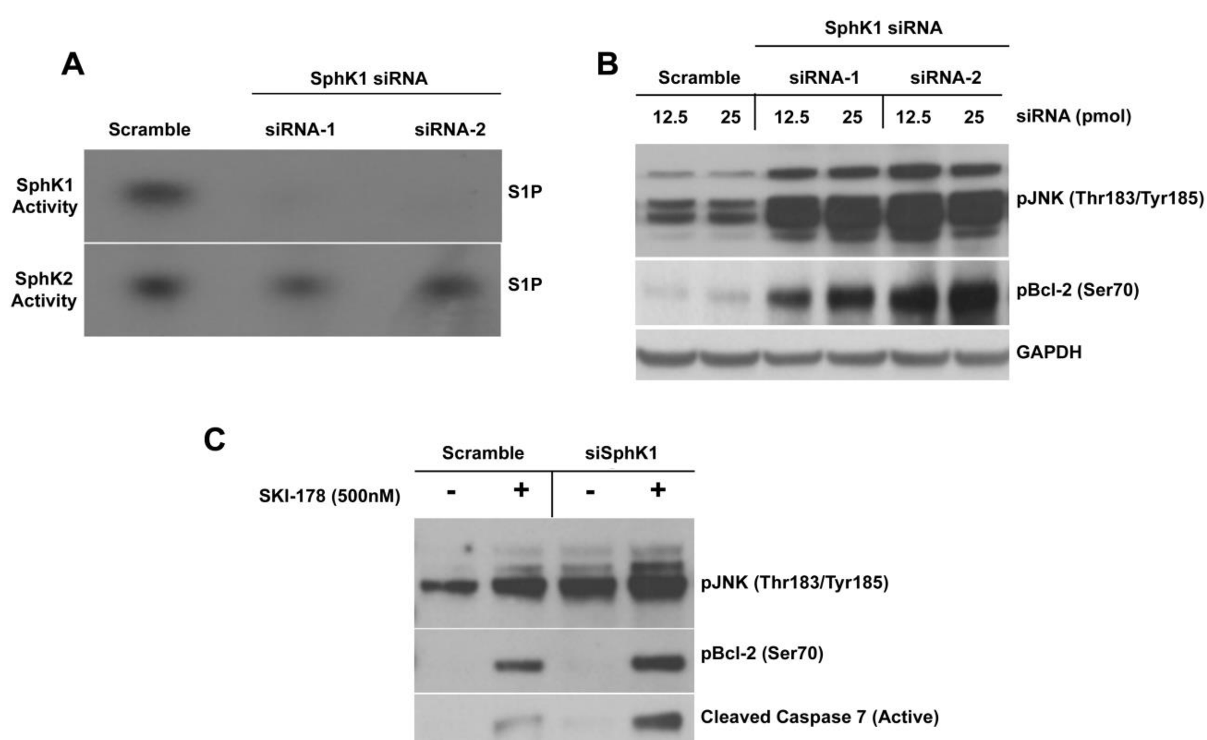


Figure 2-9. siRNA knockdown of SphK1 recapitulates the effects of SKI-178

(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.

Discussion

In this study we used 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 [218], and has become a promising target for novel therapeutic strategies [132]. We have previously established SKI-178 as a potent selective inhibitor of SphK1 through *in vitro* activity assays with purified SphK1 protein [130]. 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 24h of treatment, levels of S1P are significantly decreased compared to control whereas levels of pro-apoptotic long chain Ceramide species (C16 and C18) are increased. Interestingly, SKI-178 treatment concomitantly decreased levels of very long chain Ceramide species which, recent studies suggest, have anti-apoptotic properties [52, 236, 237]. 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 [259]. The majority of very long chain Ceramide species are produced specifically by Ceramide Synthase 2 (CerS2) [260]. 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. have demonstrated that S1P inhibits CerS2

activity [261]. Since 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. recently demonstrated that induction of apoptotic signaling stimulates the formation of long chain Ceramide species [262]. 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 anti-apoptotic Ceramide species, respectively, presents a unique 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 caspase 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 [263-265]. Furthermore, activation of these MAPKs play an important role in Ceramide induced apoptotic cell death [266, 267]. Therefore, as SKI-178 treatment induces accumulation of pro-apoptotic 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 anti-apoptotic Bcl-2 at

Ser70. Although JNK has been linked to the phosphorylation of Bcl-2 in mitotically arrested cells [239, 241, 268], there is conflicting evidence in the literature implicating CDK1 as the kinase responsible for this effect [208, 247]. Our results concur with the latter findings, demonstrating that CDK1 is required for SKI-178-induced Bcl-2 phosphorylation. We do however find that JNK activity is important for cell cycle progression from G2 into mitosis and that JNK inhibition, prior to the onset of mitosis, abrogates SKI-178 mediated cell death by preventing entry into mitosis where SKI-178 mediated cell death occurs. This is consistent with previous finding that JNK activity peaks during late G2 and inactivation of JNK prior to mitosis inhibits the expression of Aurora kinase B and phosphorylation of Histone H3 at ser10 [251, 269].

Our findings further suggest a link between SphK1 inhibition, with SKI-178, and prolonged activation of CDK1 leading to apoptotic cell death. While it is possible that SKI-178 induces prolonged mitosis as a polypharmacological 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 $S1P_5$ receptor have been shown to co-localize to the centrosome of mammalian cells, where the authors proposed that the $S1P_5$ receptor acts as a guanine nucleotide exchange factor stimulated by localized S1P production [270]. 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 [271], and non-selective small molecule inhibitors of SphK1 and SphK2 induce substantial G2/M arrest in

melanoma cell lines [272]. While 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 [273]. This leaves cells extremely vulnerable and potentially more susceptible to apoptotic stimuli. Mitosis is tightly controlled by the spindle-assembly checkpoint which 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 pro-apoptotic pathway responsible for clearing cells that fail to exit mitosis in a timely fashion [274]. 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 [275].

Several lines of evidence indicate that extensive phosphorylation of Bcl-2 at Ser70 and other sites by CDK1 inactivates its anti-apoptotic function and is a key feature of apoptotic cell death in response to agents that induce prolonged mitotic arrest [214, 245, 246]. However, Dai et al. recently demonstrated that phosphorylation of Bcl-2 at Ser70 enhances the anti-apoptotic function of Bcl-2 by promoting Bim and Bak binding [258]. Interestingly, CDK1 mediated phosphorylation of Bcl-xl at Ser62 during mitotic arrest has more consistently been shown to play a pro-apoptotic role, at least in part by disrupting its ability to bind and inhibit pro-apoptotic Bax [211, 212]. Furthermore, CDK1 mediated phosphorylation of Mcl-1 at Thr92 and its subsequent proteasomal degradation during prolonged mitotic arrest has also been shown to play a key role in

linking mitotic arrest to the induction of apoptotic cell death [210]. Our results clearly show that during SKI-178-induced prolonged mitosis, these anti-apoptotic Bcl-2 family members undergo these post-translational 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 anti-apoptotic effects or induces apoptosis, pBcl-2 (ser70) is a clear marker 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 anti-apoptotic Bcl-2 family members for survival. Although many leukemic cells are highly dependent on Bcl-2 [276], the other anti-apoptotic Bcl-2 family members, Mcl-1 and Bcl-xl, have overlapping functions and can compensate for Bcl-2 inhibition [277-279]. 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 up-regulation of Mcl-1 [280]. Similarly, high levels of Bcl-xl expression confers resistance to Mcl-1 inhibitors [279]. 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 [208-210]. 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 anti-apoptotic 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 [281]. Unfortunately, a major problem in the treatment of leukemic cancers is the development of resistance to such chemotherapeutic agents [282]. Much of this resistance has been attributed to the overexpression of MDR1 [283, 284]. Taken together, our results demonstrate that SKI-178 induces apoptotic cell death in multiple leukemic cell lines, including multi-drug resistant cell lines. These results highlight the promising potential of developing SKI-178 as a therapeutic strategy, not only for AML, but also an array of cancer types including those with multidrug resistance. Further studies are underway to examine the *in vivo* efficacy of SKI-178 in animal models.

Chapter 3

SKI-178, a Single Agent that Functions as a Dual Target Inhibitor of SphK1 and Microtubule Dynamics

Abstract

We recently published on the detailed mechanism-of-action (MOA) of SKI-178, a cytotoxic agent originally developed as a novel Sphingosine kinase 1 (SphK1) selective inhibitor. We demonstrated that SKI-178 induced apoptotic cell death results from sustained cyclin-dependent kinase 1 (CDK1) activation during prolonged mitotic arrest. Based on evidence in the literature suggesting a link between SphK1 activity and mitotic arrest, we originally attributed much of the MOA to SphK1 inhibition. However, in light of recent reports claiming that SphK1 inhibition alone is not sufficient to induce apoptotic cell death; we considered the possibility that SKI-178 may have additional targets that contribute to its cytotoxic effects. Based on the striking similarly between the MOA of SKI-178 and various agents that disrupt microtubules we considered the possibility that SKI-178, in addition to being a SphK1 selective inhibitor, also affects normal microtubule dynamics. We hypothesize that SKI-178 mediated apoptotic cell death is due to its ability to work as a microtubule targeting agent (MTA), and that its concomitant inhibition of SphK1 sensitizes cells to this effect. Herein we present evidence that SKI-178 does indeed function as MTA, preventing the polymerization of the microtubule network. Furthermore, we demonstrate that independent of SKI-178, inhibition of SphK1 alone sensitizes cells to vincristine, a MTA known for its ability to disrupt microtubule polymerization. We also definitively confirm that in cells, SKI-178 is able to directly target-engage both SphK1 and tubulin proteins at concentrations known to induce apoptotic cell death in a panel of cancer cell lines. Together, these results establish SKI-178 as a dual target inhibitor with promising chemotherapeutic potential.

Introduction

We previously developed SKI-178 as a novel Sphingosine kinase 1 (SphK1) selective inhibitor that is able to induce apoptotic cell death in a broad panel of cancer cell lines including multidrug resistant HL-60/VCR cells [130, 285]. Furthermore, we recently uncovered the detailed apoptotic mechanism-of-action (MOA) of SKI-178 whereby we clearly demonstrate that cell death is the direct result of prolonged cyclin-dependent kinase 1 (CDK1) activation during mitotic arrest. 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 [285]. Based on supporting evidence in the literature to suggest a link between SphK1 inhibition and mitotic arrest [270, 271], we originally hypothesized that the apoptotic MOA of SKI-178 was predominantly due to the inhibition of SphK1. For example, SphK1, Sphk2, and S1P receptor 5 (S1PR5) are known to co-localize to the centrosome of mammalian cells [270]. Furthermore, pharmacological or molecular inhibition of SphK has been shown to block mitotic exit [271], and small molecule inhibitors of SphK induce G2/M arrest in melanoma cell lines [272].

Interestingly, in recent years, evidence has been accumulating in support of the fact that SphK1 inhibition alone is not always sufficient to induce growth arrest or apoptotic cell death. For example, some of the most potent and selective inhibitors of SphK1 have shown little to no effect on cancer cell growth or induction of apoptotic cell death, despite their ability to result in large decreases in cellular S1P: Sphingosine ratio [168]. Furthermore, it has been suggested that the cytotoxic effects of other SphK1

selective inhibitors, such as SKI-II and SK1-I, are at least in part due to off-target effects [286]. In fact, SKI-II has recently been shown to directly inhibit dihydroceramide desaturase (Des1) [150] which has since proven critical for its anti-cancer properties as analogs of SKI-II that lack the capacity to inhibit Des1 have no effect on cell survival [287]. In light of these conflicting reports, we considered the possibility that SKI-178 may have additional targets, other than SphK1, that contribute to its cytotoxic effects.

Based on the striking similarly between the MOA of SKI-178 and various agents that disrupt microtubules we considered the possibility that SKI-178, in addition to being a SphK1 selective inhibitor, also affects normal microtubule dynamics. Microtubules are important during all stages of the cell cycle, but proper microtubule dynamics is particularly important during mitosis where it is required for the orientation and segregation of chromosomes. During the prometaphase stage of mitosis, a cell cycle checkpoint termed the spindle-assembly-checkpoint (SAC) is activated at the kinetochore of chromosomes that have yet to attach to microtubules extending from the spindle apparatus. Activated SAC proteins, such as BubR1 and Mad2, inhibit the activity of anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase with the main function of transitioning a cell from metaphase to anaphase [179, 180]. Only once all kinetochores are properly attached to microtubules does the APC/C get activated. Once activated, APC/C ubiquitinates specific proteins, such as securin and cyclin B1, and targets them for degradation [181]. Degradation of securin frees a protease called separase to cleave and dissolve cohesion, the protein complex holding sister chromatids together [182]. Cyclin B1 is the cyclin partner of CDK1, and together they form an active heterodimer that functions as a critical mitosis-promoting-factor required for both entry

into and progression through mitosis [183]. Once stripped of cyclin B1 by the activity of the APC/C, CDK1 undergoes a conformational change that inhibits its kinase activity [184]. Even if a single kinetochore is unattached to a microtubule of the spindle apparatus, APC/C remains inactive, CDK1 remains activated, and cells can't exit out of mitosis (Figure 3-1) [185].

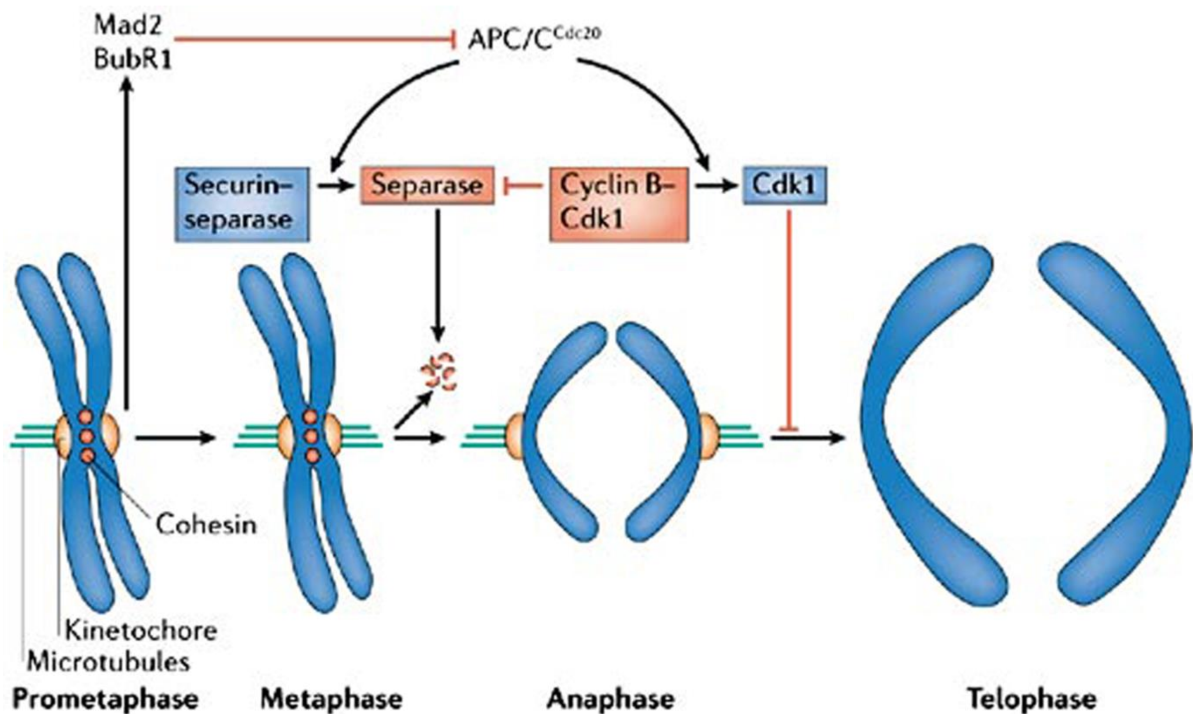


Figure 3-1. SAC regulation of the APC/C and mitotic exit.

During the prometaphase stage of mitosis, SAC proteins including Mad2 and BubR1 inhibit the activity of APC/C to ubiquitinate securin and cyclin B1 and therefore prevent the onset of anaphase and mitotic exit. Once all kinetochores have attached to microtubules during metaphase, the SAC is satisfied and APC/C gets activated. Activated APC/C ubiquitinates securin and cyclin B1, signaling for the onset of anaphase and mitotic exit. Taken from peters et al. [181].

This critical role microtubules play during mitosis and cell division make them excellent targets for the development of chemotherapeutic drugs aimed at rapidly

dividing cancer cells. In fact, agents that target microtubules, referred to here as microtubule-targeting agents (MTA), are one of the most successful classes of anti-cancer drugs [186, 187]. MTAs are a group of chemically diverse chemotherapeutic agents that can be divided into two major sub-classes: microtubule stabilizing or destabilizing agents. The former typically binds to polymerized microtubules and prevents their disassembly, while the latter often binds to one of two domains in free tubulin monomers and prevents their assembly into polymerized microtubules [175]. Despite their differences, both classes of MTA ultimately disrupt the dynamic equilibrium of the mitotic spindles, causing sustained mitotic arrest and ultimately apoptotic cell death through the sustained activation of CDK1.

Based on the striking similarity between the MOA of MTAs described above and SKI-178 described in detail in Chapter 3, we hypothesized that SKI-178, in addition to being a SphK1 inhibitor, also disrupts microtubule polymerization. Furthermore, we proposed that the concomitant inhibition of SphK1 sensitizes cells to this effect. Herein we demonstrate that SKI-178 does indeed function as a microtubule targeting agent and provide evidence that SphK1 inhibition can sensitize cells to the effects of MTAs.

Materials and Methods

Reagents: Reagents were purchased as follows: SKI-178 (N'-[(1E)-1-(3,4-dimethoxyphenyl)ethylidene]-3-(4-methoxxyphenyl)-1H-pyrazole-5-carbohydrazide) (ChemBridge Corporation, San Diego, CA), vincristine (Thermo Fisher Scientific, Waltham, MA), Colchicine (Enzo Life Sciences, Farmingdale, NY), Paclitaxel (Sigma-

Aldrich, St. Louis, MO), MG132 (Sigma-Aldrich, St. Louis, MO) and PF-543 (Selleck Chemicals, Houston, TX), antibodies against SphK1, pBcl-2(Ser70), Cleaved caspase-7, and Tubulin (Cell Signaling Technology, Danvers, MA), SphK1 (pSer225) (ECM Biosciences, Versailles, KY), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

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, Virginia, USA) and were maintained in IMDM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% FBS (Denville Scientific, South Plainfield, NJ, USA). His_{6X}-SphK1 overexpressed in HEK293 cells as described in [288] were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS. All cell lines were maintained at 37°C with 5% CO_2 in a humidified incubator.

Whole cell analysis of tubulin polymerization: Microtubules were fixed and stained as previously described [289]. Cells were treated in 24 well plates with vincristine, paclitaxel, SKI-178, or PF-543 for 18h. Cells were pelleted by centrifugation at 600 x g for 5min. Cell pellets were resuspended and fixed with 1ml 0.5% glutaraldehyde in microtubule stabilizing buffer (80mm PIPES (pH 6.8) 1mm $MgCl_2$, 5 mm EDTA, and 0.5% Triton X-100) for 10 min at room temperature. Glutaraldehyde was quenched with 0.7ml of 1mg/ml $NaBH_4$ in PBS and cells were subsequently pelleted at 1,000 x g for 7min. Cell pellet was re-suspended in 20µl of 50µg/ml RNase A in antibody dilution solution (PBS (pH 7.4), 0.2% Triton X-100, 2% bovine serum albumin (BSA), 0.1% NaN_3) and incubated at 4°C overnight. The next morning, 5µl of a 1:50 dilution of anti-α-tubulin-FITC antibody in antibody dilution solution was added to the 20µl

suspention to achieve a final antibody dilution of 1:250 and incubated in for 3h protected from light. Samples were further diluted in 200µl of 50µg/ml PI in PBS. Cells were analyzed by flow cytometry on a BD FACSCalibur (BD Biosciences, San Jose, California, USA) and results were analyzed by ModFit LT (Verity Software House, Topsham, Maine, USA).

***In vitro* tubulin polymerization assay:** Inhibition of tubulin polymerization was measured using the Cytoskeleton HTS-Tubulin Polymerization Assay Kit (Cytoskeleton, Inc., Denver, CO) 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 $MgCl_2$, and 1 mm GTP). 10 µl of 10× 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 cells with GFP-tagged tubulin: U251 human glioblastoma cell lines were provided by Dr. James Connor (Penn State College of Medicine, Hershey, PA) and were maintained in DMEM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% FBS (Denville Scientific, South Plainfield, NJ, USA). U251 cells were plated on 10cm culture dishes and transfected with 10µg of pAcGFP1-Tubulin plasmid in 200µl of Lipofectamine 200 reagent (Invitrogen, Carlsbad, CA). The pAcGFP1-Tubulin plasmid contains cDNA encoding a fusion protein consisting of the monomeric green florescent protein (GFP) and α-tubulin. The plasmid also contains a kanamycin resistant gene. The pAcGFP1-Tubulin plasmid was provided by Dr. Christopher Yengo (Penn State College of Medicine, Hershey, PA). After overnight

transfection, transfected cells were selected using 500µg/ml G-418 for 72h to select for cells that express the kanamycin resistant gene.

Western blot analysis: This was performed as previously described [224] with some modifications. Briefly, whole cell lysates were harvested in 100µL 1X RIPA buffer (20mM Tris-HCL (pH 7.5), 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM sodium orthovandate, 30mM sodium fluoride, and complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany)). Lysates were centrifuged (≥10,000g) for 15min 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 1h 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, Massachusetts, USA).

Analysis of apoptosis: HL-60 cells were treated with PF-543, vincristine, colchicine, or a combination of vincristine/colchicine with PF-543 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): This was performed as described in [290] with some minor modification. Briefly, His6x-SphK1 overexpressing HEK293 cells were treated with either vehicle control or SKI-178 for 16h, washed with PBS, and 100µl 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 x 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.

Results

SKI-178 directly engages SphK1 protein in cells

In previous studies we have established the SphK1 inhibitory potential of SKI-178 [130, 285]. However, many of these conclusions are based on the indirect monitoring of downstream cellular responses or the assumption that SKI-178's ability to bind and inhibit SphK1 activity *in vitro* will directly correlate with its binding in intact cells. Unfortunately, monitoring downstream accumulation of sphingosine-1-phosphate (S1P) or depletion of ceramide as indicators of SphK1 inhibition is complicated by other interconnecting pathways. Furthermore, SKI-II, an established SphK1 selective inhibitor, is actually a weak kinase inhibitor of SphK1 but is a potent promoter of SphK1 proteasomal degradation [133]. Moreover, PF-543, currently one of the more potent inhibitors of SphK1, was recently shown to induce SphK1 proteasomal degradation in addition to its ability to inhibit SphK1 kinase activity [287]. Therefore, we explored the possibility that SKI-178 also has the capacity to induce SphK1 degradation. Due to the limitations of commercially available antibodies in detecting endogenous SphK1, we employed HEK293 cells that overexpress His-tagged SphK1 (His6x-SphK1). SphK1 proteasomal degradation was measured by treating His6x-Sphk1 cells with SKI-178, PF-543, or SKI-II alone and in combination with the proteasome inhibitor MG132 for 16h. The effects of these compounds on SphK1 protein degradation was assessed by Western botting (Figure 3-2A). Consistent with previous reports [133, 287], MG132 treatment alone leads to an increase in SphK1 levels above that of untreated cells, suggesting a

proteasomal-dependent post-translational turnover of basal SphK1 in healthy untreated cells. Also consistent with the literature, both SKI-II and PF-543 completely ablate SphK1 protein levels in a proteasomal-dependent manner [133, 287]. Unexpectedly, SKI-178 had little to no effect on SphK1 expression levels. While these studies demonstrate that SKI-178 does not induce proteasomal SphK1 degradation, they don't exclude the possibility that SKI-178 directly binds and inhibits the kinase activity of SphK1.

To confirm that SKI-178 directly binds SphK1, we employed a recently developed cellular thermal shift assay (CETSA) to monitor direct binding of SKI-178 to SphK1 in a relevant cellular context [290, 291]. 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 His6x-Sphk1 cells were treated with SKI-178 for 16h, after which multiple aliquots of the cell suspension were heated to different temperatures ranging from 51.0-63.2°C. Cells were subsequently lysed and centrifuged to separate soluble fractions from precipitated denatured proteins. The binding of SKI-178 to SphK1 should alter its melting curve by either stabilizing or destabilizing the protein. The presence of SphK1 in the soluble fraction was examined by Western blotting. As shown in Figure 3-2B, relative to control, cells treated with SKI-178 show a marked increase in the accumulation of total His-tagged SphK1 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 [81], we confirmed that SKI-178 is also able to bind to active pSphK1 (Ser225) at all temperatures examined. These data provide direct evidence that in cells, SKI-178 is able to target engage SphK1 including its Ser225 phosphorylated active form.

Knowing the temperature at which SphK1 denatures and precipitates allows us to employ a variation of these studies termed isothermal dose-response fingerprint ($ITDRF_{CETSA}$) [291]. With this experimental design, the stabilization or destabilization of a protein can be monitored as a function of increasing drug concentration [290]. This allows for determining concentrations of SKI-178 required for engaging SphK1 in cells. To this end, His6x-Sphk1 cells were again treated for 16hs with a 1:2 serial dilution of SKI-178 and subsequently heated to 60°C. Based on the previously described CETSA data, it was known that 60°C is an appropriate temperature to denature SphK1 or active pSphK1 (Ser225) protein in vehicle treated cells. As seen in Figure 3-2C, SKI-178 dose dependently binds and significantly stabilizes both His-tagged SphK1 and active pSphK1 (Ser225). Although this effect starts to decline around 630nM, we are still able to see signs of stabilization as low as 160 nM. The concentrations of SKI-178 required to bind SphK1 correlate well with the published cytotoxic IC_{50} of SKI-178 which ranges from about 400-800nM depending on the cell line [285]. Therefore, SKI-178 directly binds and inhibits SphK1 at concentrations required to induce apoptotic cell death. Together, these results further establish SKI-178 as a relevant inhibitor of SphK1 kinase activity in cells.

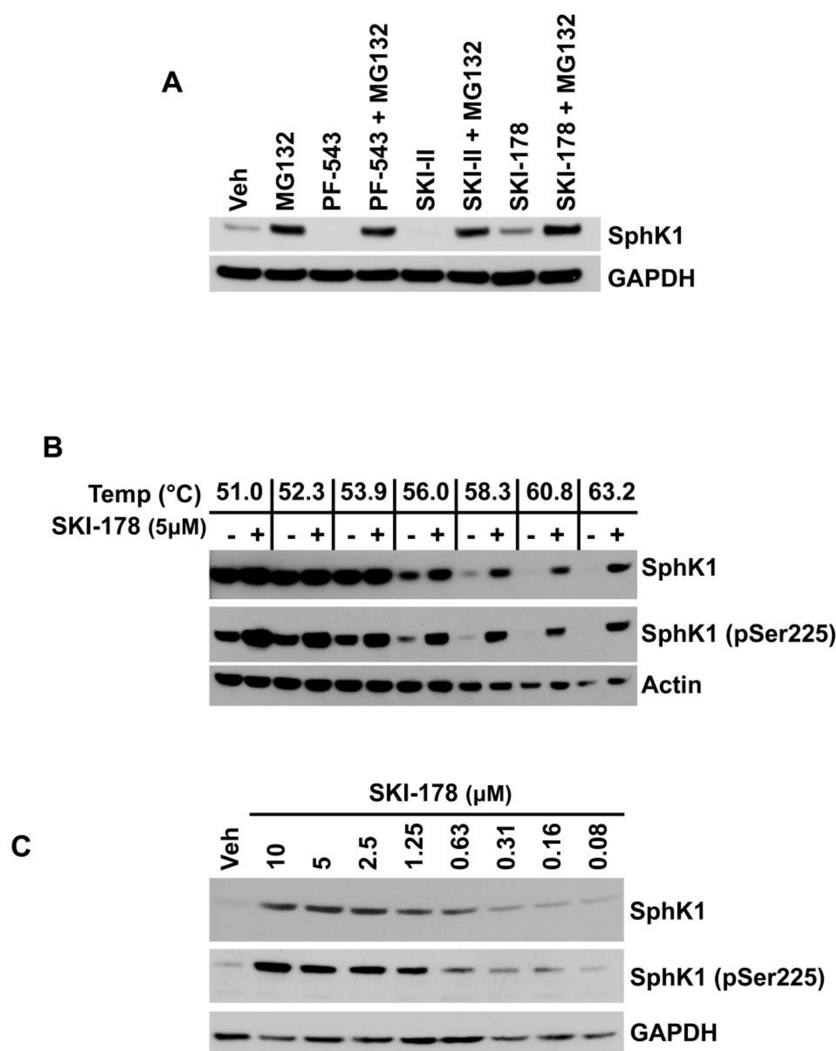


Figure 3-2. SKI-178 Directly Engages SphK1 Protein in Cells.

(A) His6x-Sphk1 cells lines were treated for 16h with PF-543 (5μM), SKI-II (10μM), or SKI-178 (5μM) alone and in combination with MG132 (10μM). Western blot analysis was performed on whole cell lysates using indicated antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) CETSA was performed on His6x-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. Similarly, (C) CETSA was used as described in the materials and methods to generate an isothermal dose-response fingerprint showing the stabilization effect of SKI-178 at different concentrations. The stabilization effect of SKI-178 on SphK1 and active SphK1 at

different concentrations was evaluated by Western blot using indicated antibodies. GAPDH was used as a loading control.

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SKI-178 directly impairs microtubule assembly

SphK1 has long been considered a promising chemotherapeutic target and extensive efforts have been made to develop novel SphK1 selective inhibitors for cancer therapeutics. While certain SphK1 inhibitors, such as SKI-II and SK1-I, are able to inhibit cell growth both *in vitro* and *in vivo*, there is evidence to suggest that this is the result of off-target effects of these compounds [286, 287]. Furthermore, recent reports have shown that some of the most potent and selective inhibitors of SphK1 failed to inhibit growth or induce apoptotic cell death, despite their ability to significantly decrease the cellular S1P:Sphingosine ratio [168]. Therefore, although the data presented above corroborate the SphK1 inhibitory potential of SKI-178, it would be imprudent to ignore the possibility of off-target effects. 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 SphK1 inhibitor, also works as a microtubule targeting agent.

Microtubules are a vital component of the cytoskeleton and are essential for a variety of cellular processes. Although microtubules are important for all stages of the cell cycle, microtubules constituting the mitotic spindle are extremely dynamic and cells are particularly sensitive to microtubule disruption during mitosis. Therefore, microtubules have become a prime target for a group of anticancer drugs termed

microtubule-targeting agents (MTA). This class of chemotherapeutics can be divided into two major sub-classes: microtubule stabilizing or destabilizing agents. The former typically bind to polymerized microtubules and prevents their disassembly while the latter often bind to one of two domains in tubulin dimers and prevents their assembly into polymerized microtubules [292, 293]. Despite their differences, both classes of MTA ultimately 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 [247]. Similarly we observed a prolonged CDK1 activation in response to SKI-178 treatment of AML cell lines. Therefore, with these subsequent studies we tested the hypothesis that SKI-178 also functions as a MTA and that its concomitant inhibition of SphK1 sensitizes cells to this effect.

To visually examine the effects of SKI-178 on microtubule dynamics, U-251 glioblastoma cells over-expressing GFP-tagged tubulin were treated with either SKI-178 or PF-543, a non-cytotoxic but highly potent inhibitor of SphK1. As shown in Figure 3-3A, SKI-178, but not PF-543, significantly disrupts the normal 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 degree of tubulin polymerization using flow cytometry [289]. As would be expected, relative to control, the MTA vincristine decreased tubulin fluorescence, while paclitaxel increased fluorescence due to their abilities to destabilize or stabilize microtubule polymerization respectively. While PF-543 had no effect on microtubule polymerization, SKI-178 destabilizes microtubule polymerization similar to that of vincristine (Figure 3-3B). The fact that PF-543 has no effect on microtubule

polymerization confirms that SphK1 inhibition alone 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 3-3C, 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 and inhibit tubulin polymerization similar to that of vincristine. However, its ability to bind tubulin *in vitro* may not necessarily correlate with its ability to bind tubulin in cells. Therefore, to confirm direct binding of tubulin by SKI-178 in a cellular context we again employed the cellular thermal shift assay (CETSA) described in more detail above. His6x-Sphk1 cells were similarly treated with SKI-178 for 16h and multiple aliquots of the cell suspension were subsequently heated to temperatures ranging from 58.3-63.5°C. The soluble fractions of cell lysates were examined by Western blotting. Unlike SphK1 (Figure 3-2B), SKI-178 binding to tubulin destabilizes the protein and hence we observe a significant decrease in the accumulation of tubulin in the soluble fraction at all temperatures examined (Figure 3-3D). These results, in combination with the SphK1 CETSA data shown in Figure 3-2B provide direct evidence that SKI-178 is able to target engage both SphK1 and tubulin proteins in cells, suggest that SKI-178 might be a dual target inhibitor: binding and disrupting the functions of two relevant chemotherapeutic targets.

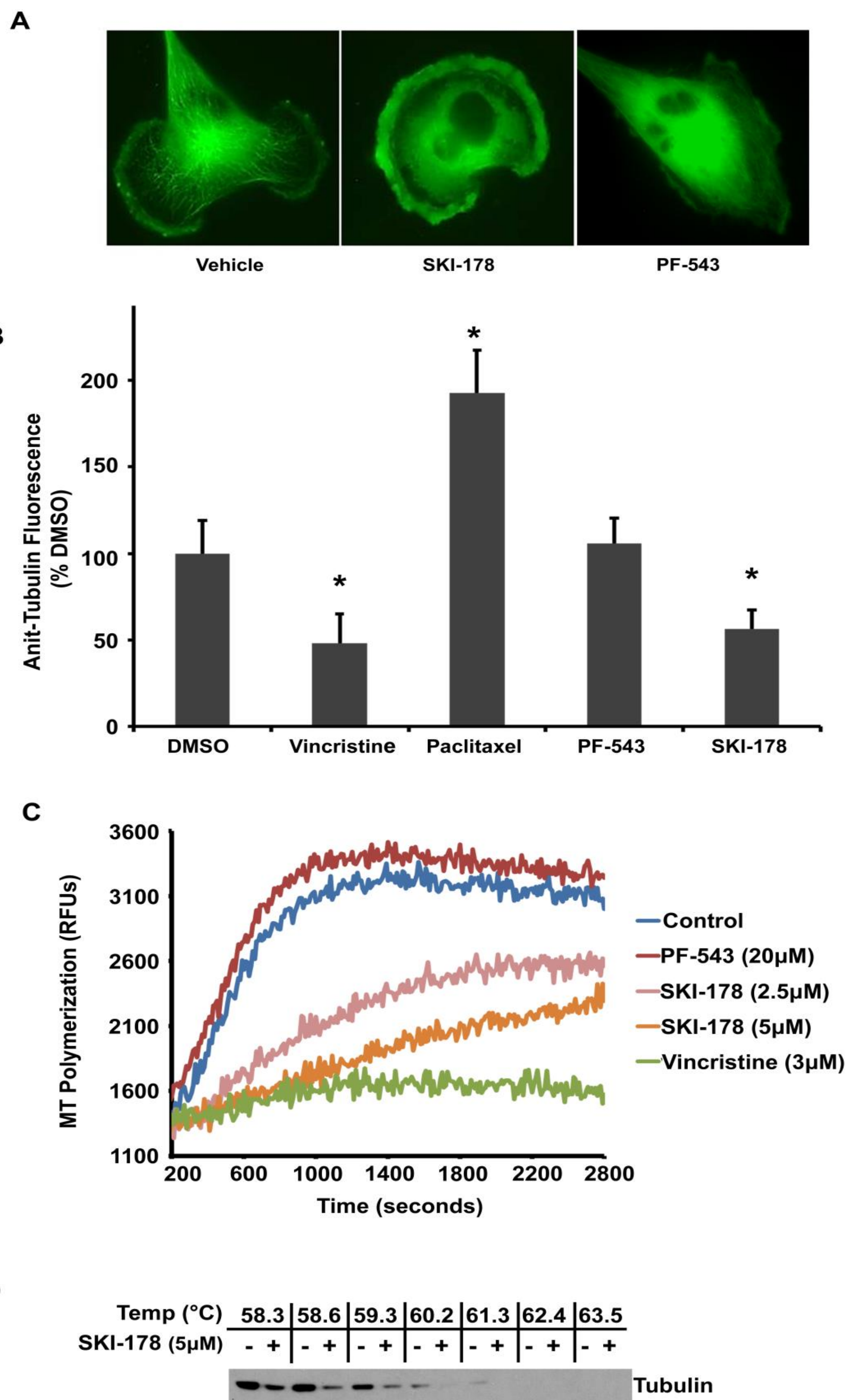
A
Vehicle
SKI-178
PF-543
B
Anit-Tubulin Fluorescence (% DMSO)
0
50
100
150
200
DMSO
Vincristine
Paclitaxel
PF-543
SKI-178
*
C
MT Polymerization (RFUs)
Time (seconds)
1100
1600
2100
2600
3100
3600
200
600
1000
1400
1800
2200
2800
Control
PF-543 (20μM)
SKI-178 (2.5μM)
SKI-178 (5μM)
Vincristine (3μM)
D
Temp (°C)
58.3
58.6
59.3
60.2
61.3
62.4
63.5
SKI-178 (5μM)
Tubulin

Figure 3-3. 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 fluoresce microscopy. (B) Whole cell analysis of tubulin polymerization was performed on HL-60 cells after 18h treatment with vincristine (200nM), paclitaxel (100nM), SKI-178 (5µM), or PF-543 (10 µM) for 18hs. Error bars represent standard deviation of the mean (n=3). Asterisks indicate significant: *,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 (20µM), SKI-178 (2.5µM), SKI-178 (5µM), and vincristine (3µM) for 60 min. Tubulin polymerization (i.e. lengthening of microtubules) was assayed in terms of absorbance at 340nM. 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. (D) CETSA was performed on His6x-Sphk1 cells as described in materials and methods. The destabilization effect of SKI-178 on tubulin at various temperatures was evaluated by Western blot using indicated antibodies.

SphK1 inhibition sensitizes cells to the effects of microtubule disruption

The data described above suggests that SKI-178 functions as a dual target inhibitor of both SphK1 and microtubule dynamics. However, owing to recent findings that potent and highly selective inhibitors of SphK1 exert little to no effect on cell viability [166, 168], the question remains as to whether SphK1 inhibition by SKI-178 contributes to its cytotoxic effects. Therefore, to demonstrate that SphK1 inhibition could sensitize cells to microtubule disruption independently of SKI-178, we used vincristine in combination with PF-543, the most potent and selective inhibitor of SphK1 currently available. We hypothesized that SKI-178 on its own 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 percent of total apoptosis was quantified by Annexin V staining. Our results show that while there was no statistical induction of

apoptotic cell death with either 6nM vincristine or 5µM PF-543, in combination we see a more than 10-fold increase in the percent of total apoptotic cells (Figure 3-4A).

In our previous publication we 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 [209, 241] which has likewise been linked to prolonged CDK1 activity [208]. Therefore we considered the possibility that SphK1 inhibition with PF-543 could act cooperatively with vincristine to enhance these effects. In Figure 3-4B we show that in HL-60 cells, treatment with a non-cytotoxic dose of vincristine (3nM) induces Bcl-2 phosphorylation. However, when combined with 6.25µM 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 Figure 3-4A 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 colchicine, another MTA that, like vincristine, also induces apoptotic cell death through disruption of microtubule polymerization [294]. We again treated HL-60 cells with non-cytotoxic doses of either colchicine or PF-543 alone or in combination. The percent of total apoptosis was quantified by annexin V staining. While 50nM colchicine alone led to an approximate 2-fold increase in apoptotic cell death, the combination with a non-cytotoxic dose of PF-543 almost doubled this effect (Figure 3-4C). These results support the hypothesis that SphK1 inhibition can sensitize cells to the effects of various MTAs.

The fact that SKI-178 exerts both of these effects as a single agent is therefore a novel and promising chemotherapeutic approach.

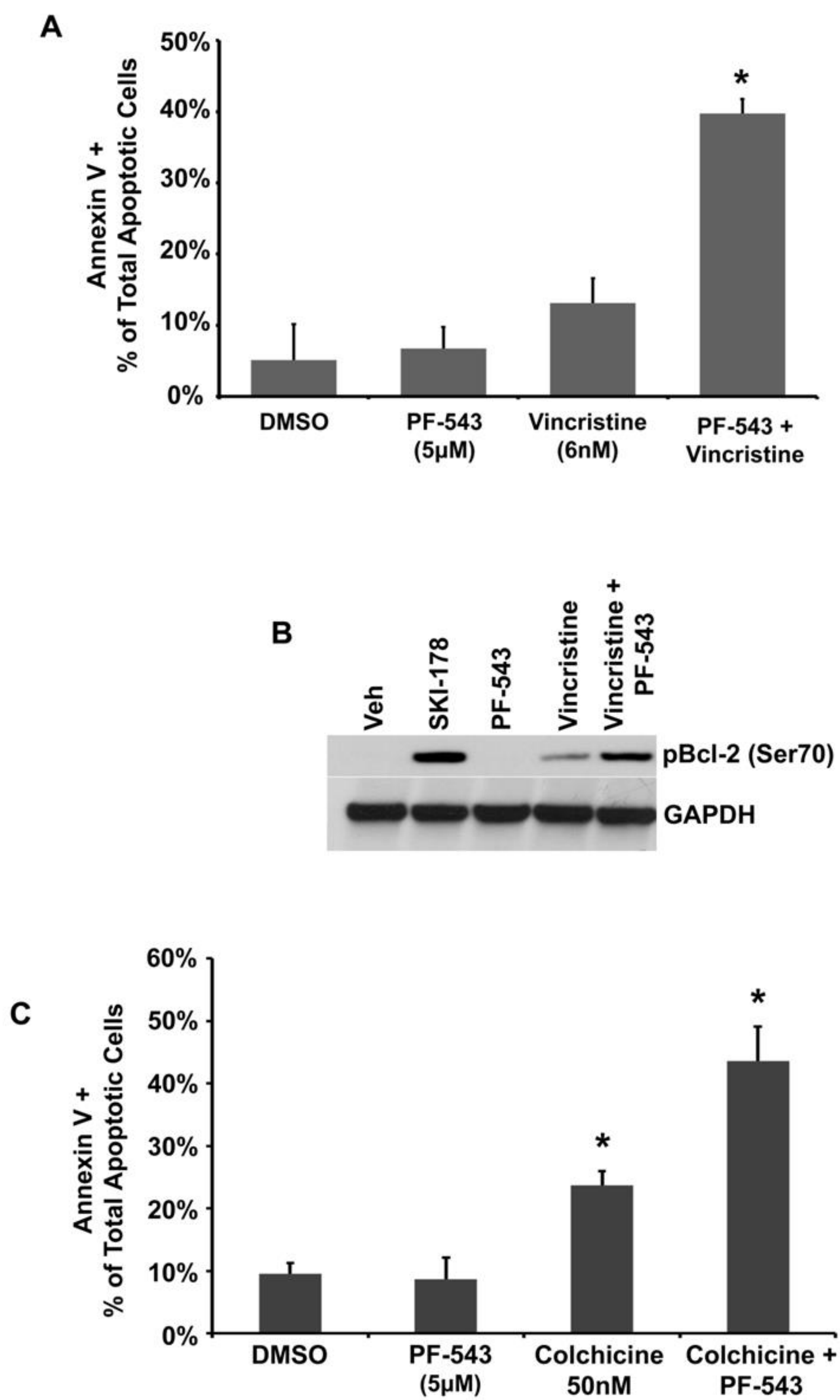
A
Annexin V +
% of Total Apoptotic Cells
50%
40%
30%
20%
10%
0%
*
DMSO
PF-543 (5µM)
Vincristine (6nM)
PF-543 + Vincristine
B
Veh
SKI-178
PF-543
Vincristine
Vincristine + PF-543
pBcl-2 (Ser70)
GAPDH
C
Annexin V +
% of Total Apoptotic Cells
60%
50%
40%
30%
20%
10%
0%
*
*
DMSO
PF-543 (5µM)
Colchicine 50nM
Colchicine + PF-543

Figure 3-4. SphK1 inhibition sensitizes cells to the effects of microtubule disruption.

(A) HL-60 cells treated with PF-543 (5µM) and vincristine (6nM) alone and in combination for 48h. Apoptosis was quantified by Annexin V-positive staining. Asterisks indicate significant: *,P< 0.05 relative to DMSO control based on a two-tailed Student's *t* test. (B) HL-60 cells treated for 16h with PF-543 (6.25µM) and vincristine (3nM) alone and in combination. Western blot analysis was performed on whole cell lysates using indicated antibodies. (C) HL-60 cells treated with PF-543 (5µM) and colchicine (50nM) alone and in combination for 48h. Apoptosis was quantified by Annexin V-positive staining. Asterisks indicate significant: *,P< 0.05 relative to DMSO control based on a two-tailed Student's *t* test.

Discussion

Microtubule-targeting agents (MTA) are a successful class of anti-cancer drugs that have shown therapeutic benefit in a panel of cancer types. They are often referred to as antimitotic agents as they exert their cytotoxic effects primarily by arresting cells in mitosis. Even though their effects on the stability and dynamic parameters of microtubules may differ, at the cellular level, all agents in this class eventually lead to cell cycle arrest in mitosis which subsequently triggers cell death through mitochondrial dependent apoptosis [186]. As previously shown, SKI-178 induced apoptotic cell death is also the result of prolonged mitotic arrest and eventual mitotic cell death [285]. Therefore we tested and confirmed that SKI-178 is in fact a microtubule targeting agent. Hence, SKI-178 can be classified as a bifunctional inhibitor of both SphK1 and normal microtubule dynamics.

Although much of the MOA of SKI-178 could be attributed to its effects on microtubules, it would be imprudent to ignore its effects on SphK1 inhibition. There is substantial evidence in the literature demonstrating that bioactive sphingolipid metabolites, such as ceramide in particular, are able to sensitize cells to the actions of MTAs. For example, Sietsma et al. [295] demonstrate that PDMP, a well-known inhibitor of ceramide glycosylation, highly sensitizes neuroblastoma cells to either paclitaxel or vincristine, and that this effect likely involves the sustained elevation of ceramide levels. In support of this, other studies have shown that direct addition of ceramide enhanced paclitaxel-mediated growth inhibition of Jurkat leukemic cells [296] and synergistically induced pancreatic cancer cell death [297]. As SKI-178 is known to induce the

accumulation of ceramide by inhibiting SphK1, it's likely that this effect sensitizes cells to the concomitant disruption of microtubules. If the MOA of SKI-178 is dependent on both SphK1 inhibition and disruption of microtubule dynamics, we would expect to see similar results if we disrupted these two targets independently of SKI-178. We demonstrate using two independent inhibitors of SphK1 and microtubule dynamics, PF-543 and vincristine respectfully, that a non-cytotoxic dose of these two agents in combination synergistically enhances apoptotic cell death in HL-60 AML cell lines. We confirmed that this effect is not restricted to vincristine and can be replicated using another MTA, colchicine. PF-543 is considered one of the more potent and selective inhibitors of SphK1. However, relative to other known SphK1 selective inhibitors, PF-543 shows little to no cytotoxic effects [168]. We confirm that unlike SKI-178, PF-543 has no effect on microtubule polymerization. Overall, these data support our hypothesis that SKI-178 alone is able to exert similar effects as these two agents in combination. As further evidence that SKI-178 is able to directly bind to and inhibit SphK1 activity and tubulin polymerization, we demonstrated that SKI-178 target engages both SphK1 and tubulin at concentrations known to induce apoptotic cell death.

Future studies are required to uncover the intricate molecular details behind this synergistic effect. However, there is enough evidence in the literature to speculate that synergy between SphK1 inhibition and microtubule disruption is likely due to the accumulation of ceramide [295-297]. Furthermore, we speculate that the interplay between these two pathways converges on the activities of Bcl-2 family proteins. It is well documented that MTAs lead to the prolonged activation of CDK1 which ultimately phosphorylates and inhibits anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-xl, and Mcl-

1). In fact, the MOA of SKI-178 induced apoptotic cell death relies heavily on this mechanism of cell death. On the other hand, bioactive sphingolipids such as sphingosine and ceramide, which are enhanced by SphK1 inhibition, have been shown to play a role in the activities of pro-apoptotic Bcl-2 family members such as Bak and Bax [298-300]. Therefore, it's interesting to speculate that SKI-178 inhibits the activities of anti-apoptotic Bcl-2 proteins and activates the activities of the pro-apoptotic Bcl-2 family members simultaneously.

An alternative mechanism for how SphK1 inhibition might sensitize cells to microtubule damage is through further prolonging mitotic arrest. There is evidence in the literature to suggest that prolonging mitotic arrest, which can be achieved through multiple avenues, is able to sensitize cells to MTAs [301, 302]. For example, a recent study by Sinnotte et al. shows that inhibition of TRIM69A, a protein that binds to mitotic centrosomes and supports centrosome clustering, enhances the sensitivity of tumor cells to paclitaxel [302]. To this end, recent findings have shown that SphK1 and SphK2 are both localized to the centrosome and their activity may be essential for coordinating molecular events required for proper bipolar spindle formation [270]. Therefore, it's possible that inhibition of SphK1 by SKI-178 disrupts centrosome clustering. This effect on centrosome formation could further prolong the mitotic arrest induced by microtubule disruption.

Regardless of the underlying mechanism, our results clearly demonstrate that SphK1 inhibition sensitizes cells to the effects of MTAs. MTAs are some of the most successful clinically available chemotherapeutic agents and have shown benefits for both solid tumors and some hematological malignancies (reviewed in [281]. Despite their

success, adverse side effects and the tendency for cancers to acquire resistance to many MTAs has greatly affected their applicability in the clinical setting. Resistance to MTAs has largely been attributed to the increased efflux of drugs via transmembrane proteins such as the multidrug-resistant protein 1 (MDR1). We have previously demonstrated that SKI-178 is not a substrate for drug efflux proteins such as MDR1 and confirmed its cytotoxic effect toward multidrug resistant HL-60/VCR AML cells [285]. Furthermore, it is our hypothesis that being a dual target inhibitor will help to overcome some of the limitations with regards to adverse side effects. In these studies we have clearly demonstrated that SphK1 inhibition synergizes well with microtubule disruption. SKI-178 has the benefit of simultaneously targeting both SphK1 and microtubule dynamics as a single agent. Therefore, it would eliminate the need to combine multiple chemotherapeutics, each with their own unique adverse side effects. Taken together, these findings suggest that SKI-178 represents a promising chemotherapeutic with dramatic improvements over traditional MTAs.

Chapter 4

Summary and Future Directions

Summary and Final Considerations

SKI-178 was originally developed as a novel small molecule, sphingosine kinase 1 (SphK1)-selective inhibitor, and was shown to be cytotoxic toward a broad panel of cancer cell lines [130]. Nonetheless, the mechanism underlying SKI-178 induced cell death had not yet been elucidated. Therefore, the overarching goal of this research study was to uncover the detailed molecular mechanism-of-action (MOA) of SKI-178 induced apoptosis. Using human acute myeloid leukemia (AML) cell lines as a model, the studies presented in Chapter 2 provide evidence that SKI-178 induces intrinsic mitochondrial-dependent apoptosis as a result of prolonged mitotic arrest. Specifically, the sustained activation of cyclin dependent kinase 1 (CDK1) (Figure 2-6E-F, Figure 2-7) during mitotic arrest simultaneously phosphorylates and inhibits various anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-xl, and Mcl-1, which undergoes subsequent proteolytic degradation (Figure 2-8B-C). These Bcl-2 family proteins act as important mediators of intrinsic apoptosis by regulating mitochondrial outer membrane permeability (MOMP) and ultimately the release of pro-apoptotic factors such as cytochrome c into the cytosol. In addition to the anti-apoptotic Bcl-2 family proteins listed above, a second functional class of Bcl-2 proteins, including Bax and Bak, exert a more pro-apoptotic effect [303]. The anti-apoptotic Bcl-2 proteins actually exert their anti-apoptotic functions in part by binding and neutralizing these pro-apoptotic Bcl-2 family proteins [304-307]. Therefore, it is presumed that SKI-178 mediated inhibition of anti-apoptotic Bcl-2 proteins frees pro-apoptotic Bax and Bak to form membrane associated homo-oligomers that permeabilize the outer mitochondrial membrane. While

there is substantial evidence in the literature to support this assumption [209, 277, 306], many contradictory reports have surfaced, particularly regarding the functional significance of Bcl-2 phosphorylation at serine 70 [258]. Therefore, it's important to note that only anti-apoptotic Bcl-2 proteins were examined in these studies and that particular emphasis was placed on Bcl-2 and its phosphorylation at serine 70. To avoid misinterpretation and obtain a clearer picture of the signal transduction pathway involved in SKI-178 induced apoptosis, it would be prudent to confirm activation of Bak and/or Bax.

While there may be gaps in our understanding of the detailed downstream signaling, the overall findings presented in Chapter 2 clearly demonstrates that prolonged activation of CDK1 during mitotic arrest underlies SKI-178 induced apoptotic cell death. Based on supporting evidence in the literature linking SphK inhibition to mitotic arrest [270-272], it was originally hypothesized that the apoptotic effects of SKI-178 were directly linked to Sphk1 inhibition. However, the marked similarity between the MOA of SKI-178 and microtubule targeting agents (MTA) greatly challenged this hypothesis. Therefore, the studies outlined in Chapter 3 tested the revised hypothesis that SKI-178 functions as a polypharmacological agent, targeting both SphK1 and the microtubule network. We confirm that SKI-178 does in fact inhibit microtubule polymerization similar to that of other known microtubule destabilizing agents such as vincristine (Figure 3-3B-C). These results confirm that, in addition to being a SphK1 inhibitor, SKI-178 also acts as a MTA. However, because the mechanism of SKI-178 induced apoptosis is so similar to that of other known MTAs, it's easy to speculate that SphK1 inhibition likely plays little to no role in SKI-178 induced cell death. Dissecting the independent roles of

SphK1 inhibition and microtubule disruption in SKI-178 induced apoptosis is complicated by the lack of tools to effectively mask the effect of SKI-178 on either target alone. Therefore, we used a more selective SphK1 inhibitor, PF-543, to demonstrate that SphK1 inhibition can sensitize cells to microtubule disruption, independently of SKI-178. As seen in Figure 3-4A-B, non-cytotoxic doses of PF-543 sensitized cells to vincristine, supporting the hypothesis that SphK1 inhibition by SKI-178 likely enhances the apoptotic effect induced by SKI-178 mediated microtubule disruption. It's interesting to note, however, that the concentration of PF-543 required to sensitize cells to vincristine is about 1000 fold higher than its published IC_{50} for SphK1 inhibition [168]. Furthermore, while PF-543 is known to be more than 130 fold more selective for SphK1 over SphK2, the IC_{50} for SphK2 inhibition is well below the 5μM concentration used in these studies [168]. This would suggest that inhibition of both SphK1 and SphK2 may be required to sensitize cells to vincristine.

SKI-178 was originally identified as a SphK1 selective inhibitor using assays with SphK-isoform selective buffer conditions [130]. These selective conditions are based on the observation that buffers containing either high salts (NaCl or KCl) or Triton X-100 detergent have opposing effects on the activities of either SphK1 or SphK2. While high salt buffers markedly inhibit SphK1 activity, they conversely stimulate the activity of SphK2. On the other hand, buffers containing 0.5% Triton X-100 inhibit SphK2 activity while significantly stimulating the activity of SphK1 [78]. This system allows for evaluation of the selectivity of potential SphK inhibitors by observing their effects on either SphK isoenzyme independently. The pitfall of this assay, however, is the potential for differential effects of these buffer conditions on the SphK inhibitors themselves. For

example, high salt concentrations may precipitate certain inhibitors out of solution, leading to potentially false interpretations that they don't affect SphK2 activity. Therefore, more conclusive assays are needed to confirm the selectivity of SKI-178 toward SphK1. A more appropriate alternative may be high performance liquid chromatography (HPLC) based assays that don't require the use of these particular buffer conditions. Consequently, while SKI-178 is currently considered to be a SphK1 selective inhibitor, the possibility remains that it is in fact a pan inhibitor of both SphK1 and SphK2.

Future Directions:

We have come a long way in understanding the complex mechanism behind SKI-178 induced apoptotic cell death and in clearly establishing SphK inhibition as a means for sensitizing cells to MTAs. Nevertheless, important questions still remain. As described above, there are gaps in the overall understanding of SKI-178 induced cell death, particularly with regards to the downstream effects on Bcl-2 family protein inhibition and the overall SphK isoenzyme selectivity of SKI-178. Nonetheless, the results presented in Chapters 2-3 clearly demonstrate that (1) SKI-178 induced apoptosis is a consequence of prolonged CDK1 activity during mitotic arrest, (2) SKI-178 functions as a microtubule disrupting agent, and that (3) SKI-178's ability to simultaneously inhibit SphK likely sensitizes cells to the effects of this microtubule disruption. However, the details surrounding how SphK inhibition leads to this sensitization remains elusive. Therefore, the next step in the continuation of this work would be to understand the

molecular mechanism behind how SphK inhibition enhances the effects of MTAs. This knowledge not only would enhance our understanding of the apoptotic mechanism of SKI-178, but also could be directly applicable as a means to improve the efficacy of current clinically approved MTAs. Described below are four potential hypotheses based on the current understanding of the data presented herein, as well as data from the literature. Experiments are currently or will soon be underway to investigate these potential hypotheses in hopes of uncovering the true mechanism behind how SphK1 sensitizes cells to the effects of MTAs.

(1) SphK1 Inhibition Increases Expression of Pro-apoptotic Bcl-2 Family Proteins

Our first theory is that the interplay between the effects of SphK1 inhibition and microtubule disruption converges on controlling the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family proteins. A crucial step in committing a cell to intrinsic apoptotic cell death is mitochondrial outer membrane permeabilization (MOMP), which results in the release of multiple pro-apoptotic factors that influence downstream physiological changes of apoptotic cell death. The Bcl-2 family-proteins are not only key players in governing the commitment of cells to apoptotic death, but also are crucial effectors of MOMP itself. Therefore, the delicate balance between cell survival and apoptosis is tightly controlled by the subcellular location, expression, and activities of all Bcl-2 protein family classes. As both SphK1 inhibition and MTAs are known to affect the expression and/or activation of this protein family, we propose a mechanism whereby SphK1 inhibition sensitizes cells to the effects of microtubule disruption by tipping the

balance between pro-and anti-apoptotic Bcl-2 proteins even further to promote more cell death.

It is well documented that MTAs phospho-regulate and inhibit anti-apoptotic Bcl-2 proteins through prolonging the activity of CDK1, a cyclin B1 dependent serine/threonine kinase active during mitosis [308]. CDK1 mediated phosphorylation of Bcl-2 and Bcl-xL inhibits their anti-apoptotic effects [208, 309], whereas phosphorylation of Mcl-1 results in its proteasomal degradation and inactivation [209, 210]. In addition to CDK1 mediated inhibition of anti-apoptotic Bcl-2 proteins, studies also demonstrate prolonged CDK1 activity hyper-phosphorylates Bim, a member of the pro-apoptotic BH3-only Bcl-2 family [310]. CDK1 mediated phosphorylation of Bim precedes cell death induced by paclitaxel, and siRNA silencing of Bim reduces the sensitivity of cells to paclitaxel [310]. Therefore, this hyper-phosphorylation of Bim likely enhances its pro-apoptotic effects, further coupling mitotic arrest to apoptotic cell death.

While MTAs lead to post translational modifications that alter the activities of Bcl-2 proteins, there is growing evidence in the literature to suggest that SphK1 inhibition alters expression of Bcl-2 family proteins. Some of the earliest findings show that overexpression of SphK1 in endothelial cells promotes survival, partially by up-regulating anti-apoptotic Bcl-2 and down regulating pro-apoptotic Bim [311]. This effect on Bim expression was recently validated in gastric cancer cell lines, whereby SphK1 overexpression induced cell survival and conferred resistance to apoptosis through suppressing Bim expression [312]. More recent studies support these earlier findings, demonstrating that siRNA depletion of SphK1 decreased Bcl-2 levels while significantly increasing Bax expression [313]. In addition to molecular inhibition of SphK1,

pharmacological SphK1 inhibitors, such as SKI-II, have also been shown to significantly upregulate the expression of pro-apoptotic Bax while decreasing expression of anti-apoptotic Bcl-2 proteins in glioma and gastric cancer cell lines [314, 315]. These studies are further supported by the findings that ceramide accumulation is also shown to increase expression of pro-apoptotic Bcl-2 proteins while concomitantly downregulating anti-apoptotic Bcl-2 [316].

In addition to the transcriptional effects on Bcl-2 family proteins with SphK1 inhibition, there is substantial evidence supporting a role for ceramide in regulating the activities of various Bcl-2 family proteins. For example, direct interactions between ceramide and activated Bax have been demonstrated using yeast mitochondrial membranes, whereby they show that increased ceramide enhanced the propensity of Bax to permeabilize these membranes [299]. Furthermore, cell permeable C_2-ceramide was shown to induce a conformational change in Bax, promoting its activation in prostate cancer cell lines [317]. The idea that ceramide induces apoptosis via a Bax-dependent pathway is supported by the findings that over-expression of anti-apoptotic Bcl-2 proteins inhibits ceramide induced apoptosis [318, 319]

(2) Accumulation of Ceramide Enhances Mitochondrial Outer Membrane Permeabilization

Inhibition of SphK1 leads to the accumulation of ceramide, a pro-survival sphingosine-based lipid known to play a major role in regulating several cellular processes including growth inhibition, differentiation, senescence, and apoptosis [10]. A

net increase in intracellular ceramide levels is observed in response to numerous chemotherapeutic agents [18, 320]. Of the many roles of ceramide in apoptosis induction, a particularly important role is the ability of a specific ceramides, such as C_{16}-ceramide, to self-assemble into large stable channels within the mitochondrial outer membrane that allow for the release of cytochrome c into the cytosol from the inter-membrane space [321, 322]. Our previous hypothesis described a mechanism whereby SphK1 inhibition enhanced the apoptotic effects of MTAs by further upregulating pro-apoptotic Bcl-2 proteins, ultimately leading to greater cytochrome c release as a result of enhanced Bax and/or Bak channel formation. Here, we describe a variation of this hypothesis whereby we propose that the inhibition of SphK1 leads to the accumulation of ceramide, which enhances the apoptotic effects induced by MTAs by forming additional channels in the mitochondrial membrane.

The notion that ceramide accumulation sensitizes cells to various MTAs, such as paclitaxel and vincristine, has been well documented [295-297], but the molecular mechanisms underlying this effect has yet to be fully elucidated. Mitochondrial outer membrane permeabilization (MOMP), which allows for the release of proteins, such as cytochrome c, into the cytosol from the inter-membrane space is a key initiation step in apoptosis. Two types of outer membrane channels have been identified. The first, and more widely accepted, are channels formed from the oligomerization of Bax and Bak proteins [323, 324]. The second are channels formed from the self-assembly of ceramide monomers [49, 321]. While the role of ceramide in inducing apoptosis is widely acknowledged [325], the belief that ceramide directly forms channels in mitochondrial outer membranes has only recently gained acceptance [326]. This stems from the long

standing belief that lipids function to help regulate proteins and are simply incapable of organizing themselves into functional machines [327]. A unique feature of ceramide, however, is that it is capable of forming amide linages, a key molecular structure normally found in proteins that allows ceramide to form these structural channels [327].

As ceramides appear to be capable of spontaneously self-associating into channels [328], regulatory mechanisms must exist to avoid uncontrollable cell death. Interestingly, Bcl-2 family proteins that are known for their roles in regulating and executing Bax/Bak channel formation also function to regulate ceramide channels. For example, recent findings demonstrate that anti-apoptotic Bcl-2 and Bcl-xl also directly lead to the disassembly of ceramide channels [300]. More extensive studies focused on Bcl-xl demonstrating that the hydrophobic grove of Bcl-xl binds to apolar ceramide tails, which disruptes ceramide channel formation and assembly [326, 329]. While ceramides of varying fatty acyl chain lengths including C_2-, C_8-, C_{16}-, and C_{24}-ceramides are capable of forming outer mitochondrial membrane channels in *in vitro* studies [321, 329, 330], it's mainly the long chain ceramides (i.e. C_{16}-, and to some extent, C_{18}-, and C_{20}-ceramides) that appear to be physiologically relevant [327]. In fact, very long chain ceramides such as C_{24}-ceramide actually interfere with C_{16}-ceramide channel formation [331]. It's interesting to note that while the exact mechanism remains elusive, SKI-178 significantly increases levels of long chain ceramide species (C_{16}-C_{18}), while concomitantly decreasing very long chain ceramide species (C_{22}-$C_{24.1}$) (Table 2.2).

(3) SphK1 Inhibition Further Prolongs Mitotic arrest

An alternative mechanism for how SphK1 inhibition might sensitize cells to microtubule damage is through further prolonging mitotic arrest. The efficacy of MTAs hinges upon their ability to couple mitotic arrest to cell death. As previously described, MTAs induce mitotic arrest by disrupting the dynamic instability of microtubules, thereby preventing the spindle assembly checkpoint (SAC) from being satisfied. Until the SAC is satisfied, it will continue to inhibit the activity of the anaphase promoting complex/cyclosome (APC/C) and therefore, cells remain arrested in mitosis. Paclitaxel is an example of a MTA that is used as a first-line chemotherapeutic agent in a panel of cancer types, including breast and ovarian tumors [175]. While nearly all cancer cell lines display mitotic defects upon treatment with paclitaxel, the outcome of this effect can be quite variable within and among various cancer cell lines [332]. For example, many cells treated with MTAs undergo dramatic mitotic arrest, which is eventually resolved by cell death. However, subsets of cells undergo mitotic slippage, a process whereby cells aberrantly exit mitosis, despite the presence of misaligned chromosomes and an intact SAC [333]. Mitotic slippage results in the formation of multinucleated cells that will either die in the subsequent interphase, arrest in interphase, or enter into a subsequent cell cycle [185, 334]. Therefore, mitotic slippage is proposed as a mechanism to promote survival of cells treated with MTAs such as paclitaxel [302].

There is evidence in the literature to suggest that further prolonging mitotic arrest, which can be achieved through multiple avenues, is able to sensitize cells to MTAs [301, 302]. For example, inhibiting Cdc20, an activator of the APC/C, or overexpressing cyclin

B1 significantly prolongs the time slippage prone cells arrest in mitosis, ultimately increasing the percentage of cell death during mitosis [334]. Furthermore, a recent study by Sinnott et al. shows that inhibition of TRIM69A, a protein that binds to mitotic centrosomes and supports centrosome clustering, enhances the sensitivity of tumor cells to paclitaxel [302]. It has been proposed that prolonging the duration of mitotic arrest allows for further accumulation of pro-apoptotic signals [210, 302].

Although a role for SphK1 in mitosis has yet to be definitively described, recent findings have shown that SphK1 and SphK2 are both localized to the centrosome, and that their activity may be essential for coordinating molecular events required for proper bipolar spindle formation [270]. In support of this, pharmacological SphK inhibition and RNAi mediated SphK1 downregulation have both shown to block mitotic exit in breast carcinoma cell lines [271] and induce substantial G2/M arrest in melanoma cell lines [272]. Therefore, we hypothesize that inhibition of SphK1 by SKI-178 may disrupt centrosome clustering; further prolonging the duration of mitotic arrest induced by MTAs.

A useful model for investigating this hypothesis would be HCC366 NSCLC cells as they are notoriously intrinsically resistant to paclitaxel, despite its ability to significantly disrupt their polar spindle formation [302]. Studies show that HCC366 cells are able to survive for at least 72 hours after paclitaxel treatment, and 40-50% of treated cells are capable of colony formation despite significant mitotic damage [302]. These cells would allow for detailed molecular studies to uncover the role of SphK1 inhibition during mitotic arrest. For example, time lapsed live-cell imaging of HCC366 cells stably expressing GFP tagged histone H2B (GFP-H2B) can be used to manually quantify the

duration of time spent in mitosis [335]. This will also allow for the visualization of micro-nucleated cells. This methodology could then be used to observe whether pharmacological inhibition or RNAi mediated knockdown of SphK1 prolongs the mitotic transition time of HCC366, and whether this prolonged mitosis sensitizes cells to MTAs.

This proposed hypothesis implies that SphK1 plays a supportive role in cell division, particularly in cells undergoing mitotic stress induced by exposure to MTAs. If proven, this hypothesis would suggest the synthetic lethality of SphK1 inhibition when combined with microtubule disruption. This could be the result of tumor cells becoming dependent upon SphK1 activity for progression through mitosis under mitotic stress conditions. The idea that cells rely on SphK1 through a non-oncogene addiction mechanism has been previously proposed [99] based on the observation that SphK1 is overexpressed in most cancer types despite the lack of any known mutations in the SphK1 gene. These findings would expand upon this previously proposed mechanism for SphK1's oncogenic activity by demonstrating that tumor cells, under condition of mitotic stress, become dependent upon SphK1 activity for mitotic progression.

All three above mentioned hypotheses (outlined in Figure 4.1) are not necessarily mutually exclusive and there is no reason they couldn't all be contributing to the overall MOA of how SphK1 inhibition by SKI-178 sensitizes cells to its microtubule disruption effects. However, the model best supported by our data is this final hypothesis that SphK1 inhibition sensitizes cells to microtubule disruption by further prolonging mitotic arrest. For example, Figure 3-4B shows that vincristine treatment at a non-cytotoxic dose leads to an increase in Bcl-2 phosphorylation at Ser70 but had little to no effect on cell death (Figure 3-4A). However, when combined with a non-cytotoxic dose of PF543, Bcl-

2 phosphorylation is dramatically amplified (Figure 3-4B) with a concomitant increase in apoptosis (Figure 3-4A). These results indicate that the ability of SphK1 inhibition to sensitize cells to MTAs likely involves enhancing the activity of CDK1, a mitosis specific kinase responsible for this phosphorylation of Bcl-2. Therefore, while the possibility remains that SphK1 inhibition sensitizes cells to the effects of microtubule disruption by enhancing the expression of pro-apoptotic proteins and/or promoting ceramide channel formation, these effects can't explain the increase in Bcl-2 phosphorylation which likely occurs through enhancing CDK1 activity by further prolonging mitotic arrest.

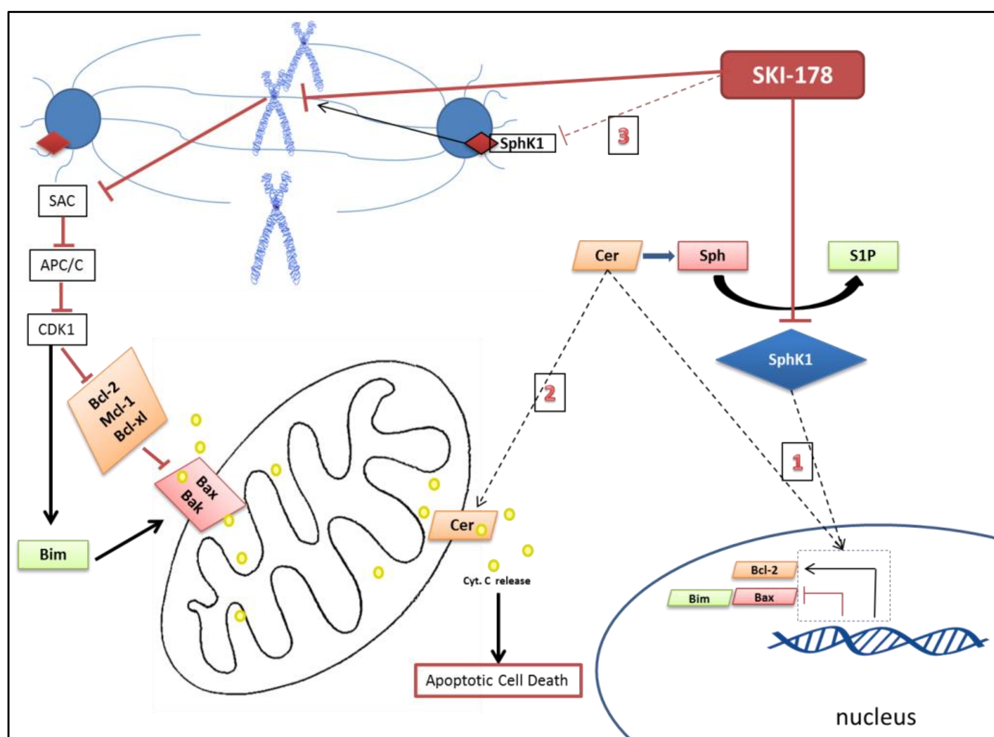

Figure 4-1. Proposed models for how SphK1 inhibition by SKI-178 may sensitize cells to its microtubule disruption effects

SKI-178 functions as a microtubule targeting agent inducing apoptotic cell death as a consequence of prolonging CDK1 induced inhibitory phosphorylation of anti-apoptotic Bcl-2 proteins. Furthermore, SKI-178 simultaneously inhibits SphK1 which

sensitizes cells to the effects of microtubule disruption. The details surrounding how SphK1 inhibition leads to this sensitization remains elusive but three potential hypotheses could explain this phenomenon: (1) SphK1 inhibition increases expression of pro-apoptotic Bcl-2 family proteins, (2) the accumulation of ceramide by SphK1 inhibition enhances mitochondrial outer membrane permeabilization, and (3) the inhibition of SphK1 could further prolong mitotic arrest

Chapter 5

Significance, Implications, and Clinical Relevance

Clinical Significance of the Polypharmacological MOA of SKI-178:

Cancer drug discovery over the previous decades has been dominated by the search for magic bullets, i.e. highly potent and selective drugs with one molecular target. This is based on the notion that high selectivity would reduce the likelihood of potentially hazardous off-target effects and provide information regarding the likely mechanism-of-action (MOA) [336]. This paradigm assumes a direct cause and effect relationship between the activity of a particular target and the disease phenotype. Indeed, high selectivity is a valuable drug characteristic when a single target acts as the molecular driver of cancer pathogenesis. Chronic myeloid leukemia (CML), for example, is often driven by a very specific chromosomal translocation, the Philadelphia chromosome, which results in a specific fusion gene that codes for an abnormal, constitutively active protein, BCR-Abl, that drives unregulated cell growth [337]. Therefore, a few targeted agents, such as imatinib, a kinase inhibitor of BCR-Abl used for the treatment of CML [338], or erlotinib and gefitinib, epidermal growth factor receptor (EGFR) inhibitors for the treatment of non-small-cell lung cancer (NSCLC) [339, 340] have dramatically improved treatment of certain cancer types. Despite their initial successes however, the effectiveness of these targeted therapies is often hindered by the rapid onset of resistance, particularly in patients with advanced stages of disease. In addition to numerous other factors, resistance to targeted kinase inhibitors often results from the expansion of cells with mutated forms of the target protein [339, 341-343]. Furthermore, most cancers are not governed by a single molecular target. In fact, most harbor abnormalities in multiple oncogenic or tumor suppressive proteins [344] and rely on redundant signaling pathways,

if one pathway is somehow inhibited [345, 346]. Therefore, the reality is that selective treatment strategies aimed at inhibiting a single chemotherapeutic target are generally not curative or even particularly effective considering the high rates of resistance. Furthermore, the therapeutic potential of many targeted based therapies is hindered by adverse side effects when used at clinically relevant concentrations.

Contrary to the dogma governing drug discovery for much of the late 20th century, drug promiscuity, particularly with regards to polypharmacology (i.e. drugs with multiple relevant targets), is now being viewed in a more positive light [347]. Historically, multi-targeted drugs are viewed as promiscuous agents with unpredictable biological activities and inadvertent side effects. Nowadays, however, single agents able to specifically but simultaneous target multiple therapeutically relevant targets are gaining popularity in the field of drug discovery [348-350]. In fact, many commonly used drugs available in the market are known to have polypharmacological effects [351]. Aspirin, for example, has been used for decades as an anti-inflammatory agent targeting the COX enzymes. Recent finding now show that it also functions as a chemo-preventive agent, likely through its ability to target other kinases such as IKK and ERK [352]. The notion that simultaneously hitting multiple targets is a superior treatment strategy for improving efficacy and overcoming resistance is well established. However, much of this is based on the use of specific drug combinations to simultaneously block multiple disease relevant targets. For example, combined administration of drugs targeting mitogen-activated protein kinase (MAPK), VEGFR, EGFR, PI3K and others have been successfully tested in clinical trials and shown enhanced therapeutic efficacy [353]. Ideal

drug combinations will simultaneously block multiple disease relevant targets using agents that are individually active but act synergistically in combination [354].

Polypharmacological drugs have numerous potential advantages over combination therapies [354]. The use of a single agent with multiple activities is likely to have a more predictable pharmacokinetic and safety profile compared to administering a combination of multiple agents. In addition, it guarantees the presence and simultaneous efficacies in target tissue, reduces the risk of negative drug-drug interactions or unforeseen toxicities, and leads to a higher likelihood of patient compliance compared to the complicated treatment regimens often seen with combination therapy. Despite these advantages, there is discernable difficulty with shifting the focus of drug discovery towards a “one-drug-multiple-target” approach. For example, there is no clear methodology to the discovery and development of these polypharmacological agents. Most polypharmacological agents discovered to date have arisen largely out of serendipity [336]. The successful rational design of polypharmacological agents is limited by numerous factors. For one, linking multiple pharmacophores to hit different targets will likely yield compounds with higher molecular weights, which may limit their efficacy *in vivo* [336]. Furthermore, designing methods for screening and optimizing drug candidates with multiple targets will likely be a challenging task.

Despite the ongoing search for more selective chemotherapeutic agents, systemic chemotherapy remains the backbone of cancer treatment. For example, agents that disrupt mitotic spindle assembly by targeting microtubules are some of the most commonly used anti-cancer drugs. Taxol® (paclitaxel) is FDA approved for the treatment of ovarian, breast, and NSCLC among others. It is also commonly used off-label for the treatment of

numerous other solid tumors such as cervical, prostate, and head and neck cancers as well as hematological malignancies like leukemia and lymphoma [355]. The vinca alkaloids, including vincristine and vinblastine, are approved for the treatment of acute lymphoblastic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, and various solid tumors such as rhabdomyosarcoma, neuroblastoma, and Wilms' tumor [356]. Despite their unprecedented success, the clinical efficacy of microtubule targeting agents (MTA) still has several limitations. The most notable being high levels of neurological [357] and myeloid [358] toxicities as well as the common emergence of drug-resistance.

One of the most common factors associated with resistance to MTAs is the overexpression of ABC transporter proteins such as multidrug resistant associated protein-1 (MDR1) and P-glycoprotein (Pgp) that interfere with intracellular drug accumulation [359]. Poor chemotherapeutic responses in patients with a range of different cancer types correlates with MDR or Pgp expression, particularly in patients that have previously received chemotherapy [360, 361]. These findings led to the development of various ABC transporter inhibitors in hopes that these agents would improve the clinical success of MTAs. Unfortunately, the use of both first- and second-generation ABC transporter inhibitors has been met with an overwhelming lack of clinical success owing to unanticipated toxicities, variable pharmacokinetic interactions, and non-specific targeting [362, 363]. Therefore, the search for new microtubule targeting agents should screen for those that aren't substrates of these ABC transporters. ABC transporter overexpression, however, is not the only clinical mechanism of resistance to MTAs. Another mechanism of paramount concern is the selective overexpression of certain β-tubulin isotypes. Class III β-tubulin in particular is highly

insensitive to the binding of MTAs. This is hypothesized to involve the intrinsic dynamic properties of class III β-tubulin, or possibly differential binding of MTAs to class III β-tubulin [364]. Despite the limited understanding of the underlying mechanism, high levels of class III β-tubulin is associated with resistance to paclitaxel [365, 366], docetaxel [367], as well as vinca alkaloids [368]. Interestingly, agents that bind to the colchicine binding site appear to circumvent this class III β-tubulin resistance [369, 370], indicating a need for novel agents binding to this site. A third type of resistance is found in acute myeloid leukemia (AML) patients and is specific to members of the vinca alkaloid family. While vincristine is proven effective against acute lymphoblastic leukemia (ALL) and many solid tumors, AMLs often found to be inherently resistant to vincristine [371]. This resistance is attributed to the inherent capacity of leukemic myeloid cells to produce myeloperoxidase. Myeloperoxidase is a pro-inflammatory peroxide enzyme found most abundantly in the granules of myeloid cells to help them carry out their antimicrobial activities [372]. The ability of myeloperoxidase to oxidize vincristine has been demonstrated in both cell free systems as well as AML cell lines [373]. In support of these findings, myeloperoxidase inhibitors blocked vincristine oxidation, and siRNA mediated downregulation of myeloperoxidase restored the sensitivity of AML cells to vincristine [374].

Implications and Clinical Relevance

Our data presented in Figure 3-4 clearly demonstrates that Sphingosine kinase 1 (SphK) inhibition, using PF-543, sensitizes cells to the effects of MTAs such as

vincristine. These data suggest that the addition of a SphK selective inhibitor may improve the therapeutic index for MTAs currently in clinical use by limiting the aforementioned neurological and myeloid toxicities often seen with MTAs at higher concentrations. Furthermore, while there is always an ongoing search for newer, safer, and more effective MTAs, the delays and barriers of translating promising molecules into approved drugs can take years. Therefore, any strategy that may improve the efficacy of currently approved drugs is worth considering. Although not directly tested in these studies, fingolimod is a clinically approved agent known to inhibit SphK1 that could directly enter clinical trials with approved MTAs. Unfortunately, there are many limitations to MTAs that would not be reversible or improved with the addition of a SphK1 inhibitor. For example, the emergence of drug resistance which often results from overexpression of MDR1 can't be overcome with the addition of a SphK inhibitor. Furthermore, AML patients who don't respond to vincristine as a result of its degradation by myeloperoxidase will also not be sensitized with the addition of a SphK inhibitor. While SphK1 inhibition does appear to sensitize cells to the effects of MTAs, it can't overcome problems of acquired or inherent resistance to MTAs. Therefore, from a therapeutic standpoint, many cancer types may not respond to this treatment combination. Fortunately, owing to problems with resistance and toxicity with MTAs, many "next generation" mitosis-specific agents have been developed that don't actually target microtubules, but rather target proteins that primarily function during mitosis [375]. Among these include aurora kinase inhibitors, polo-like kinase (PLK) inhibitors, and kinesin spindle protein (KSP) inhibitors [213]. Although MTAs tend to induce mitotic cell death owning to the fact that cells are particularly sensitive to disruption of normal

microtubule function during this stage, numerous studies have confirmed that MTA disrupt both mitotic and interphase microtubules at similar concentrations [202]. In fact, neurological toxicity, a major limitation in the use of MTAs, is proposed to stem from the disruption of interphase microtubules in resting neurons [202]. Mitosis associated proteins on the other hand are highly regulated and their expression is largely restricted to mitosis. Therefore, inhibitors of these mitosis associated proteins ultimately disrupt normal mitotic functions, similar to that of MTAs, but without the additional disruption of interphase microtubule functions. As a result, these agents are likely to be more specific for actively dividing cells, and thus should be associated with less nonspecific toxicity. Based on their similarities to MTAs, there is a high likelihood that SphK1 inhibition will sensitize cells to these protein inhibitors as well. This is an interesting hypothesis that if proven would open doors to even more promising treatment combination strategies to overcome some of the above mentioned limitations of MTAs.

Despite the promise of these combination strategies, SKI-178 as a single agent is probably the most promising therapeutic approach. As a single agent, SKI-178 exerts the same effect as these agents in combination. For example, the work presented in Table 2-2, in addition to previously published data [130], clearly establishes SKI-178 as a bona fide SphK inhibitor. While additional studies may be needed to confirm the isoenzyme selectivity of SKI-178, it's clear that SKI-178 induces alterations in intracellular levels of bioactive sphingolipid metabolites that are consistent with SphK inhibition in cells. Furthermore, we confirmed that SKI-178 is able to directly decrease tubulin polymerization *in vitro* and significantly destabilize microtubule polymerization in cells (Figure 3-3B). Therefore, SKI-178 is a promising polypharmacological agent that exerts

its cytotoxic effects by targeting two important chemotherapeutic targets. As a MTA, SKI-178 has numerous characteristics that stand out as an improvement over many currently approved agents such as vincristine and paclitaxel. For one, we have clearly established that SKI-178 induces apoptotic cell death in highly multidrug resistant cell lines, and therefore is not a substrate for multidrug resistant proteins (Figure 2-1C-D). Furthermore, it is highly effective at inducing apoptotic cell death in a range of AML cell lines, indicating that it is likely not degraded by myeloperoxidase enzymes. Very preliminary evidence obtained by other members of this lab suggests that SKI-178 binds to the colchicine binding site (Figure 5-1). As described above, agents binding to the colchicine binding site on tubulin appear to circumvent resistance resulting from the overexpression of class III β-tubulin. Lastly, the concomitant inhibition of SphK1 by SKI-178 will likely improve its therapeutic window and reduce toxicity. Therefore, SKI-178 is a promising alternative to currently available MTAs.

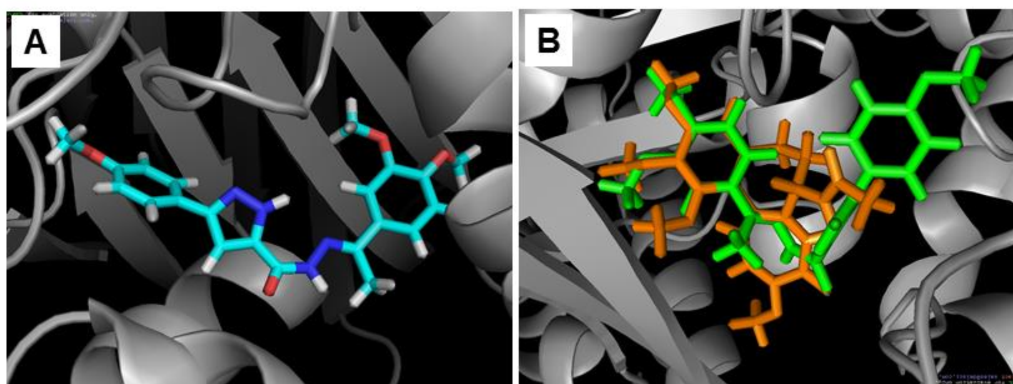

Figure 5-1. SKI-178 docking studies

SKI-178 docked to (A) SphK1 and (B) α/β tubulin dimer (SKI-178 is shown in green and colchicine in brown). Docking was carried out using GLIDE (Schrodinger LLC).

Furthermore, recent work done in the lab has evaluated the efficacy of SKI-178, *in vivo*, using three mouse models of AML. The first is a retroviral transduction model of the MLL/AF9 t(9;11)(p22;23) translocation. The results show that SphK1 is necessary for the development and progression of MLL/AF9-driven AML and that SKI-178 is effective at inducing complete remission of AML in this model system. Using a separate model it was also shown that SKI-178 significantly extends survival of mice xenografted with human MOLM-13 (MLL/AF9^{+}, FLT3-ITD^{+}) AML cell lines. Lastly, SKI-178 was also shown to significantly extend survival of mice xenografted with human patient AML cells. Together these finds further support the potential of SKI-178 as a promising chemotherapeutic strategy for the treatment of AML and possibly numerous other cancer types.

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Publications and Manuscripts

1. T.E. Dick, J.A. Hengst, Laura M. Geffert, Stephen Matthews, Jong K. Yun, SKI-178, a Single Agent that Functions as a Dual Target Inhibitor of SphK1 and Microtubule Dynamics. (manuscript soon to be sumbitted)
2. T.E. Dick, J.A. Hengst, T.E. Fox, A.L. Colledge, V.P. Kale, S.S. Sung, A. Sharma, S. Amin, T.P. Loughran, Jr., M. Kester, H.G. Wang, J.K. Yun, 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 352 (2015) 494-508
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