Inhibition of Sphingosine Kinase 2 Down-regulates the Expression of c-Myc and Mcl-1 and Induces Apoptosis in Multiple Myeloma

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Running title: sphingosine kinase in myeloma

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Key Points:

1. Sphingosine kinase 2 is over-expressed in myeloma cells and contributes to myeloma cell survival and proliferation.

2. Sphingosine kinase 2-specific inhibitor promotes proteasome degradation of Mcl-1 and c-Myc and inhibits myeloma growth in vitro and in vivo.
ABSTRACT:

Sphingolipid metabolism is being increasingly recognized as a key pathway in regulating cancer cell survival and proliferation. However, very little is known about its role in multiple myeloma (MM). We investigated the potential of targeting sphingosine kinase 2 (SK2) for the treatment of MM. We found that SK2 was over-expressed in MM cell lines as well as in primary human bone marrow CD138+ myeloma cells. Inhibition of SK2 by SK2-specific shRNA or ABC294640 (a SK2 specific inhibitor) effectively inhibited myeloma cell proliferation and induced Caspase 3-mediated apoptosis. ABC294640 inhibited primary human CD138+ myeloma cells with the same efficacy as with MM cell lines. ABC294640 effectively induced apoptosis of myeloma cells even in the presence of bone marrow stromal cells. We further found that ABC294640 down-regulated the expression of pS6 and directed c-Myc and Mcl-1 for proteasome degradation. Additionally, ABC294640 increased Noxa gene transcription and protein expression. ABC294640 per se did not affect the expression of Bcl-2, but acted synergistically with ABT-737 (a Bcl-2 inhibitor) in inducing myeloma cell death. ABC294640 suppressed myeloma tumor growth in vivo in mouse myeloma xenograft models. Our data demonstrated that SK2 provides a novel therapeutic target for the treatment of MM. Study registered to www.clinicaltrials.gov: NCT01410981.
INTRODUCTION:

Multiple myeloma (MM) is the second most common hematological malignancy in the United States where it accounts for about 11,000 deaths annually.\textsuperscript{1,2} The overall outcome and survival of patients with MM have significantly improved over the last decade, largely due to the use of several highly active agents (i.e., thalidomide, lenalidomide, and bortezomib) and the incorporation of high dose chemotherapy supported with autologous hematopoietic stem cell transplantation. MM, however, remains an incurable disease. Patients may relapse within months after autologous hematopoietic stem cell transplantation. Furthermore, nearly all MM patients will eventually develop resistance to currently available agents. There is an unmet medical need for the development of novel therapeutic agents for this disease. It is particularly important to develop new agents that do not share similar mechanism of action with proteasome inhibitors or immunomodulatory drugs because most of the refractory/relapsed MM patients would have exposed to those agents during their course of treatment.

Sphingolipids are an extremely diverse group of water insoluble molecules that include ceramides, sphingoid bases, ceramide phosphates and sphingoid base phosphates. In addition to supporting the structure and fluidity of the lipid bilayer, sphingolipid metabolites function as second messengers and hormones, and regulate cytokine-mediated cell signaling.\textsuperscript{3,4} Sphingolipids are involved in a wide range of biological and pathological events including inflammation, cell proliferation, apoptosis, angiogenesis, and transformation (reviewed in\textsuperscript{5-10}). More recently, sphingolipid metabolism is being increasingly recognized as a key pathway in tumor cell survival and in cancer biology.\textsuperscript{11-18}

Among sphingolipid metabolites, ceramide, sphingosine and sphingosine-1-phosphate (S1P) are the key players for their biophysiological functions. Ceramide can be produced via hydrolyzation of
sphingomyelin in response to stimuli such as cytokines and growth factors. Ceramide is further
hydrolyzed to sphingosine. Sphingosine is then rapidly phosphorylated by sphingosine kinases (SK) to
S1P. Ceramide and sphingosine are pro-apoptotic, inducing apoptosis in tumor cells without disrupting
quiescent normal cells. In contrast, S1P is mitogenic and anti-apoptotic. A critical balance, i.e. a
ceramide:S1P rheostat, is hypothesized to determine the fate of the cell.

There is accumulating evidence demonstrating an important role of S1P in cancer cell survival, drug
resistance, adhesion, and the communication between tumor cells and the microenvironment. Most
effort has focused on developing modulators of S1P receptors, such as Fingolimod (FTY720). FTY720
was found to be able to induce apoptosis and overcome drug resistance in MM. In a fundamentally
different approach, our current study targeted SKs that catalyzes the generation of S1P. We reasoned that
SKs provide a potential site for manipulation of the ceramide:S1P rheostat. SKs have two isoenzymes:
SK1 and SK2. SK1 was found to play a key role in IL-6 induced myeloma cell proliferation and
survival. Many studies have suggested that the biological roles and localization of SK1 and SK2 are
different, and very little is known about the role of SK2 in MM. We herein examined the role of
SK2 in myeloma cell survival and determined the potential of targeting SK2 for the treatment of MM.
METHODS AND MATERIALS:

Cell lines

Cell lines\textsuperscript{36,37} used in the study were described in the Supplementary Methods.

Patient samples and isolation of primary human CD138\textsuperscript{+} myeloma cells

IRB approval, patient bone marrow aspirates, and isolation of CD138\textsuperscript{+} myeloma cells were described in the Supplementary Methods (Clinicaltrials.gov: NCT01410981).

Reagents

ABC294640 (the SK2-specific inhibitor) was synthesized and provided by Apogee Biotechnology Inc. SK1 inhibitor (SK1-II), i.e., 2-(p-Hydroxyanilino)-4-(p-chlorophenyl)thiazole, was purchased from Echelos Biosciences Inc (Salt Lake City, UT). For other reagents, please see the Supplementary Methods.

SK2-specific shRNA lentiviruses

Details of shRNA lentivirus production\textsuperscript{38} and transduction were available in the Supplementary Methods.

In vitro drug treatment, cell proliferation assay, apoptosis assay, Western blot analysis, and RT-PCR

Details of drug treatment and assays were described in the Supplementary Methods.

In vivo experiment

Details of \textit{in vivo} experiments were available in the Supplementary Methods. Briefly, MM.1S cells stably expressing luciferase (5×10\textsuperscript{5} cells/mouse) were injected via tail vein or subcutaneously into irradiated (2.5 Gy) NSG mice. Beginning at day 2 or at day 14, the mice were treated with ABC294640 (50mg/kg, i.p.) daily for ~30 days. Tumor growth was followed by bioluminescence imaging at the time-points indicated.
Statistical analysis

Details of statistical analysis were available in the Supplementary Methods.
RESULTS:

Sphingosine Kinase 2 is over-expressed in myeloma cells.

To determine the potential utility of targeting SKs for the treatment of MM, we measured the gene expression levels of SK1 and SK2 in myeloma cells. We measured SK1 and SK2 mRNA expression in a publicly available myeloma microarray dataset, in myeloma cell lines, and in freshly isolated human bone marrow CD138⁺ myeloma cells (Fig. 1). We downloaded the GSE6477 Affymetrix microarray dataset originated by Mayo Clinic. This dataset contained microarray mRNA gene profile on purified plasma cells isolated from normal control subjects (n=15) or newly diagnosed MM patients (n=73). We generated the Robust Multi-array Average (RMA)- normalized gene expression data for SK1 and SK2 and compared their expression levels between normal subjects and newly diagnosed MM patients. As shown in Fig. 1A, SK2 expression was increased in MM patients compared to normal subjects (p=0.046), whereas there was no significant difference in SK1 expression level in plasma cells between MM patients and normal subjects.

SK1 and SK2 mRNA expression levels in two B cell lines (EBV-immortalized B cell line and ATCC B lymphocyte cell line) and seven myeloma cell lines (NCI-H929, OPM1, U266, RPMI-8226, RPMI-8226-Dox40, MM.1R and MM.1S) were measured by quantitative RT-PCR. As shown in Fig. 1B, the mRNA expression level of SK2 was higher than that of SK1 in all tested myeloma cell lines except RPMI-8226-Dox40 cells. Furthermore, the mRNA expression level of SK2 was higher in all seven myeloma cell lines than that in the two B cell lines.

We also determined the SK1 and SK2 mRNA gene expression in freshly isolated primary human bone marrow (BM) CD138⁺ MM specimens. CD138⁺ plasma cells were isolated from the BM aspirates of
normal controls, MGUS (monoclonal gammopathy of undetermined significance) patients, or MM patients including amyloidosis patients. No difference in SK1 mRNA expression was observed between these three populations of patients (data not shown). Interestingly, SK2 gene expression was increased in the CD138+ cells in 10 out of 34 (29%) MM patients (Fig. 1C). We performed additional subset analyses to determine whether SK2 mRNA expression correlated with myeloma disease stage, cytogenetic profile, M protein level or BM plasma cell number. No correlation was observed with these subset analyses (data not shown).

SKs catalyze the phosphorylation of sphingosine to S1P and sphingosine derives from ceramide. To determine if the over-expression of SK2 in myeloma cells affects the levels of ceramides, sphingosine and S1P, mass spectrometry measurement of 14 different ceramides, sphingosine and S1P was performed in two B cell lines and six MM cell lines. The levels of ceramides and S1P varied highly among MM cell lines and between B cell lines and MM cell lines (data not shown). Interestingly, sphingosine level was lower in MM cells than that in B cells and the difference was statistically significant for 4 out of 6 MM cell lines we tested (Fig. 1D). The decrease in the level of sphingosine in MM is consistent with increased sphingosine kinase gene expression.

**SK2-specific shRNA inhibits myeloma proliferation and induces Caspase 3-mediated cell death.**

To determine the roles of SK2 in MM cell survival and proliferation, we used specific shRNA to knockdown SK2 expression in MM cells. Lentiviral vector expressing SK2-specific shRNA or control shRNA was constructed and used to transduce MM cell lines. Both SK2-specific shRNA and control shRNA effectively transduced MM cell lines as demonstrated by high level of DsRFP expression (Fig. 2A). SK2-specific shRNA decreased SK2 mRNA expression by ~80% (Fig. 2B). SK2-specific shRNA
effectively inhibited myeloma cell proliferation as measured by MTT assay (Fig. 2C). To further
determine the effect of SK2 on myeloma cell proliferation, we transduced OPM1 myeloma cells with
SK2-specific shRNA or control shRNA. We then labeled the cells with CellTrace Violet Cell
Proliferation dye and measured dye fluorescence intensity 7 days later (Fig. 2D). With cell division, the
dye is diluted and the fluorescence intensity is reduced. As shown in Fig. 2D, compared to control
shRNA, SK2-specific RNA inhibited myeloma cell proliferation and division. Additionally, we found
that SK2-specific shRNA activated Caspase 3 (Fig. 2E). These data suggested that SK2 plays an
important role in both cell proliferation and survival of myeloma cells, and thus provides a therapeutic
target for the treatment of MM.

**SK2-specific inhibitor (ABC294640) inhibits myeloma growth in vitro.**
We next tested the effectiveness of the SK2-selective inhibitor (ABC294640) in killing myeloma cells in vitro. ABC294640 is the most advanced, non-lipid-based oral SK2 inhibitor and shows no inhibition for
SK1 or panel of protein kinases.\(^{41}\) ABC294640 is currently undergoing single agent phase I/II clinical trial
at our institute for solid tumors. We treated 6 different MM cell lines with various concentrations of
ABC294640 and found that ABC294640 inhibited myeloma cell growth with an IC\(_{50}\) of ~30 \(\mu\)M,
including steroid resistant MM.1R cells (Fig. 3A). Among the 6 MM cell lines we tested, OPM1 appears
to be the most sensitive cell line to ABC294640 treatment whereas U266 is relatively resistant.
ABC294640 inhibited myeloma cell growth as early as 16 hours after exposure (Fig. 3B).

To determine if ABC294640 induces cytotoxic or cytostatic effects on MM cells, we cultured MM cells
with 30\(\mu\)M of ABC294640 and then quantified live cell numbers over time. As shown in Fig. 3C,
ABC294640 exhibited cytotoxic effects on the majority (7 out of 8) of MM cell lines we tested, except for
U266 cells. We further found that ABC294640 treatment induced apoptotic cell death in myeloma cells as demonstrated by Annexin V staining (Fig. 3D), Caspase 3 activation (Fig. 3E), Caspase 9 activation, and PARP cleavage (Fig. 3F). ABC294640 induced growth arrest, but no apoptosis in U266 cells (data not shown).

We also tested the anti-tumor effects of ABC294640 on primary human CD138+ myeloma cells. Human CD138+ myeloma cells were freshly isolated from myeloma patient’s BM aspirate and treated with various concentrations of ABC294640. Cell proliferation was then measured by MTT assay. ABC294640 inhibited primary human myeloma cells with the same efficacy as with the most sensitive myeloma cell lines (OPM1) (Fig. 3G), demonstrating the potential clinical utility of ABC294640 in the treatment of MM.

The effects of SK1 inhibitor (SK1-II), Myriocin (a natural product inhibitor of serine palmitoyltransferase), and FTY720 (a S1P receptor antagonist) on myeloma cell proliferation were investigated (supplementary data). Consistent with previous reports by others, FTY720 inhibited myeloma cell growth (Supplementary Figure 1A). No inhibitory effects of Myriocin or SK1 inhibitor on myeloma cells were observed (Supplementary Figure 1B and 1C). These data again support the important role of SK2 in myeloma cell proliferation and survival.

**ABC294640 up-regulates Noxa expression and promotes proteasome degradation of Mcl-1.**

We performed extensive mechanistic studies to understand the pathways through which SK2 inhibition induces myeloma cell death. Extensive studies have demonstrated an essential role of Mcl-1 in the survival of human myeloma cells. Mcl-1 is over-expressed in more than half of newly diagnosed MM patients...
and in ~81% of relapsed MM patients. Furthermore, recent studies suggested that SK1 plays a key role in Mcl-1 expression induced by IL-6. Therefore, we examined if ABC294640 could affect the expression of Mcl-1 in MM. As shown in Fig. 4A, ABC294640 treatment led to down-regulation of Mcl-1 protein expression in MM cells, with the largest reduction occurring in OPM1 cells, which is also the most sensitive cell line for inhibition of proliferation by ABC294640. ABC294640 caused minimal changes in Mcl-1 expression in U266 cells.

Protein expression is controlled by the rate of biosynthesis and degradation. We thus first determined if ABC294640 affected Mcl-1 gene transcription. MM cell lines were treated with ABC294640 for 16 hr and Mcl-1 mRNA was quantified by RT-PCR. Additionally, OPM1 cells were stably transduced with SK2-specific shRNA or control shRNA and Mcl-1 mRNA was measured. Inhibition of SK2 by either ABC294640 or shRNA did not affect Mcl-1 gene transcription (Supplementary Figure 2), suggesting that ABC294640 down-regulated Mcl-1 expression at the post-transcription level.

We next tested if ABC294640 would increase the rate of Mcl-1 degradation. To this end, we treated OPM1 cells with DMSO or ABC294640 for 3 hrs, and then added cycloheximide to inhibit new protein synthesis. Mcl-1 protein levels were measured by immunoblot every hour for 4 hrs. As shown in Fig. 4B, ABC294640 treatment significantly increased Mcl-1 degradation.

Proteasome degradation plays an important role in regulating protein stability. We thus tested if ABC294640 treatment promoted Mcl-1 degradation in a proteasome-dependent manner. OPM1 cells were treated with ABC294640 alone, proteasome inhibitor (either MG132 or bortezomib) alone or in combination of ABC294640 with proteasome inhibitor. MG132 and bortezomib partially but
reproducibly protected Mcl-1 from degradation induced by ABC294640 treatment (Fig. 4C). These data suggested that ABC294640 at least in part increased Mcl-1 proteasome degradation.

Noxa is a pro-apoptotic, Bcl-2 homolog (BH) 3-only member of the Bcl-2 family. Treatment with Bortezomib or arsenic trioxide induced up-regulation of Noxa while down-regulating Mcl-1. These studies and others suggested that Noxa and Mcl-1 form a complex and the Noxa/Mcl-1 ratio plays an important role in regulating apoptosis. We thus examined the effects of ABC294640 on Noxa expression. ABC294640 treatment increased Noxa protein expression levels by 50% to 2 fold (Fig. 4D). Additionally, ABC294640 up-regulated Noxa mRNA expression in all eight MM cell lines we tested by at least 5 fold (Fig. 4E). These data suggested that ABC294640 treatment shifted the Noxa/Mcl-1 apoptosis rheostat towards favoring cell death.

**ABC294640 down-regulates pS6 and promotes proteasome degradation of c-Myc.**

C-MYC is an important oncogene dysregulated in post-germinal center malignancies including MM. Translocation involving c-MYC was found in 19 of 20 MM cell lines and in approximately 50% of advanced primary MM tumors. MM cells are addicted to c-MYC activity and c-MYC is indispensable in myeloma development. Furthermore, constitutive activation of PI3K/AKT/mTOR pathway is a common event in MM pathogenesis and contributes to MM proliferation and survival. Our unpublished data indicated that the downstream of mTOR pathway, i.e., pS6, was highly up-regulated in nearly all myeloma patients’ bone marrow samples. Given the importance of c-Myc and pS6 in MM cell survival, we examined the effects of ABC294640 on the expression of c-Myc and pS6 in MM cells. ABC294640 treatment significantly down-regulated the expression of c-Myc and pS6 (Fig. 5A). U266 has no detectable c-Myc expression by Western blot analysis.
We performed additional studies similar to those described with Mcl-1 to understand the mechanisms through which ABC294640 down-regulated c-Myc expression. We found that ABC294640 or SK2-specific shRNA did not affect the rate of c-Myc gene transcription (Supplementary Figure 3). Cycloheximide study suggested that ABC294640 increased c-Myc protein degradation (Fig. 5B). MG132 protected c-Myc from ABC294640-induced c-Myc degradation, suggesting that ABC294640 enhances proteasome degradation of c-Myc (Fig. 5C).

**ABC294640 synergizes with Bcl-2 inhibitor in the killing of myeloma cells.**

We reasoned that combined chemotherapy incorporating drugs with different mechanisms of action is likely to be more effective in killing myeloma cells and could overcome drug resistance. To this end, we examined the anti-myeloma activity of ABC294640 in combination with other agents. We were particularly interested in combining ABC294640 with Bcl-2 inhibitor for the treatment of MM, because ABC294640 per se did not affect Bcl-2 expression (Fig. 6A). We treated MM cell lines with various concentrations of ABT-737 (a Bcl-2 inhibitor) in combination with 15µM of ABC294640. Cell proliferation was then measured by MTT assay. Combining ABC294640 with ABT-737 led to greater inhibition of MM cell proliferation (Fig. 6B). Additional ABC294640 concentrations were tested with ABT-737 and the combination index (CI) value was calculated. Fa-CI plot analysis demonstrated synergism between ABC294640 and ABT-737 in inhibiting myeloma cell proliferation (Fig. 6C).

**ABC294640 induces myeloma cell apoptosis in the presence of bone marrow stromal cells.**

BM stromal cells support the growth and survival of myeloma cells and confer them with drug resistance. We sought to test if ABC294640 could still effectively kill myeloma cells in the presence of BM stromal
cells. We co-cultured e-GFP expressing myeloma cells with HS5 BM stromal cells. We then added ABC294640 to the co-culture system and measured Annexin V+ cells gated on the eGFP+ myeloma cells. ABC294640 did not induce apoptosis of HS5 BM stromal cells (Fig. 6D). Interestingly, as shown in Fig. 6D, the percentage of Annexin V+ myeloma cells following ABC294640 treatment was quite similar in OPM1 cells alone and in OPM1 cells co-cultured with HS5 BM stromal cells. These data suggested that ABC294640 could induce myeloma cell apoptosis even in the presence of BM stromal cells.

**ABC294640 suppresses MM tumor growth in mouse xenograft models.**

The *in vivo* anti-myeloma activity of ABC294640 was assessed using mouse xenograft models. We transduced MM.1S myeloma cells with lentiviral vector expressing luciferase and generated MM.1S cell line stably expressing luciferase. We performed two series of *in vivo* experiments. In the first series of experiments, the luciferase expressing MM.1S cells were injected via tail vein (Fig. 7A) or subcutaneously (Fig. 7B) into sublethally irradiated NOD/SCID IL-2γ (NSG) mice. Two days after the tumor injection the mice were treated with ABC294640 (50mg/kg daily i.p.) or vehicle control buffer for 30 days. In our second series of experiments, MM.1S cells were injected subcutaneously into irradiated (2.5 Gy) NSG mice. ABC294640 treatment was started 14 days later when bioluminescence imaging showed tumor engraftment, and continued daily for ~1 month (Fig. 7C). Tumor growth was monitored by bioluminescence imaging at the time points indicated. As shown in Fig. 7A-7C, ABC294640 effectively inhibited myeloma growth *in vivo.*
DISCUSSION:

In the current study, we found that SK2 was over-expressed in myeloma cells, and 10 out of 34 MM patients (~29% of MM patients) had up-regulated SK2 expression (Fig. 1C). Furthermore, knockdown of SK2 gene with shRNA inhibited myeloma cell proliferation and induced cell death. These data demonstrated an important role of SK2 in myeloma pathogenesis. Using a SK2 specific inhibitor, we showed for the first time that SK2 provides a potential therapeutic target for the treatment of MM.

Additionally, we investigated the combinatorial anti-tumor effects of ABC294640 with other categories of anti-myeloma agents. We found that ABC294640 acts synergistically with Bcl-2 inhibitor (ABT-737) in killing myeloma cells. This novel combination of ABC294640 with Bcl-2 inhibitor (Fig. 7D) may be particularly important in the treatment of relapsed or refractory MM patients. This population of patients most likely have been exposed to and become resistant to currently available agents such as dexamethasone, immunomodulatory drugs, and proteasome inhibitors.

ABC294640[^1,^51-^58] is currently undergoing single-agent phase I clinical trial at our institute in patients with advanced solid tumors. Sixteen patients to date have been enrolled showing no drug-related toxicities, and the clinical trial demonstrated excellent correlation between mouse and dog PK parameters with human data. ABC294640 has shown good pharmacokinetics, oral bioavailability and biodistribution. Plasma concentrations can reach >200 µM without hematologic or major organ toxicity, about 6-7 fold higher than the IC$_{50}$ we found with MM cell lines. The anti-myeloma activity of ABC294640 seen with freshly isolated primary human CD138$^+$ cells and in our in vivo mouse xenograft models further provides justification for testing ABC294640 in a clinical setting with refractory/relapsed myeloma patients.

Mechanistically, we found that inhibition of SK2 up-regulated Noxa expression and enhanced the proteasome degradation of Mcl-1 as well as c-Myc (Fig. 7D). Additionally, ABC294640 was found to
down-regulate pS6 expression level. This finding suggested that SK2 might also play a role in mTOR signaling pathway and affect cell translation. It is currently unknown; however, whether ABC294640 affects a common upstream event that controls Mcl-1, c-Myc and pS6 pathways or if it has diverse biological activities affecting multiple pathways simultaneously. It also remains to be determined which effect, i.e., down-regulation of Mcl-1, c-Myc, or pS6, contributes to a larger extent to the killing of myeloma cells by ABC294640. We have found that ABC294640 induced apoptosis in OPM1 cells, but failed to do so in U266 cells. Interestingly, U266 cells do not express detectable level of c-Myc (Fig. 5A) and show minimal change in Mcl-1 expression after ABC294640 treatment (Fig. 4A). On the other hand, ABC294640 reduced pS6 expression in both U266 cells and OPM1 cells. These data suggested that among these 3 molecules, Mcl-1 might play a major role in mediating ABC294640-induced apoptosis.

We are currently undertaking experiments to over-express c-Myc in U266 cells or transduce c-Myc dominant negative construct into OPM1 cells to further dissect the role of c-Myc in ABC294640-mediated apoptosis in MM cells.

Treatment of proteasome inhibitors consistently, although partially reversed ABC294640-mediated down-regulation of Mcl-1 protein expression (Fig. 4C), suggesting ABC294640 promotes Mcl-1 degradation at least in part via proteasome degradation pathway. It is also possible that ABC294640 may affect other protein degradation pathway including lysosomal proteolysis- mediated protein degradation or protein translation as suggested by the down-regulation of pS6, which correlates with cell translation activities.

The relative roles of SK1 and SK2 in tumor biology have been of great interest to many investigators, and were a central issue in the selection of ABC294640 for our study. Many studies have suggested different biological roles of the two SK isozymes.5,17,32-35 Most studies have focused on SK1 because it is the
predominant isozyme in most cells, and is up-regulated in many cancers. The latter response is likely due to hypoxia because SK1 is HIF-regulated, making it difficult to discern whether overexpression is required to drive tumor growth or is a consequence of tumor growth. Target validation studies using cDNA transfection or RNA interference are inconsistent in ascribing dominance to either isozyme. For example, overexpression of SK1 has been shown to be oncogenic, while transfection with SK2 was originally reported to inhibit cell growth and to induce apoptosis. However, these effects of SK2 are not dependent on its catalytic activity, and may be mediated by its BH3 domain. Consistent with this are the observations that physiological levels of SK2 do not inhibit DNA synthesis. Published studies have also shown conflicting results on SK1 and SK2 expression. For instance, one study found that EGF induced the expression of SK1 but not SK2 in MCF-7 cells, while others reported that EGF activated SK2 in MDA-MB-453 cells. Knockdown of SK2 had more profound effects than SK1 knockdown in inhibiting glioblastoma cell growth. Our results with SK1 inhibitor did not reveal any effects of SK1 inhibitor at a concentration up to 60 µM on MM cell growth (Supplementary Figure 1S). Additional work is needed to refine our knowledge about SK1 vs. SK2 biology in MM.

The exact role of SK2 in MM pathogenesis remains to be determined. SK2 deficient MCF-7 breast cancer showed delayed tumor growth in mouse xenograft model, but the authors attributed this delay in tumor development to polarization of tumor-associated macrophages. It is unclear if the over-expression of SK2 in myeloma cells serves as a driving event to initiate the development of MM or merely reflects phenotypical changes due to other oncogene aberrations. There are no reports in the literature suggesting an increased incidence of myeloma in SK2 transgenic mice. We are currently planning to crossbreed the IL-6/c-MYC double transgenic myeloma mice with SK2 knockout mice and determine the incidence and
severity of myeloma in the IL-6/c-Myc double transgenic/SK2 knockout mice. These studies will help us further define the role of SK2 in MM development.

In summary, SK2 is aberrantly up-regulated in MM cells and inhibition of SK2 suppresses myeloma growth \textit{in vitro} and \textit{in vivo}. Inhibition of SK2 affects several pathways critical to myeloma cell survival and proliferation including Mcl-1/Noxa, c-Myc and pS6. Our study provides important preclinical data for testing SK2 inhibitor (ABC294640) in clinical settings.
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AUTHOR CONTRIBUTIONS

Contribution: J.K.V., N.A., and Y.K. initiated the research, developed the concept of the paper, designed the study, and analyzed and interpreted the data; H.C. and J.H.S. performed part of the experiments; R.S., L.C., W.C., K.G., T.M, and Y.K. consented patients and obtained primary human bone marrow samples; E.G-M and Z.W. assisted in biostatistical analyses. C.S. and B.O. provided reagents and helped design the study. J.K.V. and Y.K. wrote the manuscript.
CONFLICT-OF-INTEREST DISCLOSURE:

Dr. Charles Smith is the President and CEO of Apogee Biotechnology Corporation. All other authors declare no potential conflict of interest.
REFERENCES:


FIGURE LEGEND

Figure 1: SK2 was over-expressed in myeloma cells. A: SK1 and SK2 gene expression in GSE6477 Affymetrix microarray dataset. Publicly available Affymetrix microarray data set GSE6477 was downloaded and the RMA normalized gene expression data were generated. The SK1 and SK2 expression level between plasma cells from normal subjects (blue bar; n=15) and purified CD138+ cells from newly diagnosed MM (red bar; n=73) were compared. B: SK1 and SK2 expression in myeloma cell lines and B cell lines. RNA was extracted from two B cell lines (EBV-Immortalized B cells (IBC) and ATCC® CCL-156 B lymphocytes) and seven MM cell lines (NCI-H929, OPM1, U266, RPMI-8226, RPMI-8226-Dox40, MM.1R, and MM.1S) and RT-PCR was performed for SK1 and SK2. Relative SK1 and SK2 mRNA expression with respect to β-Actin was shown (Mean± SEM of 3 separate sets of experiments). C: SK2 expression in primary human bone marrow CD138+ cells. Primary human CD138+ cells were isolated using CD138 enrichment kit from the BM aspirates of normal subjects (n=5), MGUS patients (n=6) and myeloma patients (n=34). SK2 gene expression was normalized against β-Actin control (Each dot represented one individual patient and the two solid circles represented amyloidosis patients). D: Sphingosine level in myeloma cell lines and B cell lines. Sphingosine was measured by HPLC in freshly prepared EBV-Immortalized B cells, ATCC B lymphocytes, and 6 MM cell lines (NCI-H929, OPM1, U266, RPMI-8226, RPMI-8226-Dox40, and MM.1S). Data represented sphingosine concentration (pmole/1×10^6 cells) (Mean± SEM of one of 4 separate sets of experiments) (*: p<0.05; **: p<0.01).

Figure 2: SK2-specific shRNA inhibited cell proliferation and induced Caspase 3 activation in myeloma cells. OPM1 cells were transduced with lentiviruses expressing SK2-shRNA-DsRFP or control shRNA-DsRFP for 4h. The cells were then washed and grew in regular culture medium for additional conditions.
48h. **A: Fluorescent microscopy image showing DsRFP expression. B: Expression of SK2 mRNA in SK2-shRNA- or control shRNA- transduced OPM1 cells. C: Cell proliferation by MTT assay.** Cell proliferation was measured using MTT assay at 0h and 48h following transduction. **D: Cell proliferation by flow cytometry.** OPM1 cells transduced with SK2-shRNA or control shRNA were stained with CellTrace Violet Cell Proliferation dye and allowed to proliferate for 7 days. The dye fluorescence intensity was measured by flow cytometry. **E: Activation of Caspase 3.** OPM1 cells were transduced with SK2-shRNA viruses or control shRNA viruses. Forty-eight hrs later, the cells were stained with Fixable Live/Dead cell dye, then fixed and permeabilized, and stained with Caspase-3 antibody. Caspase 3 intensity was gated on live cell population. Data were representative of 4 separate experiments.

**Figure 3: ABC294640 inhibited cell proliferation and induced apoptosis in MM cells. A: Dose-dependent inhibition of cell proliferation by ABC294640.** Six different MM cell lines were treated with various concentrations of ABC294640 for 48h and cell proliferation was measured by MTT assay (Mean± SEM of one of 3 separate sets of experiments). **B: Time course of proliferation inhibition by ABC294640.** Six different MM cells lines were treated with 30µM of ABC294640 or DMSO for various durations. Cell proliferation was analyzed by MTT assay at the time-points indicated (Mean± SEM of one of 3 separate sets of experiments). **C: Cytotoxic effects of ABC294640 on MM cells.** OPM1 cells were treated with 30µM of ABC294640 or DMSO and total live cells were quantified at the time-points indicated (Mean± SEM of 3 separate sets of experiments). **D: Increased Annexin V+ cells by ABC294640.** OPM1 cells were treated with 30µM of ABC294640 or DMSO for 16h and cells were stained with Annexin V and 7-AAD and analyzed by flow cytometry. Data shown were representative of 3 separate sets of experiments. **E: Caspase 3 activation.** OPM1 cells were treated with 30µM of ABC294640 or DMSO for 16h and cells were then fixed, permeabilized, and stained Caspase3 antibody.
F: Caspase 9 activation and PAPR cleavage. OPM1 cells were treated with 30µM of ABC294640 (indicated as A) or DMSO control (indicated as D) for 3h, 6h, 9h and 12h and analyzed for PARP and cleaved PARP, full length Caspase-9 and cleaved Caspase -9 by western blot. β-actin was used as loading control (data were representative of 3 separate sets of experiments). G: Inhibition of primary human CD138⁺ myeloma cells by ABC294640. Primary human CD138⁺ cells were freshly isolated using CD138 enrichment kit from the BM aspirates of myeloma patients and cultured in triplicate at 1×10⁴ cells in 100 µl of RPMI1640 medium supplemented with 2 mM Glutamax and 10% fetal calf serum containing DMSO control or various concentrations of ABC294640 for 24h at 37⁰C under 5% CO2. Cell proliferation was then measured by MTT assay. OPM1 cells were similarly treated for comparison (n=3; ID#: 7244, 7452 and 7476).

Figure 4: ABC294640 enhanced Mcl-1 proteasome degradation and increased Noxa expression. A: ABC294640 down-regulated Mcl-1 protein expression. MM cells (OPM1, RPMI-8226, MM.1S, JK6L, and U266) were treated with 30µM of ABC294640 (A) or DMSO (D) for 16 hr and whole cell lysates were prepared and analyzed for Mcl-1 expression by Western blot. β-actin was used as the loading control. Data were representative of 3 separate sets of experiments. B: A294640 increased Mcl-1 protein degradation. OPM1 cells were treated with 30µM of ABC294640 or DMSO for 3 hr and then cycloheximide (100µg/ml) was added. Cells were collected at each hour after cycloheximide treatment and whole cell lysate was prepared and analyzed for Mcl-1 expression by Western blot. β-actin was used as the loading control. Graph below represented the quantification of western blots. Western blots were quantified using ImageJ. Data were representative of 2 separate sets of experiments. C: Proteasome inhibitor (MG132 and bortezomib) prevented the degradation of Mcl-1 by ABC294640. OPM1 and JK6L cells were treated with DMSO control buffer, proteasome inhibitor MG132 (1µM) or Bortezomib.
(50nM) for 1 h, followed by treatment with DMSO or 30 µM of ABC294640 for additional 6h. Whole cell lysate was prepared and analyzed for Mcl-1 expression by Western blot. Data were representative of 2 separate sets of experiments. **D: ABC294640 increased Noxa protein expression.** OPM1, RPMI8226, MM.1S, and JK6L were treated with 30µM of ABC294640 (A) or DMSO (D) for 16 hr and whole cell lysates were prepared and analyzed for Noxa expression by Western blot. **E: ABC294640 induced Noxa gene expression.** Eight MM cell lines were treated with 30µM of ABC294640 (A) or DMSO (D) for 16 hr and RNA was isolated and analyzed for Noxa gene expression by RT-PCR. Gene expression was normalized against β-actin internal control. Graphs represented the fold change of Noxa mRNA in ABC294640- treated MM cells lines compared to DMSO- treated cells. Data shown in the figure were representative of at least 2 separate sets of experiments.

**Figure 5: ABC294640 enhanced c-Myc proteasome degradation.** **A: ABC294640 down-regulated c-Myc and pS6 protein expression.** MM cells (OPM1, RPMI-8226, MM.1S, JK6L, and U266) were treated with 30µM of ABC294640 (A) or DMSO (D) for 16 hr and whole cell lysates were prepared and analyzed for c-Myc and pS6 expression by Western blot. β-actin was used as the loading control. **B: A294640 increased c-Myc protein degradation.** OPM1 cells were treated with 30µM of ABC294640 or DMSO for 3 hr and then cyclohexamide (100µg/ml) was added. Cells were collected at each hour after cyclohexamide treatment and whole cell lysate was prepared and analyzed for c-Myc expression by Western blot. β-actin was used as the loading control. Graph below represented the quantification of western blots using ImageJ. **C: Proteasome inhibitor (MG132) prevented the degradation of c-Myc by ABC294640.** OPM1 and JK6L cells were treated with DMSO control buffer or MG132 (1µM) for 1 h, followed by treatment with DMSO or 30 µM of ABC294640 for additional 6h. Whole cell lysate was
prepared and analyzed for c-Myc expression by Western blot. Data shown in the figure were representative of at least 2 separate sets of experiments.

**Figure 6:** ABC294640 acted synergistically with Bcl-2 inhibitor in inhibiting myeloma cell growth and induced myeloma cell apoptosis in the presence of bone marrow stromal cells. A. **ABC294640 did not affect Bcl-2 expression.** MM cells (OPM1, RPMI-8226, MM.1S, and U266) were treated with 30µM of ABC294640 (A) or DMSO (D) for 16 hr and whole cell lysates were prepared and analyzed for Bcl-2 expression by Western blot. B: **Combination of ABC294640 and ABT-737 led to enhanced inhibition of cell proliferation.** OPM1 cells were treated with various concentrations of ABT-737 in the absence or presence of ABC294640 (15 µM) for 48 hr and cell proliferation was measured by MTT assay.

C: **FA-CI plots showing the synergistic effect of ABC294640 and ABT-737.** Fa-CI plots for OPM1 cells revealed a synergistic inhibitory effect for ABC294640 15µM and ABT-737 at 0.1µM (indicated as 1), 0.3µM (indicated as 2) and 1µM (indicated as 3). In the Fa-CI plot, the dashed line [combination index (CI) =1] indicates an additive reaction between the two substances. Values below this dashed line imply synergism. D: **ABC294640 induced myeloma apoptosis in the presence of bone marrow stromal cells.** GFP expressing OPM1 cells were cultured on the monolayer of HS5 BM stromal cells and were treated for 8h with 30µM of ABC294640 or DMSO. The cells were stained with Annexin V and 7-AAD and Annexin V+ apoptotic cells were gated on GFP positive OPM1 cells.

**Figure 7:** ABC294640 suppressed myeloma growth *in vivo* in mouse xenograft models. A: **ABC294640 inhibited myeloma growth *in vivo* in intravenously administrated mouse xenograft model.** NSG mice were sublethally irradiated (2.5 Gy) and injected via tail vein 0.5x10^6 MM.1S myeloma cells stably expressing leuciferase. Two days later, the mice were divided randomly into 2 groups and
treated either with ABC 294640 50mg/kg i.p. (A) or control vehicle (C; PBS+ 0.3% Tween-80) for 30 days. Every 10 days the mice were imaged using Perkin Elmer Ivis 200 imager and Live Image software.

**B:** ABC294640 inhibited myeloma growth in vivo in subcutaneously inoculated mouse xenograft model. NSG mice were sublethally irradiated (2.5 Gy) and injected subcutaneously with 0.5x10^6 MM.1S myeloma cells stably expressing leuciferase. Two days later, the mice were treated either with ABC294640 50mg/kg i.p. (Mice indicated as “A”) or control vehicle (Mouse “C”) for 30 days. Tumor growth was monitored by bioluminescence imaging.

**C:** ABC294640 inhibited myeloma growth in vivo. NSG mice were sublethally irradiated (2.5 Gy) and injected subcutaneously with 0.5x10^6 MM.1S myeloma cells stably expressing leuciferase. Two weeks later, the mice were imaged using bioluminescence imaging and showed tumor engraftment (D0). The mice were then treated with ABC294640 50mg/kg i.p. or control daily for 27 days (from D0 to D27). Tumor growth was monitored by bioluminescence imaging at time-points indicated (up to one week after the discontinuation of injection, i.e., D35).

**D:** Schematic diagram of the mechanisms of action of ABC294640 in MM cells.
Figure 1

A. SK1/2 mRNA expression (RMA-normalized)

B. SK1/2 mRNA expression (relative to β-actin)

C. SK2 mRNA expression (relative to β-actin)

D. Sphingosine level (pmole/1×10⁶ cells)

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Figure 2

**A**

Control shRNA-DsRFP

SK2-shRNA-DsRFP

**B**

Relative SK2 mRNA level (% change)

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<tr>
<td>0h</td>
<td>100%</td>
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<td>48h</td>
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**C**

Cell Proliferation (fold change)

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<td>0.86</td>
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**D**

Red: control shRNA
Blue: SK2-shRNA

**E**

Cell division

Red: control shRNA
Blue: SK2-shRNA

Caspase 3

- 51.5%
- 21.1%
Figure 3
Figure 4
Figure 5
Figure 6

A

<table>
<thead>
<tr>
<th></th>
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<th>MM.1S</th>
<th>U266</th>
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<tr>
<td></td>
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B

Cell proliferation (% change)

- ABC294640 (0μM)
- ABC294640 (15μM)

C

Combination Index

Fractional effect

D

Annexin V

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Figure 7

SK2 shRNA or inhibitor

C-MYC degradation

NOXA overexpression

Mcl-1 degradation

BCL2

ABT-737

Mcl-1 degradation

Apoptosis

Day 10

Day 20

Day 30

A

C

A

C

Day 10

Day 17

Day 30

A

C

A

A

ABC294640

Control

D0

D10

D20

D27

D35

Treatment

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Inhibition of sphingosine kinase 2 down-regulates the expression of c-Myc and Mcl-1 and induces apoptosis in multiple myeloma