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Biological Effects of the Pim Kinase Inhibitor, SGI-1776, in Multiple Myeloma

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Abstract

Pim kinases are constitutively active serine/threonine/tyrosine kinases that are overexpressed in hematological malignancies such as multiple myeloma. Pim kinase substrates are involved in transcription, protein translation, cell proliferation, and apoptosis. SGI-1776 is a potent Pim kinase inhibitor that has proven to be cytotoxic to leukemia and lymphoma cells. Based on this background, we hypothesized that SGI-1776 treatment would result in myeloma cytotoxicity. To test this, myeloma cell lines and primary CD138+ cells from myeloma patients were treated with SGI-1776 in a dose- and time-dependent manner and effect on cell death and proliferation, induction of autophagy, as well as changes in cell cycle profile were measured. SGI-1776 treatment resulted in limited apoptosis in cell lines (mean 30%) and CD138+ cells (<10%) as assessed by Annexin-V/PI. Limited effect was observed in cell cycle profile or growth in cell lines. However, DNA synthesis was decreased by 70% at 3 μ M (all time points) in U266 though this was not observed in MM.1S. In accordance, immunoblot analyses revealed no change in transcription (c-Myc and H3), or apoptotic (Bad) proteins that are substrates of Pim kinases. In contrast, autophagy, as assessed by acridine orange staining, was induced with SGI-1776 treatment in both cell lines (U266 25-70%; MM.1S 8-52%) and CD138+ cells (19-21%). Immunoblot analyses of autophagy LC3b marker and translation initiation proteins (phospho p70S6K and 4E-BP1) corroborated autophagy induction. These data indicate that SGI-1776 treatment in myeloma cell lines and CD138+ myeloma cells elicits its deleterious effects through inhibition of translation and induction of autophagy.

Keywords

multiple myeloma; Pim kinase inhibitor; SGI-1776; autophagy; cell death

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Introduction

Pim (pro-viral insertion site for the Moloney Murine Leukemia Virus) genes are proto-oncogenes first discovered in the early 1980's in mouse models ¹. They encode a family of three constitutively active serine/threonine/tyrosine kinases, Pim-1, Pim-2, and Pim-3. Pim kinase overexpression has been reported in several hematological malignancies such as chronic lymphocytic leukemia (CLL), mantle cell lymphoma, acute myeloid leukemia (AML), and multiple myeloma (MM) as well as several solid cancer including prostate, pancreatic, and colon cancer ². Pim-2 was elucidated to be one of the survival pathways that result in increased survival and growth of primary B-lymphocytes³. Also, Pim-2 mRNA and protein levels have been reported to be upregulated in B-CLL and non-Hodgkin's lymphoma suggesting an important role in the pathogenesis and/or progression of these malignancies⁴. It has also been found that Pim kinase-deficient mice exhibit reduced body size and impaired responses to hematopoietic growth factors and that Pim kinases are required for the efficient proliferation of peripheral T-lymphocytes⁵.

Pim kinases elicits their tumorigenic effect by phosphorylating substrates involved in many cellular processes such as transcription, translation, survival, cell cycle, signal transduction, and migration ². Overexpression of Pim-1 and Pim-2 kinases promote cell transformation through c-Myc stabilization by phosphorylating the amino acid residues Ser62 and Ser329, respectively, enhancing c-Myc transcription activity ⁶. Cooperation between c-Myc and Pim-1 is believed to be through the recruitment of Pim-1 by c-myc to the E boxes of c-Myc target genes. Pim-1 kinase then phosphorylates histone H3 at Ser10 resulting in c-Myc transcription activation⁷. Furthermore, tumorigenic synergism between c-Myc and Pim kinases has been established in mice models. Specifically, double-transgenic mice overexpressing c-Myc and Pim-1 in lymphoid tissue result in non-viable mice that develop pre-B-cell leukemia prenatally⁸. Pim-2 kinase expression indirectly regulates cap-dependent translation by sustaining phosphorylation of the translational repressor 4E-BP1 independent of the PI3K/Akt/TOR pathway⁹. An *in vitro* kinase assay was utilized to evaluate the ability of Pim-2 to directly phosphorylate 4E-BP1 at Ser65, a site that is required for cap-dependent translation initiation. Results indicated the Pim kinase inability to directly phosphorylate 4E-BP1 suggesting that a yet unidentified kinase(s) downstream of Pim-2 is responsible for this process¹⁰. The Pim kinase family also phosphorylate and inactivate proapoptotic substrates such as Bad to evade cell death. Pim-1 and Pim-2 predominantly phosphorylate Bad at Ser112, and Pim-3 largely phosphorylates Ser136/155, however some cross-phosphorylation occurs. Phosphorylation of Bad subsequently hinders its ability to bind and sequester antiapoptotic Bcl-2 proteins¹¹⁻¹⁴. Instead, phosphorylated/inactivated Bad is bound by 14-3-3 protein which then exports the proapoptotic protein from the mitochondria to the cytosol¹². Cell cycle progression, and hence proliferation, is regulated through the phosphorylation of the cyclin-dependent kinase inhibitor p21 at Thr145 by Pim-1 ¹⁵. In addition, *in vitro* experiments demonstrated that all three Pim kinase family members directly phosphorylated the cyclin-dependent kinase inhibitor p27 at Thr157 and Thr198. Phosphorylation of p27 facilitated its sequestration by 14-3-3 protein leading to cytoplasmic relocation and proteasomal degradation¹⁶. Noticeably, Pim-1 phosphorylates the cell cycle phosphatases Cdc25A and Cdc25C increasing their phosphatase activity resulting in cell cycle progression^{17,18}. Substrates involved in signal transduction such as the suppressors of cytokine signaling SOCS-1 and SOCS-3 and the chemokine receptor 4 (CXCR4) involved in migration have also been reported to be regulated by Pim-1 kinase ².

Multiple myeloma (MM) is an incurable plasma cell malignancy characterized by high levels of monoclonal immunoglobulin (paraprotein) in the serum and/or urine, accumulation of plasma cells in the bone marrow, and osteolytic lesions ¹⁹. Claudio et al conducted a microarray gene analysis in myeloma cells and concluded that Pim-2 is one of the 34-

upregulated genes involved in B-cell biology²⁰. Furthermore, the serine/threonine kinase Pim-2 is known to be overexpressed in malignant plasma cells but not in normal plasma cells providing a therapeutic index²¹. Importantly, bone marrow stromal cells and osteoclasts play a crucial role in Pim-2 upregulation resulting in MM cell survival²². Furthermore, reports in the literature suggest that pharmacologic inhibition of Pim kinases results in selective toxicity of myeloma cells^{21,22}. Pim kinase inhibitors suppress 4E-BP1 phosphorylation (an important translation regulator) in addition to decreasing Mcl-1 and c-Myc levels²². Collectively, these data suggest that Pim kinases are targets in myeloma.

SGI-1776 is an imidazo[1,2-b] pyridazine small molecule. SGI-1776 was found to be a potent ATP competitive inhibitor of Pim-1, Pim-2 and Pim-3 kinases with an IC₅₀ of 7, 363, and 69 nM, respectively²³. Since all family members have high homology at the amino acid level, the small molecule was expected to inhibit the three Pim kinases to a similar extent²⁴. In addition, SGI-1776 was also found to inhibit FLT3 (a cytokine receptor) and haspin (a serine/threonine kinase) at similar low nanomolar concentrations^{23,25}. Even though clinical trials in prostate cancer, non-Hodgkin's lymphoma, and leukemia were suspended due to cardiac toxicity, SGI-1776 still represents a proof-of-principle compound due to its potent inhibitory activity on all three Pim kinases.

Based on this we hypothesized that the Pim kinase inhibitor would result in MM cytotoxicity due to the specific targeting of the overexpressed Pim-2 kinase. Evaluation of our data indicated that SGI-1776 treatment in myeloma cells as well as bone marrow aspirates from MM patients elicits its deleterious effects through inhibition of translation and induction of autophagy rather than apoptosis.

Materials and Methods

Cell lines

MM.1S cell line was obtained from Drs. Nancy Krett and Steve Rosen (Robert H. Laurie Comprehensive Cancer Center, Northwestern University, Chicago, IL). U266 cell line was obtained from Dr. William S. Dalton (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL). Both cell lines were maintained in RPMI-1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum in the presence of 5% CO₂ at 37°C. Cells were routinely tested for Mycoplasma infection. Approximate doubling times for MM.1S and U266 are 48 and 36 h, respectively.

Primary bone marrow aspirates from MM patients

Bone marrow samples from 14 MM patients were obtained with informed consent in accordance with the Declaration of Helsinki to participate in the laboratory protocol, which was approved by the Institutional Review Board of the University of Texas M. D. Anderson Cancer Center. Samples were processed using a ficoll gradient. CD138⁺ cells were separated using a MACS magnetic separation technique (Miltenyi Biotech, Auburn, CA), leaving the remaining cells to be treated as CD138⁻ cells. CD138⁺ and CD138⁻ cells were suspended in RPMI-1640 medium (Life Technologies) supplemented with 10% human AB serum (Cambrex Biosciences, East Rutherford, NJ) in the presence of 5% CO₂ at 37°C.

Materials

SGI-1776 was provided by SuperGen (now Astex Pharmaceuticals) as a powder dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM and stored at -20°C. Rapamycin and bafilomycin A1 were purchased from Sigma (Saint Louis, MO).

Radioactive thymidine Incorporation

DNA synthesis was measured using [^3H]Thymidine incorporation (61.6 Ci/mmol; Moraveck Biochemical Inc, Brea, CA). MM cells were treated in a dose-response dependent manner for 24, 48, and 72 h. Thirty minutes before the end of incubation, the cells were labeled with [^3H]Thymidine (1 μCi) at 37°C. The labeled samples were harvested, washed with 10 mL of cold PBS, and transferred to glass fiber filters (Whatman Inc., Clifton, NJ) using a Millipore vacuum manifold (Fisher Scientific, Pittsburgh, PA). The filters were then washed twice with 5 mL of cold 0.4 N perchloric acid and rinsed with 70% ethanol. The filters were dried overnight and transferred to scintillation vials containing 7 mL of scintillation fluid. The radioactivity on the filters was quantified by a liquid scintillation counter (Packard, Ramsey, MN). Data were expressed as a percentage of untreated control.

Protein extraction and immunoblot assays

After treatment, cells were harvested, washed twice with PBS, and lysed using one tablet of Complete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN) in 10 mL of mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL). For immunoblot analysis, protein samples were electrophoresed on Criterion Bis-Tris Gels using the XT MOPS buffer kit (Bio-Rad, Hercules, CA) for 2 h at 100 V and transferred to nitrocellulose membranes at 100 V for 1 h at 4°C. Membranes were then probed using primary antibodies from the following sources: 4E-BP1, Bad, phospho-Bad (Ser112), GAPDH, and total histone H3 (Cell Signaling Technology, Danvers, MA), c-Myc (clone C33) and Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA), c-Myc (Ser62; Abcam, Cambridge, MA), histone H3 (Ser10; Millipore, Billerica, MA), LC3b (Novus, Littleton, CO), and β -actin (Sigma). Immunoblots were imaged and quantitated using the Odyssey imaging system (LI-COR Biosciences, Lincoln, NE).

Cell death assays

After MM cell were treated, cytotoxicity was assessed by Annexin-V/7-AAD or Annexin-V/Propidium Iodide (PI) binding assay. Briefly, cells were centrifuged at 1500 rpm for 5 min and washed twice with cold PBS. Samples were then resuspended in 1 $\times$ Annexin-V binding buffer and incubated with 7-AAD (or PI) and Annexin V-FITC (BD Biosciences Pharmingen, San Diego, CA) for 15 min at room temperature. Total cell death was collected and analyzed using the BD FACS Calibur system and CellQuest software (BD Biosciences), respectively. Growth inhibition was also confirmed by counting MM cells after treatment using a Coulter Counter (Beckman, Brea, CA).

Cell cycle assay

Treated MM cells were harvested, centrifuged, and washed with PBS. Cell when then resuspended in 1 mL of PBS and 3 mL of 100% ethanol while gently vortexing. Fixed cells were kept under ice for 2 h followed by centrifugation and removal of ethanol. Cells were washed with PBS and resuspended in 1 mL of cold PI solution (PBS, 0.5% Tween 20, 15 $\mu\text{g/mL}$ PI) containing RNase, DNase-free from bovine pancreas (5 $\mu\text{L/mL}$, Roche). Cell cycle arrest was also analyzed using the BD FACS Calibur system and CellQuest software.

Acridine orange staining

Acidic vesicles were visualized by acridine orange staining. After treatment, 1 mL of MM cell lines or patient samples were incubated at 37°C with 1 μL (10 mg/mL stock) acridine orange stain (Invitrogen Life Technologies, Grand Island, NY) for 15 min in the dark. Cells were centrifuged (1500 rpm for 5 min) and resuspended in 0.5 mL of RPMI-1640 medium without phenol red (Gibco Life Technologies, Grand Island, NY). Accumulation of acidic

vesicles was quantified as red/green fluorescence ratio (mean FL3/FL1) using the BD FACS Calibur system.

Statistical analysis

Plots and analyses were done using GraphPad Prism (GraphPad Software, San Diego, CA).

Results

Effect of SGI-1776 treatment on cell death and proliferation MM cell lines

U266 and MM.1S cells were treated in a dose-dependent (0.1, 0.3, 1, and 3 μ M) manner for 24, 48 and 72 h. Endogenous cell death was < 7% and < 15% in U266 and MM.1S cells, respectively. Only the 3 μ M SGI-1776 concentration had a cytotoxic effect in U266 cells resulting in approximately 25% cell death at 24 and 48 h and 60% cell death after 72 h drug incubation (Figure 1A). SGI-1776 had a minimal cytotoxic effect in MM.1S cells even at 3 μ M, cell death ranged from 20-30% at all 3 time points (Figure 1B). Analysis of growth inhibition in both cell lines resulted in a noticeable decline by approximately 28% in U266 and 41% in MM.1S when treated with 3 μ M SGI-1776 in comparison to the untreated control at 72 h (Figure 1C and Figure 1D). Though to a lesser extent, growth inhibition (25-30%) was also observed at 48 h in both cell lines at 3 μ M SGI-1776 compared to untreated control. In accordance to the doubling times of both cell lines (36 h for U266 and 48 h MM.1S), no appreciated growth inhibition was observed at the early 24 h time point. To further assess the effect of SGI-1776 on cell proliferation, DNA synthesis rate was measured by [³H]Thymidine incorporation. DNA synthesis was plotted as percent of untreated control. In U266 cells there was a dose-dependent steady decrease reaching a decline of 70% DNA synthesis with 3 μ M at 24, 48, and 72 h. This effect was not observed in MM.1S cells where endogenous DNA proliferation was maintained at all concentrations and different time points (Figure 1E and Figure 1F).

Effect of SGI-1776 treatment on autophagy in MM cell lines

Autophagy, a catabolic process involved in cell survival, is characterized by acidic vesicular organelle (AVO) formation²⁶. Acridine orange staining is a dye that accumulates in acidic organelles. Measurement of this dye by flow cytometry is used to detect AVO formation. Cells were treated with the vehicle (DMSO 0.1%) or SGI-1776 1 μ M and 3 μ M for 24, 48, and 72 h. U266 cells were treated with bafilomycin A1 for 30 min prior to acridine orange staining to block acidic vesicular formation and was used as a negative control. Similarly cells were treated with rapamycin a known inducer of autophagy as a positive control. As expected, bafilomycin A1 inhibited the fusion of autophagosomes with lysosomes (acidic vesicles) resulting in 3% – 2% acridine orange staining. Endogenous autophagy levels (DMSO treatment) were less than 15% at all-time points, which remained after cells were treated with rapamycin. Interestingly, 1 and 3 μ M SGI-1776 treatment resulted in 47% and 53% acridine orange staining at 24 h. Similarly, a 28% and 59% staining was observed for 1 and 3 μ M SGI-1776 concentrations at 48 h; and 25% and 71% at 72 h (Figure 2A). A parallel assay was done in MM.1S cell line and quantitated (Figure 2B). As with U266, MM.1S cell line also showed increased acridine orange stain in a dose- and time- dependent fashion, albeit at a lower level.

Effect of SGI-1776 treatment on autophagy proteins

Acridine orange staining demonstrated autophagy induction by SGI-1776 treatment. Hence, key proteins that monitor autophagy were evaluated by immunoblot analysis. U266 cells were treated as indicated and protein levels were measured for phosphorylation levels of AMPK (AMP-activated protein kinase) at Thr172, LC3b (main marker of autophagosome

formation²⁷, and the loading control GAPDH (Figure 2C). AMPK is a protein kinase sensitive to the levels of ATP in the cell. A decrease of the ATP cellular pool results in the phosphorylation of AMPK at Thr172 subsequently activating this kinase²⁸. SGI-1776 treatment resulted in no visible change of phosphorylated AMPK at the amino acid residue Thr172. This suggests that SGI-1776 does not affect energy homeostasis and the autophagic pathway is being activated downstream of the ATP sensor AMPK. LC3b is an autophagic marker that is cleaved and lipidated to form LC3b-II which is bound to autophagosomes. Hence, LC3b conversion (LC3b-I to LC3b-II) correlates with autophagosome formation and it is commonly used to monitor autophagy²⁹. SGI-1776 treatment resulted in lipidation of LC3-I to LC3-II as shown by the visualization of a lower band around 17 KDa in a dose- and time- dependent manner.

Effect of SGI-1776 treatment on cell cycle in MM cell lines

U266 and MM.1S cells were treated as indicated and analyzed for cell cycle profile (Figure 3A and Figure 3B). G1, S, and G2/M populations were quantitated in both cell lines. Similar experiments were performed three times and data were tabulated (Table 1). There was no change in any population in either cell line at any concentration or time point, suggesting SGI-1776 treatment in MM cells does not affect the cell cycle profile.

Effect of SGI-1776 treatment Pim kinase substrates

Effect of SGI-1776 treatment on transcription proteins—U266 and MM.1S cells were treated with SGI-1776 and immunoblot analyses were performed (Figure 4A). Pim-1 kinase phosphorylates two important proteins involved in transcription: histone H3 and c-myc^{6,7}. U266 cells treated with 3 μ M SGI-1776 for 24 h resulted in a noticeable increase in phospho c-Myc Ser62 levels (Figure 4A-I). No decrease in phosphorylation status was observed at the later time points. Similarly the SGI-1776 treatment did not decrease phosphorylation levels of H3 Ser10 in either cell line (Figures 4A-II and Figure 4A-III). Collectively, these data suggested that in MM cell lines, SGI-1776 treatment did not attenuate transcription axis.

Effect of SGI-1776 treatment on translation proteins—Since Pim kinases are involved in protein translation and acridine orange staining along with LC3b lipidation suggested autophagy, translation protein levels associated with Pim kinase as well as autophagy were evaluated. U266 cells were treated and protein levels were measured for phospho p70S6K Thr389 (activated serine/threonine kinase that regulates translation³⁰, phosphorylation status of 4E-BP1 (regulator protein targeting mRNA translation²⁷), and the loading control GAPDH (Figure 4B). The kinase p70S6K phosphorylates the S6 protein on the 40S ribosomal subunit allowing mRNA translation³⁰. SGI-1776 treatment in U266 cells resulted in a dose-dependent decrease of phosphorylation levels of p70S6K at Thr389 at 48 and 72 h incubation periods. The multiple bands of 4E-BP1 represent its hyperphosphorylated status, and stacking of the bands represents its hypophosphorylated form, resulting in translation inhibition^{31,32}. Treatment with SGI-1776 resulted in a time dependent decrease in 4E-BP1 phosphorylation levels, with a more pronounced hypophosphorylated form of 4E-BP1 appearing at the higher concentrations (1 μ M and 3 μ M) at 72 h.

Effect of SGI-1776 treatment on apoptotic proteins—MM.1S and U266 cell lines were treated with SGI-1776 and apoptotic proteins total and phospho Bad (Ser112) and Mcl-1, as well as the loading control β -actin were analyzed by immunoblot (Figures 4C-I to 4C-III). Levels of total Bad, phospho Bad (Ser112), and Mcl-1 were not affected following SGI-1776 treatment in either cell line. This suggests that the Pim kinase inhibitor elicits its cytotoxic effects mainly through autophagy activation.

Effect of SGI-1776 treatment on cytotoxicity in primary bone marrow cells from MM patients

Bone marrow aspirates were collected from 14 patients being treated for MM. CD138+ plasma cells were separated using magnetic beads and the remaining cells were classified as CD138-. Both cells, CD138+ and CD138-, were treated simultaneously with SGI-1776 at varying concentrations ranging from 1 μ M to 10 μ M for 24 h (Figure 5A and Figure 5B). Three patient samples were treated with 1 μ M SGI-1776, 8 samples with 3 μ M SGI-1776, and 2 samples with 10 μ M SGI-1776. Vehicle (DMSO 0.1%) treatment was also included when cell concentration allowed it. Endogenous cell death after 24 h in untreated conditions ranged from 2% – 60% in CD138- cells and from 10% to 99% in CD138+ cells. Cytotoxicity (determined by Annexin-V/PI or Annexin-V/7-AAD) of SGI-1776 at any concentration in CD138+ cells was minimal (less than 8% difference) when compared with the DMSO treated condition (Figure 5A).

Effect of SGI-1776 in AVO formation in primary bone marrow aspirates from MM patients

Five MM patient samples (CD138+ and CD138- cells) were left untreated or treated with bafilomycin A1, DMSO (vehicle 0.1%), SGI-1776 1 μ M and 3 μ M for 24 h. Cells were then assessed for AVO formation by measuring acridine orange levels by flow cytometry. Raw data from one patient CD138- (Figure 6A) and CD138+ (Figure 6C) are presented. AVO formation in CD138- cells after SGI-1776 dose increase treatment (approximately 12% – 15%) were similar to the levels observed in DMSO and untreated conditions (7% – 9%) (Figure 6A). However, CD138+ cells resulted in an increase in AVO formation as measured by acridine orange staining after SGI-1776 treatment compared to the vehicle control (average for untreated 7%, DMSO 5%, SGI-1776 1 μ M 21%, and SGI-1776 3 μ M 19%) (Figure 6D). However, this increase was not statistically significant (p value >0.05). Bafilomycin A1 treated cells resulted in an average acridine orange staining of 4% for CD138- and 2% for CD138+ cells hence successfully blocking AVO formation in the negative control.

Conclusions

Efforts to develop Pim kinase inhibitors have resulted in the synthesis of small molecules such as SGI-1776. Several publications document SGI-1776 as an effective cytotoxic agent in diverse malignancies including CLL²³, mantle cell lymphoma²⁵, prostate cancer³³, renal cell carcinoma³⁴, AML³⁵⁻³⁷, and MM³⁸. Furthermore, combination strategies inhibiting Pim kinase proteins with SGI-1776 were shown to potentiate the cytotoxicity of cytarabine in AML³⁷ and sunitinib in renal cell carcinoma³⁴. These published reports have suggested that in proliferating cell lines as well as replicating quiescent primary cells, SGI-1776 treatment induced apoptosis. In contrast, in MM cell lines (Figure 1A and Figure 1B) except for a three day incubation with 3 μ M in U266 cells, only 20% cell death was observed. Cell death in MM.1S cells on the other hand averaged from 20-30% at 24, 48, and 72 h exposure regardless of increasing concentrations (Figure 1B). Although apoptosis (assessed by Annexin-V/PI staining) was observed to an extent in MM cell lines after SGI-1776, it was negligible in primary CD138+ plasma cells (Figures 1A-1B and Figure 5B). For example, CD138+ cells treated with 10 μ M SGI-1776 for 24 h did not result in increased cell death compared to the vehicle control (Figure 5B). Tian et al reported that SGI-1776 triggered apoptosis in MM³⁸, however, it is important to note that cell death was assessed by means of MTT assay for MM.1S cells and CellTiter-Glo assay for CD138+ cells. The read out from these assays depend on cell number and hence are comparable to the growth inhibition assay (Figure 1C and Figure 1D) or thymidine incorporation measurement (Figure 1E and Figure 1D) performed by us. In addition, although Annexin-V/PI staining was also used by Tian et al as a second cell death measurement it was only

performed as a single experiment in MM.1S cells. Still, measured cell death by dual Annexin-V/PI staining also resulted in 39% at 3 μ M SGI-1776 for 24 h compared to our data where we measured at the same conditions an average of 30% cell death (range from 22 – 38% in three independent experiments). Collectively, these data suggest limited apoptosis in MM cells by SGI-1776.

Pim kinases phosphorylate a variety of substrates that are involved in four major processes. These include cell cycle progression, transcription activation, cell survival, and protein translation^{2,39}. Pharmacological inhibition of Pim kinases using escalating doses of SGI-1776 for 24, 48, and 72 h failed to result in cell cycle arrest determined by PI staining using flow cytometry (Table 1, Figure 3A and Figure 3B). Members of the Pim kinase family are known to promote c-Myc stabilization and transcription activation by phosphorylating the amino acid residues Ser62 and Ser329⁶. In addition, Pim-1 kinase can phosphorylate histone H3 at Ser10 resulting in c-Myc transcription activation⁷. In U266 cells treated with SGI-1776 there was a noticeable increase in phospho c-Myc Ser62 levels at 3 μ M SGI-1776 concentration for 24 h, although no change was visualized at 48 or 72 h at any concentration (Figure 4A-I). Therefore, further antibody probing will need to be performed since c-Myc is targeted for proteasomal degradation following a hierarchical phosphorylation process where Ser62 is first phosphorylated followed by phosphorylation at Thr58⁴⁰. Since no commercially available antibodies exist for phospho c-Myc Ser329, the amino acid residue Thr58 will have to be assessed for phosphorylation status to discern between activation and degradation of c-Myc. Normalization analyses were performed comparing total H3 versus phospho H3 Ser10 levels and no decrease in phosphorylation status was measured (data not shown). Thus, transcription inhibition does not appear to be a mechanism of action of SGI-1776 in myeloma since the SGI-1776 failed to decrease phosphorylation levels of H3 at Ser10 in either cell line (Figure 4A-II and Figure 4A-III).

Pim kinases phosphorylate the proapoptotic protein Bad at Ser112, Ser136, and Ser155 rendering the protein unable to bind and sequester antiapoptotic Bcl-2 proteins¹¹⁻¹⁴. Plus, the transcription factor c-Myc triggers the transcription of Mcl-1 resulting in an increased level of this antiapoptotic protein⁴¹. Consistent with the low levels of cell death, there was no significant decrease in the levels of phospho Bad or Mcl-1 in MM.1S and U266 cells treated with SGI-1776 (Figures 4CI-III).

The only axis downstream of Pim kinases that was impacted by SGI-1776 treatment was protein synthesis. As shown in Figure 4B, 4E-BP1 phosphorylation was inhibited by SGI-1776. Furthermore, phosphorylation of p70S6K was also attenuated by SGI-1776. Collectively, these effects may lead to inhibition of cap-dependent protein translation.

Similar to Pim kinase mediated protein translation, mTOR also signals for cap-dependent protein translation⁴². mTOR regulates protein synthesis by hyperphosphorylating 4E-BP1 and decreasing its affinity for the translation initiation factor eIF4E. mTOR also allows translation initiation by phosphorylating and activating the kinase p70S6K which in turn phosphorylates the 40S ribosomal subunit S6 protein³⁰. The mTOR inhibitor rapamycin is a known inducer of autophagy and treatment of MM cell lines as well as primary plasma cells with rapamycin results in autophagy induction⁴³ (Figure 2A). These data directed us to evaluate autophagy induction in myeloma cells after inhibition of Pim kinases with SGI-1776. Our results strongly suggest that the cytotoxic mechanism of SGI-1776 treatment in MM cell lines and bone marrow CD138+ cells is through inhibition of translation and induction of autophagy (Figure 2, Figure 4B, Figure 5, and Figure 6).

In this paper we show the use of SGI-1776 as a proof-of-concept strategy to inhibit Pim kinases in MM cells. While this drug is not a clinical candidate, presently there are other

Pim kinase inhibitors available. Such examples include AZD1208 developed by AstraZeneca currently in clinical trials for AML treatment and GNE-652 developed by Genentech.^{44,45} Therefore, based on our work these two drugs could represent potential candidates for the treatment of MM.

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Abbreviations

MM	multiple myeloma
AVO	acidic vacuolar organelle

Figure 1. Effect of SGI-1776 treatment on cell death and proliferation in MM cell lines U266 (Figures 1A, 1C, and 1E) and MM.1S (Figures 1B, 1D, and 1F) cells were left untreated or treated with escalating doses of SGI-1776 (0.1, 0.3, 1, 3 μ M) for 24, 48, and 72 h. Treated cells were harvested at the indicated time points, stained with Annexin-V/PI, and analyzed for cell death using flow cytometry (Figure 1A and Figure 1B). Following cell death analysis, cell growth was assessed by using a Coulter counter to enumerate cell count and determine cell growth inhibition (Figure 1C and Figure 1D). Untreated and treated cells were labeled with [³H]Thymidine for 30 minutes to quantitate DNA synthesis (Figure 1E and Figure 1F). All plots represent the average of three independent experiments $\pm$ SEM.

Figure 2A

Figure 2B

Figure 2C

Figure 2. Effect of SGI-1776 treatment on autophagy in MM cell lines

U266 (Figure 2A) acridine orange staining raw flow cytometry data was performed where cells were left untreated or treated with bafilomycin (blocks AVO formation, negative control), rapamycin (known inducer of autophagy, positive control) or SGI-1776 1 and 3 μ M for the indicated time points. Cells were harvested and analyzed for AVO formation by acridine orange staining using flow cytometry. Similar experiments were performed in both cell lines and percentage of acridine orange staining was plotted (Figure 2B). Following the above mentioned drug schedule, U266 and MM.1S cells were harvested, then immunoblot analysis was performed, and analyzed for expression of phospho AMPK (Thr172) and LC3b levels including the loading control GAPDH (Figure 2C).

Figure 3A **Figure 3B**

Figure 3. Effect of SGI-1776 treatment on cell cycle profile in MM cell lines
 U266 (Figure 3A) and MM.1S (Figure 3B) cells were left untreated or treated with SGI-1776 in a dose-dependent manner for the indicated time points. Cells were harvested, fixed with 100% ethanol, and stained with PI (15 $\mu\text{g}/\text{mL}$) following analysis of cell cycle arrest by flow cytometry. These histograms show representative cell cycle experiments in both cell lines.

Figure 4A-I **Figure 4A-II**

Figure 4A-III **Figure 4B**

Figure 4C-I

Figure 4C-II

Figure 4C-III

Figure 4. Effect of SGI-1776 treatment on Pim kinase associated transcription, translation, and apoptotic proteins in MM cell lines

U266 and MM.1S cells were left untreated or treated with escalating doses of SGI-1776 (0.1, 0.3, 1, 3 μM) for 24, 48, and 72 h. Cells were harvested, immunoblotted, and protein

levels for these conditions visualized using antibodies for transcription target proteins (Figure 4A), translation targets (Figure 4B), and survival targets (Figure 4C). Protein immunoblots include phospho and total c-Myc in U266 (Figure 4A-I); phospho and total histone H3 in MM.1S (Figure 4A-II) and U266 (Figure 4A-III); phospho p70S6K (Thr389) and 4E-BP1 in U266 (Figure 4B); and proapoptotic proteins like phospho and total Bad in MM.1S (Figure 4C-1) and U266 (Figure 4C-II) or Mcl-1 in U266 cells (Figure 4C-III).

Figure 5A

Figure 5B

Figure 5. Cytotoxic effect of SGI-1776 treatment on freshly obtained MM patient bone marrow CD138+ and CD138- cells

CD138- (Figure 5A) and CD138+ (Figure 5B) cells were isolated and purified from MM patient bone marrow samples and left untreated or treated *in vitro* with the vehicle (DMSO 0.1%), 3 μM or 10 μM SGI-1776 for 24 h. Cell death was analyzed by Annexin-V/PI staining using flow cytometry.

Figure 6A

Figure 6B

Figure 6C

Figure 6D

Figure 6. Autophagic effect of SGI-1776 treatment on freshly obtained MM patient bone marrow CD138+ and CD138- cells

CD138- (Figure 6A and Figure 6B) and CD138+ (Figure 6C and Figure 6D) cells were isolated and purified from MM patient bone marrow samples and left untreated or treated *in vitro* with bafilomycin, the vehicle (DMSO 0.1%), 3 µM or 10 µM SGI-1776 for 24 h. Cells were harvested and analyzed for AVO formation by acridine orange staining using flow cytometry. Plots represent the average (plus SD) percentage of AVO formation (acridine orange staining) of 5 MM patient bone marrow samples. The histogram shows a representative acridine orange staining experiment in both cell types from patient number 12 (Figure 6A and Figure 6C).

Table 1

Effect of SGI-1776 on cell cycle arrest

U266 and MM.1S cells were left untreated or treated with control DMSO or SGI-1776 in a dose-dependent manner for 24, 48, and 72 h. Cells were harvested, fixed with 100% ethanol, and stained with PI (15 µg/mL) following analysis of cell cycle arrest by flow cytometry. Histograms were gated and cell cycle phase arrest quantitated. Each cell cycle phase arrest number represents the average of at least three independent experiments including the corresponding SD.

U266	Cell cycle phase				MM.1S	Cell cycle phase			
SGI-1776	Hours	G1	S	G2M	SGI-1776	Hours	G1	S	G2M
0 µM	24	58 ±2.9	22 ±1.1	14 ±0.9	0 µM	24	46 ±0.7	23 ±2.9	18 ±3.5
	48	62 ±0.5	18 ±0.3	14 ±0.8		48	50 ±1.3	22 ±3.5	17 ±3.1
	72	58 ±1.2	19 ±3.1	17 ±0.4		72	55 ±6.9	19 ±1.1	15 ±0.2
0.1 µM	24	57 ±3.2	22 ±1.4	14 ±1.9	0.1 µM	24	48 ±5.1	23 ±2.5	17 ±0.6
	48	56 ±0.0	18 ±1.9	16 ±1.2		48	47 ±2.5	22 ±3.8	18 ±1.6
	72	61 ±0.7	19 ±1.0	16 ±0.0		72	50 ±1.9	20 ±4.2	17 ±1.3
0.3 µM	24	58 ±3.1	21 ±1.5	13 ±1.2	0.3 µM	24	46 ±3.2	23 ±2.3	19 ±2.8
	48	59 ±1.3	18 ±2.6	16 ±0.9		48	47 ±4.1	21 ±3.0	18 ±0.9
	72	57 ±0.1	19 ±1.7	18 ±0.5		72	48 ±1.8	17 ±1.3	17 ±0.9
1 µM	24	59 ±3.2	20 ±1.2	147 ±1.4	1 µM	24	50 ±5.8	20 ±1.6	17 ±1.1
	48	59 ±0.4	19 ±2.3	15 ±0.6		48	46 ±3.7	20 ±2.7	18 ±1.6
	72	59 ±0.3	18 ±2.3	17 ±0.0		72	48 ±3.4	16 ±0.3	16 ±0.8
3 µM	24	65 ±2.1	13 ±0.2	13 ±2.4	3 µM	24	58 ±3.4	10 ±0.7	17 ±5.2
	48	67 ±0.7	14 ±2.1	11 ±0.6		48	49 ±4.6	14 ±0.7	14 ±1.1
	72	63 ±3.4	14 ±0.4	13 ±3.0		72	47 ±4.3	13 ±0.4	13 ±1.5