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An integrin-targeted, pan-isoform, phosphoinositide-3 kinase inhibitor, SF1126, has activity against multiple myeloma *in vivo*

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Abstract

Purpose—Multiple reports point to an important role for the phosphoinositide-3 kinase (PI3K) and AKT signaling pathways in tumor survival and chemoresistance in multiple myeloma (MM). The goals of our study were: (1) to generate the preclinical results necessary to justify a Phase I clinical trial of SF1126 in hematopoietic malignancies including multiple myeloma, and (2) to begin combining pan PI-3 kinase inhibitors with other agents to augment antitumor activity of this class of agent in preparation for combination therapy in Phase I/II trials.

Methods—We determined the *in vitro* activity of SF1126 with 16 human MM cell lines. *In vivo* tumor growth suppression was determined with human myeloma (MM.1R) xenografts in athymic mice. In addition, we provide evidence that SF1126 has pharmacodynamic activity in the treatment of patients with MM.

Results—SF1126 was cytotoxic to all tested MM lines and potency was augmented by the addition of bortezomib. SF1126 affected MM.1R cell line signaling *in vitro*, inhibiting phospho-AKT, phospho-ERK, and the hypoxic stabilization of HIF1 α . Tumor growth was 94% inhibited, with a marked decrease in both cellular proliferation (PCNA immunostaining) and angiogenesis (tumor microvessel density via CD31 immunostaining). Our clinical results demonstrate pharmacodynamic knockdown of p-AKT in primary patient derived MM tumor cells *in vivo*.

Conclusions—Our results establish three important points: (1) SF1126, a pan PI-3 kinase inhibitor has potent antitumor activity against multiple myeloma *in vitro* and *in vivo*, (2) SF1126

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Conflict of interest

Dr. Durden discloses financial conflict of interest in Semafore and SignalRx Pharmaceuticals and in the SF1126 drug. The relationship between Dr. Durden and SignalRx has been internally reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies.

displays augmented antimyeloma activity when combined with proteasome inhibitor, bortezomib/Velcade®, and (3) SF1126 blocks the IGF-1 induced activation of AKT in primary MM tumor cells isolated from SF1126 treated patients. The results support the ongoing early Phase I clinical trial in MM and suggest a future Phase I trial in combination with bortezomib in hematopoietic malignancies.

Keywords

Pan-PI-3 kinase inhibitor; SF1126; multiple myeloma; IGF-1; IL-6

Introduction

Multiple myeloma (MM), also called plasma cell myeloma, is a relatively indolent yet clinically difficult neoplasm with a median survival of 3–5 years. This tumor is driven by transformed plasma B cells, and in the United States is newly diagnosed in about 20,000 patients annually [1]. The disease is multifocal, and commonly presents with pathologic bone fractures/osteolytic lesions, hypercalcemia, and serum paraproteins composed of pathologic immunoglobulin [2]. These paraproteins lead to immunodeficiency and renal failure [2]. Standard treatment is chemotherapy with or without autologous hematopoietic stem cell transplantation [2]. In almost all cases, MM ultimately relapses into a resistant form, hence new strategies are clearly needed to circumvent this fatal end-stage.

Several MM growth cytokines are known to exert their effects via the phosphoinositide 3-kinase (PI3K)/AKT axis which led us and others to the hypothesis that this is a key resistance pathway, and the expectation that PI3K inhibitors will exhibit anti-MM activity [3]. The major stimulatory cytokines believed to be involved in the plasma cell myeloma bone marrow microenvironment are insulin-like growth factor 1 (IGF-1) and interleukin 6 (IL-6) [4]. The IGF-1 receptor activates PI3K through the insulin receptor substrate (IRS) [5], which then activates the AKT/mTOR/p70 S6 kinase pathway, as well as the RAF-1/ERK pathway downstream of RAS, through the RAC/PAK-1/RAF-1 loop [6]. IL-6 has been shown to activate PI3K a ras-dependent but p85-independent pathway, and a complex STAT-3 containing pathway that is p85-dependent [5, 7]. RAS binds to the p110 subunit of PI3K to activate the PI3K-AKT signaling axis, which includes AKT, mTOR, and p70 S6 kinase and leads to cell survival [5, 7]. STAT3 has multiple effects that includes activation of Mcl-1 and Bcl-X_L, which regulate PI3K expression and promote cell survival [7]. Moreover, further evidence for role of PI-3 kinase in MM pathogenesis comes from experiments involving rescue of *PTEN*-null, dexamethasone-resistant MM cell lines to restore a functional *PTEN*/PI3K/AKT axis leading to apoptosis [8–12].

One of the most intensively studied pan PI-3 kinase inhibitor is the LY294002 compound which is cited in over 6000 publications used to implicate the PI-3K family of kinases in cell biology (www.ncbi.nlm.nih.gov/pubmed). LY294002 blocks all classes of PI3K with the following IC₅₀ values: (a) p110 , 720 nmol/L; (b) p110 , 306 nmol/L; (c) p110 , 1.6 μmol/L; and (d) p110 , 1.33 μmol/L [13, 14]. The systemic administration of LY294002 results in potent antitumor and antiangiogenic activity *in vivo* but it is toxic and has undesirable pharmacokinetic (PK) properties including water insolubility precluding measurement of plasma half-life in live animals [15]. This compound is not viable as a drug, so we converted it to SF1126, which is an integrin ligand conjugated water soluble prodrug that exhibits acceptable PK in preclinical models, while retaining the activity profile of the parent compound [16]. SF1126 is cleaved at physiologic pH which leads to the extended liberation of LY294002, allowing for a half-life of > 1 hour. Moreover, SF1126 binds specific integrins within the tumor compartment, which is designed to enhance delivery of the active compound to the tumor vasculature and tumor. The capacity of SF1126 to accumulate in

tumor tissue and inhibit U87MG and PC3 tumor growth was enhanced *in vivo* by the RGDS integrin ($\alpha 3 / \beta 1$) binding component, exhibiting increased activity compared with a false RADS-targeted prodrug, SF1326. SF1126 has exhibited favorable PK in a recent human solid tumor clinical trial along with activity against additional B-cell malignancies (CLL and DLBCL) with 5 patients deriving clinical benefit [17].

Herein, we now examine the activity of SF1126 in multiple myeloma systems with a focus on three major points: **1)** to determine if SF1126 has antitumor activity against MM, **2)** to examine the effect of combining SF1126 with the proteasome inhibitor, bortezomib (Velcade®), which is used clinically in MM with considerable benefit [18], and **3)** to confirm the pharmacodynamic activity of SF1126 in primary patient derived MM tumor cells *in vivo*. Perifosine is a membrane lipid raft inhibitor that suppresses the PI3K-AKT, MEK, Cdk2, MAPK/Erk and SAPK/JNK pathways[19]. This drug enhances the activity of bortezomib in MM, but it targets many more signaling nodes than does SF1126, potentially resulting in a greater risk of adverse effects [20, 21].

We show that SF1126 has potent activity against human MM *in vitro* and *in vivo*, that it specifically blocks the activation of AKT, ERK, p70 and S6 kinase. Moreover, SF1126 opposed the hypoxic stabilization of HIF-1 in MM cells, and induced apoptosis through the cleavage of caspase 3. The addition of bortezomib significantly enhanced the *in vitro* anti-MM effects of SF1126. *In vivo* studies demonstrated that SF1126 strikingly inhibited MM tumor growth and angiogenesis. Our studies with primary MM materials provide evidence of *in vivo* pharmacodynamic knockdown of AKT in human patient tumor cells.

Materials and methods

Reagents

Antibodies specific for PTEN, AKT, phospho-S⁴⁷³-AKT, ERK, phospho-44/42-ERK, p70 S6 kinase, phospho-p70 S6 kinase, cleaved caspase 3, and PCNA were purchased from Cell Signaling Technology, Danvers, MA. An antibody against HIF-1 was procured from BD Transduction Laboratories. The MM.1S and MM.1R multiple myeloma cell lines were obtained from Steve Rosen (Robert H. Lurie Comprehensive Cancer Center, Chicago, IL). The RPMI 8226 cell line was purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin, at 37° C in an incubator with 5% CO₂. All other chemicals were purchased from Sigma unless otherwise stated.

Cytotoxic assays

Multiple human MM cell lines were plated (5×10^4 cells/well) in phenol-free RPMI in 96 well plates, and treated with various concentrations of SF1126 for 24, 48 or 72 hrs. Following incubation, 10 μ l 12 mM MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5diphenyl-2H tetrazolium bromide) reagent was added, and four hours later MTT, 100 μ l 0.01 M HCl-SDS (1 g SDS in 10 ml 0.01 HCl) was added and the plates incubated for 16 hours. Absorbance was measured at 570 nm. A representative graph of the absorbance at each concentration, relative to that of the highest absorbance, is shown in Figure 2A. The 50% inhibitory concentration (IC₅₀) was calculated for each cell line using GraphPad Prism software (GraphPad Software, San Diego, CA), based on a sigmoidal dose-response curve fit.

Biochemical assays

Western blotting revealed the effect of SF1126 on AKT, ERK, and HIF-1 activity. MM.1R cells ($5-10 \times 10^6$) in 10% FBS-PBS were treated with 10 μ M of other PI3K inhibitors including SF1101 (also known as LY294002), SF1126 (LY294002 with RGDS-targeting

moiety), and/or SF1326 (LY294002 with RADS-moiety). For the HIF-1 stabilization experiments, MM.1R and RPMI 8226 cells were placed in low oxygen (1%; 4 hours at 37° C). For IGF-1 experiments, MM.1R or RPMI 8226 cells were grown at a concentration of $5\text{--}10 \times 10^6$ cells in 10 ml serum free medium for 16 hours, then incubated with SF1126 (10 μM) and/or bortezomib (10 nM) for 30 minutes or 20 hours at 37° C. The cultures were then either stimulated with IGF-1 (100 ng/ml) for 30 minutes or given PBS control. For all experiments, at the end of treatment, lysates were resolved by 10% SDS-PAGE and probed for phospho-AKT, AKT, phospho-ERK, ERK (Cell Signaling Technology Inc., Beverly, MA), and/or HIF1 (BD Transduction Laboratories, San Jose, CA). Total protein was quantified (Protein Assay Kit, Bio-Rad) and individual bands were visualized by chemiluminescence.

Apoptosis assay

To assess whether caspase-3, an enzyme involved in the effector phase of apoptosis, is a target of SF1126, cleaved caspase-3 was detected by immunocytochemistry. After treatment with 5 μM SF1126, MMR cells were centrifuged to a microscope slide at 600rpm for 5 minutes using a Cytospin 4 Cytocentrifuge (Thermo Shandon, Waltham, MA). Adherent MM.1R cells were fixed with 4% paraformaldehyde, incubated with polyclonal anti-human cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology Inc., Danvers, MA) at a final dilution of 1:100 at 4°C overnight. Horseradish peroxidase-labeled streptavidin-biotin provided visualization (Santa Cruz Biotechnology, Santa Cruz, CA). Slides were counterstained with hematoxylin, and controls were incubated with a nonspecific rabbit IgG instead of primary antibody. Additionally, MM.1R cells were incubated with SF1126 for 24, 48, and 70 hours, then cells were harvested and clarified lysates were immunoblotted as described above, using a polyclonal rabbit antibody against cleaved caspase 3.

Animal and Human subjects

Athymic female mice (CD-1 *nu/nu*, 20–25 grams) were obtained from the NIH/NCI repository and housed under specific pathogen-free conditions, according to AAALAC guidelines. All studies were carried in strict accordance with approved institutional IACUC protocols. The human studies were performed under the auspices of the relevant human subjects institutional review boards according to U.S. Food and Drug Administration (FDA) and Health Canada guidelines (U.S. Clinical Trials.gov Identifier NCT00907205). All subjects were over 18 years of age and provided informed consent. Subjects were deemed eligible if they had relapsed or refractory myeloma with at least 2 prior lines of therapy.

Xenograft implantation and SF1126 treatment

MM.1R cells at a density of 1×10^7 cells in 100 μl PBS medium were injected subcutaneously into the right flank of mice. Tumor growth was monitored twice weekly by caliper measurements. Tumor volume was calculated using the formula $V = (A \times B^2)/2$, where A is the length and B is the width. Animals bearing tumors 100–125 mm^3 in size were randomized into 2 groups to receive either vehicle (acidified sterile water diluent) or SF1126. The dosing regimen was 50 mg/kg 3 times weekly for 3 weeks, given subcutaneously in the left flank. The entire experiment was repeated three times. No untoward effects were noted in mice treated with SF1126 or vehicle.

Immunohistochemistry

MM.1R tumors were harvested from treated mice and either frozen in OCT blocks or fixed in 10% buffered formalin and processed into paraffin. Sections of 4 μm thick frozen tumor tissue were stained with rat anti-mouse CD31 antibody for detection tumor microvasculature. Paraffin-embedded tissue sectioned at 5 μm was stained overnight with

mouse anti- PCNA (PC10). Horseradish peroxidase-labeled streptavidin-biotin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) provided visualization and slides were counterstained with hematoxylin. Negative controls were incubated with a nonspecific mouse IgG instead of primary antibody. The percentage positive nuclear immunostaining (PCNA) was the labeling index, determined by counting 500 cells in ten fields at 40x magnification.

Correlative pharmacodynamics (PD) studies with primary patient derived MM cells

We sought to determine whether PI3K was inhibited in MM patients that were treated with SF1126 using *ex vivo* methodology developed with cell lines and adapted from the whole blood assay developed by Chow *et al.*, 2005 [22]. Patient characteristics were as follows. The majority of the subjects were male (7), the median age was 63 (49–70) with a median of 5 prior treatments. All subjects had documented refractory disease with bone marrow aspirates showing plasma cell percentages of 30–90%.

Serial bone marrow (BM) sampling was performed at screening (pre-dosing) and day 1 (4 h post-dosing) of treatment cycles 1 and 2. Whole BM was subjected to red blood cell (RBC) lysis and the cellular BM constituents resuspended in stem span H3000 defined serum free medium and aliquoted into FACS tubes. FACS identified samples with > 5% CD138+ MM cells which were then further analyzed in two main test conditions: **1)** Samples received either LY294002 to a final concentration of 10 μ M, or solvent. The solvent treated samples established whether there was constitutive activation of AKT in fresh patient samples. **2)** Samples received either IGF-1 to a final concentration of 50 ng/ml, or solvent. All samples were then incubated at 37°C for 30 minutes, then then fixed by adding methanol-free formaldehyde (final conc. = 4%) for 10 min, washed in cold buffer, and resuspended in cold freezing medium consisting of 10% glycerol, 20% fetal bovine serum in RPMI tissue culture medium. Samples were stored at –20°C and paired samples were analyzed together. For intracellular phospho-specific antibody staining, thawed cells were washed and permeabilized with 50% methanol in 0.9% NaCl and incubation on ice for 10 min. Cells were then washed and labeled with anti-CD138-FITC (PharMingen, San Diego, CA) and anti-phospho-AKT-PE (Cell Signaling Technology, Inc., Danvers, MA) by incubating at room temperature for 15 min. Flow cytometry was performed on a FACS Caliber flow cytometer (BD Biosciences, San Jose, CA) and analyzed using Cellquest software (BD Biosciences).

Statistical analyses

We employed classical parametric statistical considerations; the *in vitro* data were analyzed initially using an ANOVA followed by individual test group comparisons (unpaired, two-sided t-test). For the *in vivo* data we determined that using a two-sided t-test, at a type I error rate of 5%, we had 80% power to detect a significant differences in tumor volumes after treatment assuming mean difference = $-0.66 \times \text{SD}$ (SD = tumor volume standard deviation). P values lower than 0.05 were declared as significant.

Results

In vitro cytotoxicity of SF1126

IGF-1 predominantly activates the PI3K-AKT pathway while IL-6 predominantly activates the RAS/ERK and the JAK/STAT pathway[5, 7]. The proteasome inhibitor bortezomib (PS-341) inhibits IL-6-mediated phosphorylation of ERK as well as AKT, and triggers apoptosis in myeloma cells[23]. In order to determine if inhibition of the AKT axis alone had a significantly different effect versus combined inhibition of the AKT and ERK axes together, we tested several myeloma cell lines to determine their sensitivity to the SF1126 prodrug compound *in vitro*, as a single agent and in combination with bortezomib. Figure

2A depicts the *in vitro* response to treatment with IC₅₀ values indicated in Figure 2B. The IC₅₀ values for SF1126 in MM.1S, MM.1R, and RPMI 8226 cells at 48 hours were 8.89, 11.67, and 11.90 μ M, respectively (Fig. 2B). The IC₅₀'s for SF1101 (LY294002) in these cell lines were 7.174, 9.04, and 5.83 μ M. In most cell lines examined, the IC₅₀'s for the two compounds were similar, though in a few cases there were differences in cytotoxicity (Fig. 2B). The relative cytotoxicity in MM.1S cells from a 48 hour exposure to 10 μ M SF1126 alone, 5 nM bortezomib alone, and the combination of SF1126 with bortezomib is shown in figure 2C. The data show that there was an increased cytotoxic effect of the combination of SF1126 and bortezomib versus either drug alone. These results have been confirmed by measuring Annexin V staining in primary MM tumor cells treated *ex vivo* with SF1126 or a combination of SF1126 plus bortezomib (unpublished observation).

Biochemical characterization of SF1126 activity

We tested several myeloma cell lines for phosphorylation of AKT, ERK, and other downstream targets of AKT, such as MDM2, and survivin, following treatment with the novel pan-isoform PI3K inhibitor, SF1126, with or without bortezomib. MM.1S and MM.1R cells showed significantly decreased phosphorylation of AKT, MDM2, and ERK after treatment with SF1126 or SF1101, indicating effective blockade of the PI3K-AKT and RAS/ERK axes (Fig. 3). Furthermore, phospho-p70 S6 kinase was decreased in MM.1R cells after treatment with SF1101 or SF1126, but not with RGDS peptide, suggesting that mTOR was suppressed by PI3K blockade, and that this effect was due to the LY294002 moiety rather than the RGD moiety of SF1126 (data not shown). Expression of survivin was not affected by SF1126 treatment, (Fig. 3).

The effect of SF1126 treatment on MM.1R cells with SF1126 and/or bortezomib is shown by the Western blot of Figure 4A. AKT phosphorylation was decreased after 30 minutes with SF1126 alone but not by bortezomib alone, and at 20 hours SF1126 and bortezomib given both singly and together virtually abolished pAkt expression. PTEN expression was present in both MM.1R and RPMI 8226 cell lines but the RPMI8226 line did not express p-Akt (Fig. 4A inset). In MM.1R cells serum starved and stimulated with IGF-1 p-Akt expression was robust, and decreased after 30 minutes with SF1126 alone but not by bortezomib alone [Fig. 4B (i)]. At 20 hours SF1126 reduced pAkt, while bortezomib abolished it, and both compounds together abolished pAkt. These findings were further substantiated in serum starved RPMI 8226 cells stimulated by IGF-1 and treated with SF1126 and/or bortezomib [Fig. 4B (ii)]. Without IGF-1 no pAkt was expressed, with IGF-1 pAkt expression was robust but almost abolished by SF1126 alone, while Bortezomib alone had no detectable effect on pAkt expression. At 20 hours SF1126 virtually abolished pAkt and the effect of bortezomib alone was similar. Taken together these studies imply that in cells resistant to dexamethasone and which express PTEN (MM.1R), and in cells without PTEN expression (RPMI8226), the PI3K inhibitor SF1126 caused an immediate and cumulative suppression of pAkt levels, while bortezomib given by itself took time to generate a strong impact on AKT phosphorylation.

Caspase cleavage and HIF-1 α expression *in vitro*

AKT activation leads to phosphorylation of anti-apoptotic targets Bad and procaspase 9 in many cell types, including myeloma cell lines[24]. Inhibition of the PI3K axis by LY294002 or wortmannin causes caspase-dependent apoptosis in myeloma cell lines[10]. As cleavage of caspase 3 to its effector fragments is a direct consequence of caspase 9 activation, we examined myeloma cell lines for caspase 3 cleavage in response to inhibition of PI3K-AKT. Of the non-treated MM.1S cells, 5.9% were positive for cleaved caspase-3 while 12.1% of the cells treated with 1 μ M staurosporine were positive. The MM.1S cells treated with 5 μ M and 50 μ M SF1126, 8.3% and 11.4% respectively showed positive staining for cleaved

caspase-3, equating to a 1.4-fold and 1.9-fold increase over the non-treated cells. The pattern was similar in MM.1R cells, with a 3-fold and 2.7 fold increase in caspase 3 cleavage in cells treated with 5 μ M and 50 μ M SF1126 respectively (Fig. 5A). The data indicate that SF1126 has specific effects on cell survival, which is consistent with its inhibition of the PI3K-AKT axis.

In order to further substantiate the caspase results, we used immunoblot techniques with the same antibody to cleaved caspase 3 on MM.1R cells treated with 25 μ M or 50 μ M SF1126 for 8, 24, 48 and 72 hours. This technique revealed that caspase 3 cleavage was present at peak levels with both 25 μ M and 50 μ M by 24 hours, and that this effect tapered off over time (Fig. 5B). This implies that while the expression of apoptotic proteins was less at the lower dose of SF1126, there was still a proapoptotic effect by 24 hours. Thus, SF1126 was effective in inducing apoptosis over 24 hours even at the lower dose.

HIF-1 accumulation is associated with chemotherapy and radiotherapy resistance[25–28]. Interestingly, in the dexamethasone-sensitive cell line MM.1S, HIF-1 was not significantly expressed under normoxic or hypoxic conditions. HIF-1 was not significantly expressed by the dexamethasone-resistant cell line MM.1R under normoxic conditions, but was highly expressed under hypoxic conditions. SF1126 blocked HIF-1 accumulation in MM.1R myeloma cells *in vitro* under hypoxic conditions (Fig. 5C). RPMI 8226 cells showed a similar pattern of *in vitro* HIF-1 expression under hypoxic conditions, which was abrogated by SF1126 treatment (Fig. 5D).

Antitumor activity of SF1126 *in vivo*

We found that SF1126 has potent anti-tumor and anti-angiogenic activities *in vivo*. MM.1R xenografts significantly responded to SF1126, and by 34 days the SF1126 treated tumors had a mean volume that was approximately eightfold less than the controls (>90% tumor volume growth inhibition, $p < 0.02$) (Fig. 6A; representative data of three repetitions). While this study did not include the measurement of pharmacokinetic indices, the results strongly suggest that tumor penetration and dosing obtained with SF1126 were effective, and clearly sufficient to elicit a marked biologic response. The tumors were well-established at the onset of treatment and showed a greater than 90% inhibition of growth but only slight regression in terms of volume. This data is congruent with the results obtained with staining for PCNA, which demonstrated significant inhibition (25% versus 7%) of cellular proliferation with treatment by SF1126 relative to controls (Fig. 6B). The *in vivo* data suggest that SF1126 mainly suppressed further tumor expansion via inhibition of tumor cell proliferation. Future studies may investigate whether a specific tumor compartment, *i.e.*, cancer stem cells, is primarily inhibited by SF1126.

We performed immunohistochemical staining for the endothelial marker CD31 in mouse xenografts in order to examine whether there was a change in the microvessel density (MVD) in response to SF1126 treatment *in vivo*. SF1126 demonstrated potent antiangiogenic activity *in vivo* against myeloma-induced angiogenesis in the MM.1R xenograft models as measured by a striking decrease in microvessel density ($p < 0.007$) (Fig. 6C). These data suggest that in addition to the suppression of cell proliferation (PCNA staining) the anti-tumor effect of SF1126 was also associated with inhibition of angiogenesis (CD31 staining).

SF1126 patient studies – toxicity and correlative pharmacodynamics

SF1126 did not appear to modify disease progression and no grade 4 drug-related toxicities were noted. Toxicities were not dose limiting and were generally non-specific as described by the Phase I trial observations published by Mahadevan *et al.*, 2012 [17]. One-third of

patients experienced grade 2 nausea/vomiting and no grade 4 drug-related toxicities were noted. Constitutional symptoms included fatigue and loss of appetite. Although preclinical studies demonstrated a rise in blood glucose one hour post infusion, this was not seen in any patients receiving drug. The dose limiting toxicity is still undefined. Median number of cycles was 1 (0.4–2.5), with one patient achieving stable disease (urinary protein stabilized following rapid rise prior to study initiation). All patients were taken off study due to progression.

BM samples for PD analysis by multiparameter flow cytometry were obtained from 6 of 8 MM patients enrolled onto a dose-escalation Phase I study of SF1126. Patients were enrolled in dose cohorts ranging from 90 to 1110 mg/m² administered twice weekly on a 28 day cycle. Pretreatment BM aspirates were obtained at screening and serial bone marrow (BM) samples were to be collected on day 1 of cycles 1 and 2 four hours post-dosing with SF1126. BMs for PD analysis were evaluable for 3 of the 6 patients. The three non-evaluable patients included 2 for whom serial BMs were not provided, and 1 patient whose screening BM had insufficient CD138+ myeloma cells for analysis. For the patients with evaluable BM, their PK data was acceptable and similar to that acquired in the solid tumor trial. This is in contrast to LY294002 which is only soluble in 100% DMSO and does not have a measurable plasma half-life. The current study indicated the following; **a)** SF1126 is rapidly cleared post-infusion; **b)** PK of the active SF1126 hydrolysis product (LY294002/SF1101) shows $t_{1/2}$ ~1.1–1.5 hours as it is gradually cleaved from the prodrug to attain dose levels of 90–570 mg/m²; **c)** AUC values at doses 140 mg/m² exceed those found effective in mouse xenograft studies [17].

In vivo inhibitory effects of SF1126 on the pathway were demonstrated in BM. Figure 7A summarizes the essential steps in the phosphoflow analysis of patient derived BM, while figure 7B shows results from pretreatment and following the initiation of therapy with SF1126 (1110 mg/m²) cycles 1 and 2, on day 1. The bottom FACS histogram of figure 7B reveals that after two cycles of SF1126 the patient's BM CD138+ cells were suppressed in terms of their ability to phosphorylate AKT under conditions of IGF-1 stimulation. For this patient (002) the CD138+ cells isolated prior to dosing with SF1126 demonstrated a lack of constitutive AKT activity as *ex vivo* treatment with LY294002 did not suppress endogenous downstream AKT phosphorylation. However, the pathway could be activated *ex vivo* by IGF-1 as demonstrated by a 7.3 fold increase in AKT phosphorylation over unstimulated cells. After one patient dose of SF1126 the *ex vivo* IGF-1 stimulation induced only a 4.4 fold increase in AKT phosphorylation, and after the 9th dose only a 1.9-fold increase, suggesting that there was partial inhibition of PI3K *in vivo* at the administered dose of SF1126. Serial samples from the other two evaluable patients failed to demonstrate a response to IGF-1 and were therefore not informative.

Discussion

The PI3K-AKT axis is a key node of convergence for multiple upstream tyrosine kinase-associated receptors associated with growth factors, cytokines, antigens, and costimulatory molecules [3]. It in turn activates AKT, which mediates cell proliferation, the cell cycle, and apoptosis via an array of kinases and transcription factors including but not confined to MDM2, NF- κ B, FK-HR, BAD, GSK3, and mTOR [3, 21, 29]. Multiple tumor types exhibit activation of the PI3K-AKT axis which is known to mediate growth and drug resistance in myeloma cells[21]. Moreover the direct PI3K suppressor, PTEN is functionally inactivated in several MM lines and in some cases of advanced MM[30]. Hence PI3K represents an attractive therapeutic target in MM despite well-recognized and significant pharmacokinetic (PK) and pharmacodynamic (PD) challenges involved in its targeting[21].

In the context of the PI3K-AKT axis, PI3K inhibitors, and MM, this study initially added to the characterization of SF1126 as a candidate PI3K inhibitor by further validating PI3K as a target, and providing measures of *in vivo* PK/PD. A heterogeneous array of 18 MM cell lines were all inhibited *in vitro*, albeit with some variability, providing further evidence for PI3K as a key target in MM, and demonstrating significant single agent activity exerted by SF1126. Furthermore, SF1126 caused sustained knockdown of IGF-1 triggered phospho-AKT in MM.1S, MM.1R, and RPMI8226 MM cell lines, regardless of PTEN expression, which is strong evidence for PI3K inhibition. We subsequently developed an *ex vivo* methodology to detect and quantify the inhibition of PI3K in MM cells derived from patients treated with SF1126, and found that such were suppressed in terms of their ability to phosphorylate AKT in response to IGF1 stimulation *in vitro*. Although the exact influence of PK on PD is complex and difficult to define, a PK profile that satisfies well-established criteria is necessary to achieve desired PD, *i.e.*, engagement with the intracellular target and the desired biological effect[31]. In the human studies, the active SF1126 hydrolysis product attained effective dosing that exceeded levels found to inhibit MM tumors in our preclinical models [17].

Dexamethasone is one of the most effective therapeutic agents in MM, and dexamethasone resistance is a critical clinical problem [32]. Interleukin-6 (IL-6) protects MM cells from glucocorticoid-induced apoptosis, suggesting one resistance mechanism is represented by the IL-6/PI3K/Akt pathway [33]. There is also *in vitro* evidence that dexamethasone resistant cell lines are inhibited by PI3K blockade. Therefore we tested SF1126 with dexamethasone sensitive and resistant lines, MM1.S and MM1.R, respectively and found that SF1126 suppressed both cell lines and the IC₅₀ values between the two were generally close. This highlights the possibility the PI3K inhibition may be used concurrently with dexamethasone and may help to overcome resistance to dexamethasone. This important topic should be systematically addressed in preclinical models and may form the basis of a future study.

Recently, evidence of resistance to PI3K inhibition has raised concerns that sustained and profound suppression of PI3K may be required with single agents, and this could increase the risk of toxicity in normal tissues [21]. And blocking the PI3K-AKT circuit alone, even with high doses, may not prevent the occurrence of resistance. It has been suggested that stimulation of the RAS-ERK pathway independent of PI3K may allow partial escape from PI3K inhibition [34]. This may explain the non-uniform sensitivity of different myeloma cell lines to PI3K blockade with SF1126 and provides a rationale for combined therapy. Furthermore, the bone marrow (BM) and other microenvironments that are infiltrated by MM cells variously participate in the pathogenesis of MM by promoting cell proliferation, survival, migration, and drug resistance [3]. Clinically, significant elevation of tumor microvascular density (MVD) is seen in myeloma bone marrow biopsies and inversely correlates with prognosis [35]. So targeting only the tumor cell compartment may be insufficient to achieve significant and durable disease control.

SF1126 was designed to address the factors described above and in this study we also began the exploration of combined agent treatment. Two major mitogenic and anti-apoptotic signaling pathways are the mitogen-activated protein kinase (MAPKK; also known as MEK) and PI3K-mTOR cascades [21]. SF1126 blocks PI3K-mTOR, and the degradation of pro-apoptotic proteins is prevented by bortezomib (Velcade®), which in the monotherapy setting has proved effective for MM patients [18]. Hence, we explored exposing MM cells to both SF1126 and bortezomib *in vitro*, and found that the combination was significantly more potent than either drug alone. In addition we sought to determine if SF1126 inhibited the tumor stroma, as it is targeted to the stromal endothelial compartment via an RGD ligand for

v 3 and 5 1 integrins. Other reports describe an antiangiogenic effect of LY294002 in terms of VEGF, HIF-1, and p53 [36–38].

In vivo, SF1126 did impact the tumor stromal compartment as it appeared to either directly or indirectly inhibit angiogenesis. This aligns with an extensive literature demonstrating that PI3K is a key signaling node for the induction of angiogenesis [39]. Moreover, multiple reports demonstrate that the parent compound, LY294002, and other PI3K inhibitors suppress angiogenesis [40]. SF1126 treatment has previously been found to be associated with the inhibition of angiogenesis *in vivo* [16]. In the present study CD31-positive microvessel density (MVD) in whole tumor sections of subcutaneous MM.1R xenografts in the SF1126-treated group was strikingly diminished. Most MM cell lines constitutively secrete significant quantities of VEGF, and small-molecule VEGF receptor inhibitors have been shown to inhibit myeloma cell growth and survival[41]. AKT activation increases angiogenesis through the upregulation of HIF-1 and VEGF, via both mTOR-dependent and mTOR-independent pathways[42, 43]. In the present study hypoxic stabilization of HIF-1 was detected by immunoblot analysis. In MM.1R and RPMI 8226 cells under hypoxic conditions, SF1126 decreased the accumulation of HIF-1. Our data further indicate that treatment of myeloma cells with SF1126 blocked the activity of mTOR, with decreased phosphorylation of p70 S6 kinase (data not shown) and decreased stabilization of HIF-1. While the exact mechanism is not known, the effect of SF1126 on HIF-1 stabilization and subsequent production of VEGF suggests a direct link between PI3K inhibition and the inhibition of angiogenesis, as demonstrated by decreased MVD in our mouse xenografts.

The role of PI3K in MM tumor cell pathophysiology and the effects of its inhibition still remain to be further investigated, particularly at the mechanistic level. In this context our data indicates that SF1126 inhibited MM tumor cell proliferation, as indicated by PCNA staining. Moreover, we also have initial evidence that SF1126 supported the pro-apoptotic pathway proteins p53 and Caspase-3. PI3K-AKT activates MDM2, promoting its translation to the nucleus with the subsequent ubiquitination/degradation of p53, leading to inhibition of apoptosis. Suppression of PI3K-AKT activity by PTEN protects p53 from MDM2 activity, sensitizing tumor cells to chemotherapy[38]. Induction of p53 has multiple proapoptotic effects, in that it activates transcription of BH3-only proteins, promotes binding of p53 to Bcl-X_L, and promotes the oligomerization of proapoptotic proteins Bak and Bax[44]. Our immunoblot data demonstrate that treatment of MM cell lines MM.1S and MM.1R with SF1126 suppressed MDM2 phosphorylation (Figure 3B). Accordingly, our immunocytochemistry and immunoblot data also show that activated Caspase-3 increased in a dose-dependent manner with SF1126 treatment. Caspase-3 is activated from by cleavage of two pro-caspases with subsequent dimerization. So SF1126 appears to act in MM by inhibiting tumor cell proliferation, promoting apoptosis, and by inhibiting the stromal endothelium.

Conclusions

SF1126 has potent activity against MM cells *in vitro* and *in vivo*. The addition of bortezomib which has shown positive effects in MM patients and which inhibits the degradation of proapoptotic proteins, increased cytotoxicity versus either drug alone. In mouse xenograft models, we found that treatment with SF1126 appeared to affect the tumor microenvironment by inhibiting angiogenesis, possibly by the suppression of HIF-1 stabilization. *In vitro* and in human subjects SF1126 treatment knocked down phospho-AKT in MM cells. Moreover MDM2 phosphorylation was suppressed by SF1126 and Caspase-3 cleavage was increased in a dose dependent manner. Taken collectively these results, (1) provide further insights into the biological activity of SF1126, (2) support continued Phase I/

II trials with SF1126 in MM as a single agent, and (3) suggest that future trials may include evaluation of SF1126 combined with bortezomib.

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Structures of SF1126, SF1326, SF1101, RGDS, SF1110, and SF1111 (Zwitterionic salt forms depicted)

Figure 1. Chemical structures of SF1126 and SF1101

The structures of SF1126 and SF1101 (LY294002), as well as related compounds, are shown. SF1326 is an RAD conjugated prodrug that undergoes cleavage to SF1101 at same rate as SF1126 used as an *in vivo* nonintegrin targeted control[16]. Both SF1126 and SF1326 cleave *in vitro* and *in vivo* to yield the active PI3 kinase inhibitory moiety, SF1101 (LY294002).

Figure 2A

Figure 2B

MTT Assay for the MM1.S myeloma cell line treated with SF1126 and Bortezomib

Figure 2C

Figure 2. Measurement of SF1126 cytotoxicity by MTT assay in multiple myeloma cell lines

A. A graph of MTT activity in human myeloma cell lines treated with SF1126 at various concentrations for 48 hours. SF1126 effectively induced cytotoxicity in multiple human myeloma cell lines. **B.** A chart of the 50% inhibitory concentration (IC_{50}) determined for treatment with SF1126 and SF1101 (LY294002) on the human myeloma cell lines shown in 2A. SF1126 and SF1101 IC_{50} 's were similar for the most part. **C.** A chart of the relative cytotoxicity of a 48 hour exposure of MM.1S cells to 10 μ M SF1126 alone, 5 nM bortezomib alone, and 10 μ M SF1126 in combination with 5 nM bortezomib. The effectiveness of either drug alone was enhanced by addition of the second drug.

Figure 3. Signaling pathway effects (PI3K-AKT and RAS/MAPK) in MM after treatment with the pan-PI-3 kinase inhibitor, SF1126

Western blot analysis of signaling pathways in whole cell lysates, and NT represents no treatment in both figures A and B. These data suggest that high levels of p-AKT and p-ERK (compare between MM.1S and MM.1R cell lines, lane 1 and 5 in figure A and lane 1 and 4 in figure B) may have been responsible for the development of dexamethasone-resistance in plasma cell myeloma. Data show that the PI3K inhibitor significantly blocked phosphorylation of AKT, MDM2 and phosphorylation of ERK.

Figure 4. A novel pan-PI3K inhibitor, SF1126 blocks IGF-1-stimulated phosphorylation of AKT in MM

A. MM.1R cells were treated concurrently with SF1126 (10 μ M) alone or with the proteasome inhibitor bortezomib (10 nM). **B (i).** MM.1R cells starved in serum free medium for 16 hours and then pretreated with SF1126 (10 μ M) alone, bortezomib (10 nM) alone, or SF1126 (10 μ M) plus bortezomib (10 nM) at indicated time points (30 minutes and 20 hours) prior to stimulation with IGF-1 (100 ng/ml) for 30 minutes. **B (ii).** RPMI8226 cells starved in serum free medium for 16 hours and then pretreated as for B(i). In all cases - actin was the loading control, and in 4B(ii) although not necessary, AKT was also included as a loading control. The data shows that IGF-1-induced AKT activation was significantly blocked by prior treatment with SF1126 either for 30 minutes or 20 hours in both MM.1R and RPMI8226 cell lines. Bortezomib however, markedly blocked AKT phosphorylation only at the later time point (20 hours exposure).

Figure 5A

Figure 5C

Figure 5D

Figure 5. The pan-isoform PI3K inhibitor SF1126 promotes apoptosis and inhibits hypoxia-induced HIF-1 stabilization

A. MM.1S and MM.1R cells were treated with 1 μM Staurosporine (a protein kinase inhibitor used as a positive control), 5 μM SF1126 and 50 μM SF1126 for one hour. The number of cleaved Caspase-3 positive cells was determined for each cell and treatment type. The data shows that MM.1S cells exhibit little difference from SF1126 vs. staurosporine, while MM.1R cells are more sensitive to the effects of SF1126 in inducing apoptosis. **B.** Cleaved caspase 3 Western Blot shows that the amount of caspase 3 cleavage is dose and time-dependent, with a stronger band at 50 μM SF1126, and peak caspase 3 cleavage at approximately 24 hours. **C.** Hypoxic- stabilization of HIF-1 is significantly higher in MM.1R cell line as compared to the normoxic control and SF1126 inhibits this stabilization. This data may imply that the high level of HIF-1 is the cause of resistance in the MM.1R cell line. **D.** Higher doses of SF1126 also significantly inhibited hypoxia-induced HIF-1 stabilization in RPMI 8226 cells. Hypoxic- stabilization of HIF-1 was significantly blocked by prior treatment with SF1126 at both 20 and 40 μM concentrations (lanes 3 and 4).

A

Figure 6B

Figure 6C

Figure 6. SF1126 demonstrates antitumor efficacy in a human xenograft model and blocks angiogenesis

A. Suppression of growth of MM.1R xenograft in subcutaneous mouse model by pan-isoform PI3K inhibitor, SF1126. Data plotted are mean $\pm$ SEM for tumor volume. Growth rate of tumors *in vivo* was significantly less when compared with vehicle treated mice (n= 8, $p < 0.01-0.02$). Experiment repeated three times, representative data shown. **B.** In the MM.1R tumor model, treatment with SF1126 reduced proliferation, *i.e.*, decreased PCNA-positive cells (arrow). Bars indicate quantitation of PCNA-positive proliferating cells as described in Materials and Methods. Error bars are SD of mean (n=8, $p < 0.02$). **C.** PI3K inhibitor, SF1126, blocked angiogenesis. Bar diagrams quantify CD31-positive microvasculature (arrow) as described in Materials and Methods. Strikingly, the SF1126 treated tumors had significantly less CD31 vascular staining than controls. Error bars represent SD of mean (n=8, $p < 0.007$).

Figure 7A

Figure 7B

Figure 7. Pharmacodynamic knockdown of AKT kinase activity by SF1126 in primary MM cells

A. Schematic representation for methods used for multiparametric phospho-AKT FACS analysis of MM patients bone marrow aspirates (BMA). BM was collected from patients 24 hours following the administration of SF1126. CD138 cells were stimulated *ex vivo* with IGF1 and/or treated *ex vivo* with LY294002 as an internal control. Cells were permeabilized, stained for phospho-AKT, and then analyzed using multi-parameter FACS.

B. Flow cytometry histograms of the gated CD133+ MM tumor cells in a BM sample obtained from patient 002 which was subjected to phospho-AKT analysis. We show results from pretreatment and following the initiation of therapy with SF1126 (1110 mg/m²) cycles 1 and 2, on day 1. The bottom FACS histogram shows that after two cycles of SF1126 the patient's BM CD138+ cells were suppressed in terms of their ability to activate AKT under conditions of IGF-1 stimulation *ex vivo*.