

p38 α -Selective MAP Kinase Inhibitor Reduces Tumor Growth in Mouse Xenograft Models of Multiple Myeloma

SATYANARAYANA MEDICHERLA, MAMATHA REDDY, JING YING MA, TONY A. NAVAS, LINGYUN LI, AARON N. NGUYEN, IRENE KERR, NIRUPAMA HANJARAPPA, ANDREW A. PROTTER and LINDA S. HIGGINS

Scios Inc, Fremont, CA 94555, U.S.A.

Abstract. Multiple myeloma (MM) is a clonal plasma cell malignancy, which is currently incurable. Therefore, new mono- or combined therapy treatment regimens in the early and advanced phases of MM are urgently needed to combat this disease. Recently, p38 mitogen-activated protein kinase (MAPK) has been implicated as playing an important role in MM. Therefore, the effect of a p38 α -selective MAPK inhibitor, SCIO-469 (indole-5-carboxamide, ATP-competitive inhibitor), or its structural analog, SD-282 (indole-5-carboxamide, ATP-competitive inhibitor) was examined in mouse xenograft models of MM using human RPMI-8226 or H-929 plasmacytoma inocula. Oral treatment with SCIO-469 (10, 30, 90 mg/kg) twice daily was initiated in mice with palpable tumors of RPMI-8226 origin, a condition that mimics early human myeloma disease. In mice with palpable tumors, 14 days of SCIO-469 treatment significantly reduced RPMI-8226 tumor growth in a dose-dependent manner. A significant dose-dependent reduction in RPMI-8226 tumor growth was also observed when SCIO-469 oral treatment at doses of 10, 30 and 90 mg/kg twice daily was initiated in mice with tumors of pronounced size, a condition that mimics advanced human myeloma disease. In a similar set of studies employing the SCIO-469 analogue SD-282 at 90 mg/kg/bid orally, histological assessment at the end of the study demonstrated a significant reduction in RPMI-8226 tumor growth and angiogenesis. SD-282 treatment was additionally shown to significantly reduce expression of heat-shock protein-27 (HSP-27) and phospho-p38 in the tumor cells. Furthermore, co-administration of SCIO-469 with dexamethasone elicited antitumor properties in dexamethasone-sensitive H-929 tumors at much lower than the typically effective doses of dexamethasone, suggesting its potential for combined therapy.

In conclusion, p38 inhibitors reduced human myeloma cell growth in vivo both at early and advanced phases of the disease. The current study also provides evidence of potential for co-therapy with dexamethasone.

Human multiple myeloma (MM) is a neoplasm of the B-cell lineage. MM remains incurable with current treatment approaches, including high-dose therapy and autologous stem cell transplantation (1). Bortezomib and thalidomide recently provided major advances in the treatment of this disorder, having demonstrated efficacy in all phases of the disease (2, 3). The mechanisms of action of these two approaches reveal clues to the pathogenesis of MM which can be exploited in discovering novel and much needed new therapies.

The significant activity in treating MM of thalidomide (2, 3), which has anti-inflammatory properties, has provided strong support for the importance of cytokines, particularly interleukin-6 (IL-6), and other inflammatory mediators in MM (4). These inflammatory factors produced in the marrow-myeloma cell microenvironment are thought to be important for supporting survival, proliferation, and possibly metastasis of myeloma cells (5). p38 Mitogen-activated protein kinase (MAPK) activation leads to the production of IL-6 and other inflammatory mediators implicated in MM pathophysiology, and p38 MAPK has been shown to be activated in the myeloma cell microenvironment (6-9).

The proteasome inhibitor, bortezomib, has been shown to have direct cytotoxic effects on MM cells (10). It was the first proteasome inhibitor to enter clinical trials in cancer patients (11-13), based on the results of preclinical studies showing that this novel agent directly inhibited the proliferation of myeloma cells, induced their apoptosis and abrogated paracrine tumor growth in patients with advanced relapsed and/or refractory MM (14). Since then, combination studies of bortezomib with various agents, including dexamethasone, DNA-damaging drugs, thalidomide and lenalidomide, have been designed and are currently ongoing in patients with both relapsed/refractory and newly diagnosed disease. Thus, while bortezomib and dexamethasone alone or in combination offer an alternative

Correspondence to: Satyanarayana Medicherla, Fellow, Schering Plough Biopharma, 901 California Ave., Palo Alto, CA 94304, U.S.A. Tel: +1 6504961255, e-mail: satya.medicherla@spcorp.com

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treatment in the face of resistance to conventional chemotherapy (11-13), propensity for patients to develop resistance to current therapies (including dexamethasone) supports the hunt for novel therapeutics. Recent literature suggests an important function of p38 MAPK in the generation of signals that are critical in controlling normal and malignant hematopoiesis and survival *via* cytokines and growth factors (7, 15, 16). Specific inhibitors of p38 α MAPK are also known to block production in the bone marrow of major inflammatory cytokines such as IL-6, vascular endothelial growth factor (VEGF), and interleukin-1 β (IL-1 β), which promote the proliferation, survival, and drug-resistance of MM cells (17-19).

Recently, we reported that the p38 α -selective inhibitor SCIO-469 (indole-5-carboxamide, ATP-competitive inhibitor) ameliorates myeloma bone disease in a mouse model (20). SCIO-469 is a new molecular entity which has been tested in clinical studies involving a variety of healthy volunteer and patient populations, including those with MM. We have also shown that SCIO-469 acts both on MM cells and the bone marrow microenvironment and in combination with bortezomib significantly enhances the toxicity of bortezomib, reducing MM tumors *in vivo* in a murine xenograft model (21).

In this study, the *in vivo* dose-response effects of SCIO-469 and its structural analogue SD-282 on early (palpable tumor) and advanced (pronounced tumor) phases of tumor growth in human RPMI-8226 plasmacytoma xenograft murine models were examined and the mechanisms of its *in vivo* antitumor activity were characterized. Since dexamethasone is a foundation of several successful combination therapy regimens (22), the role of SCIO-469 in reducing MM tumor growth in combination with dexamethasone was also studied in a dexamethasone-sensitive H-929 tumor model.

Materials and Methods

p38 α Selective inhibitor. SCIO-469 and its structural analogue SD-282 are functionally similar small molecules from the same class of indole-5-carboxamide, ATP-competitive inhibitors of p38 kinase. SD-282 lacks a methyl group that is found on SCIO-469 on the N-1 nitrogen of the indole. SCIO-469 and SD-282 (23, 24) has an *in vitro* IC₅₀ of 9 and 2 nM for the inhibition of p38 α ; and 10 and 15-fold selectivity for p38 α over p38 β . They have at least 2,000-fold selectivity for p38 α over an *in vitro* panel of 20 other kinases (including other MAPKs). No significant affinity was detected in a panel of 70 enzymes and receptors. In pharmacokinetic studies of the two inhibitors, the overall exposure in mice as measured by AUC has also been shown to be similar (data not shown). The pharmacokinetic studies in mice revealed that administration of SCIO-469 and SD-282 at 30 and 90 mg/kg resulted in 3 and 8 μ M circulating concentrations, respectively, at 30 min while trace amounts were detected at 8 h (20, 24). The mice in the studies reported here were treated twice daily by oral gavage with 10, 30 or 90 mg/kg of SCIO-469 or SD-282 as solution in 1% PEG at a dosing volume of 10 ml/kg.

Animals. Six-week-old male triple immune-deficient BNX mice were obtained from Charles River (Wilmington, MA, USA). They were maintained in a pathogen-free environment in the Scios animal resources facility. All the animal studies were conducted according to protocols approved by the Animal Ethics Committee at Scios. All mice received antibiotic (Eneroxofloxacin) as prophylaxis against infection.

Cells and culture conditions. RPMI-8226 or H-929 human MM cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA), 2 mM L-glutamine (Life Technologies, Inc., Grand Island, NY, USA), 50 units/ml penicillin, and 50 μ g/ml streptomycin (Life Technologies, Inc.). The cells were washed twice and resuspended in RPMI-1640 at a density of 3 \times 10⁶ cells/100 μ l or 3 \times 10⁷ cells/100 μ l before injection into the mice.

Xenograft murine myeloma models. The mice were inoculated subcutaneously into the right flank with a known number of RPMI-8226 or H-929 MM cells in 100 μ l of RPMI-1640, together with 100 μ l of matrigel basement membrane matrix (Becton Dickinson, Bedford, MA, USA) on day 0. A pilot study was conducted in which the growth kinetics of tumors were determined in mice inoculated with 100 μ l of different concentrations of RPMI-8226 or H-929 cells. It was found that 3 \times 10⁶ cells/100 μ l per mouse caused palpable tumors (volume ~200 mm³) and 3 \times 10⁷ cells/100 μ l per mouse caused pronounced tumors (volume ~800 mm³) at around day 9 of the study, at which point the mice were randomly assigned into treatment groups each consisting of ten mice. Digital caliper measurements of the longest perpendicular tumor diameter were taken to calculate tumor volume [formula=4/3 \times (width/2)² \times (length/2)]. Tumor volume and body weights were monitored every 3 days. The animals were sacrificed at the end of the study, at which time the tumors were excised, weighed and subjected to histological analysis.

Experimental studies.

i) Effect of SCIO-469 on the growth of RPMI-8226 MM palpable tumors. The BNX mice were inoculated with 3 \times 10⁶ RPMI-8226 MM cells and from day 9 they were treated with vehicle twice daily (1% PEG, group 1), with SCIO-469 at 10 mg/kg twice daily (group 2), with SCIO-469 at 30 mg/kg twice daily (group 3), or with SCIO-469 at 90 mg/kg twice daily (group 4). All treatments continued for 14 days.

ii) Effect of SCIO-469 on the growth of RPMI-8226 MM pronounced tumors. The BNX mice were inoculated with 3 \times 10⁷ RPMI-8226 and from day 9 they were treated with vehicle twice daily (1% PEG, group 1), with SCIO-469 at 10 mg/kg twice daily (group 2), with SCIO-469 at 30 mg/kg twice daily (group 3), or with SCIO-469 at 90 mg/kg twice daily (group 4). All the treatments were continued for 15 days.

iii) Combined effect of SCIO-469 and dexamethasone on the growth of dexamethasone-sensitive H-929 MM palpable tumors. Before initiation of this combination study, a dose-response study was conducted in the H-929 MM model with palpable tumors at different doses of SCIO-469 (10, 30 and 90 mg/kg twice daily orally) or dexamethasone (0.15, 0.5, 1 or 4.5 mg/kg once a day orally). It was found that SCIO-469 at 30 mg/kg or dexamethasone at 0.5 mg/kg marginally reduced tumor growth and these doses were selected for the combination study to explore potential combination effects.

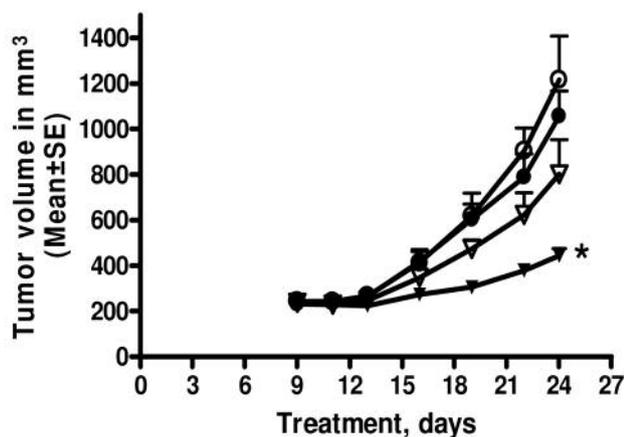


Figure 1. Effect of SCIO-469 on the growth of RPMI-8226 MM palpable tumors. The BNX mice were inoculated with 3×10^6 RPMI-8226 cells. On day 9, when tumors were palpable, vehicle or SCIO-469 was administered twice daily orally. Treatments: Vehicle (open circles), SCIO-469 at 10 mg/kg (closed circles), SCIO-469 at 30 mg/kg (open triangles), and SCIO-469 at 90 mg/kg (closed triangles), ($n=10$ /group). * $p < 0.01$ versus vehicle group, calculated by nonparametric Bonferroni multiple comparison test.

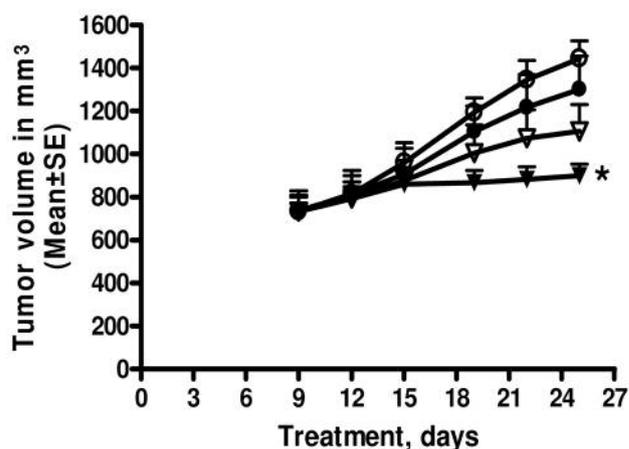


Figure 2. Effect of SCIO-469 on the growth of RPMI-8226 MM pronounced tumors. The BNX mice were inoculated with 3×10^7 RPMI-8226 cells. On day 9, when tumors were pronounced, vehicle or SCIO-469 was administered twice daily orally. Treatments: Vehicle (open circles), SCIO-469 at 10 mg/kg (closed circles), SCIO-469 at 30 mg/kg (open triangles), and SCIO-469 at 90 mg/kg (closed triangles), ($n=10$ /group). * $p < 0.01$ versus vehicle group, calculated by nonparametric Bonferroni multiple comparison test.

The BNX mice were inoculated with 3×10^6 H-929 MM cells and from day 9 they were treated with vehicle (1% PEG, group 1), with SCIO-469 at 30 mg/kg twice daily (group 2), with dexamethasone at 0.5 mg/kg once a day (group 3), or with SCIO-469 at 30 mg/kg twice daily plus 0.5 mg/kg dexamethasone once a day (group 4) for 16 days.

iv) Effect of SD-282 on angiogenesis, apoptosis, HSP-27 and phospho-p38 in RPMI-8226 palpable tumors. The BNX mice were inoculated with 3×10^6 RPMI-8226 MM cells and on day 9 they were treated with vehicle (1% PEG, group-1) or with SD-282 at 90 mg/kg twice daily orally (group 2) for 15 days. Angiogenesis was examined by determining the number, size, and intensity of newly formed micro-vessels by staining for CD-34 (Cat# CL8927; Cedarlane, Burlington, NC, USA). Formalin-fixed and paraffin-embedded tumor sections were also subjected to H&E, phospho-p38 (Cat# 9216; Cell Signaling, Danvers, MA, USA), and heat-shock protein-27 (HSP-27) (Cat# MS-101-R7; Lab Vision, Fremont, CA, USA) immunohistochemistry (IHC). Phospho-p38 and HSP-27 IHC analyses were evaluated by a semi-quantitative scoring method based on the distribution and intensity of the positive staining cells. Samples were scored as 0, 1, 2, 3, or 4, indicating no, minimal, mild, moderate or strong staining, respectively, of phospho-p38 or CD-34 in 10 positively stained "hot fields" under $\times 20$ magnification with the aid of Image Pro Plus imaging system (Applied Scientific Instrumentation, Eugene, OR, USA).

Statistical analysis. Student's *t*-test or a non-parametric Mann-Whitney, or Bonferroni multiple comparison test wherever applicable, was used to determine the significance of difference between the vehicle and the SCIO-469- or SD-282-treated groups. The differences were considered statistically significant when $p < 0.05$. All the statistical analyses were performed using Prism version 3.02 (GraphPad Software, San Diego, CA, USA).

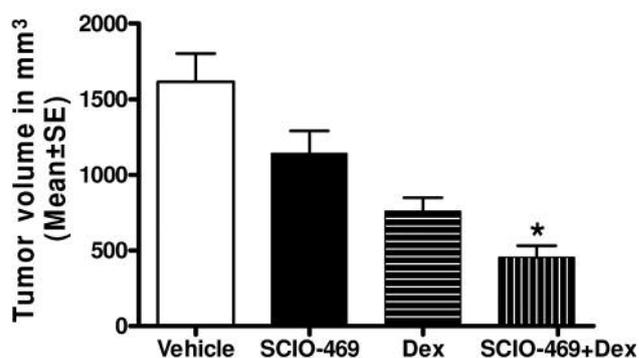


Figure 3. Effect of SCIO-469 in combination with dexamethasone on the growth of H-929 MM palpable tumors. The BNX mice were inoculated with 3×10^6 H-929 cells. On day 9, when tumors were palpable, vehicle, SCIO-469 at 30 mg/kg twice daily, 0.5 mg/kg dexamethasone once daily, or both SCIO-469 and dexamethasone were administered.

Results

The p38 α inhibitors SCIO-469 and SD-282 alone or together with dexamethasone had no impact on the body weights in the mice with palpable or pronounced tumors (data not shown).

i) Effect of SCIO-469 on the growth of RPMI-8226 MM palpable tumors. In the mice with palpable tumors, SCIO-469 treatment dose-dependently reduced tumor growth as measured by tumor volume kinetics (Figure 1). SCIO-469 also dose-dependently reduced weight of the palpable tumors

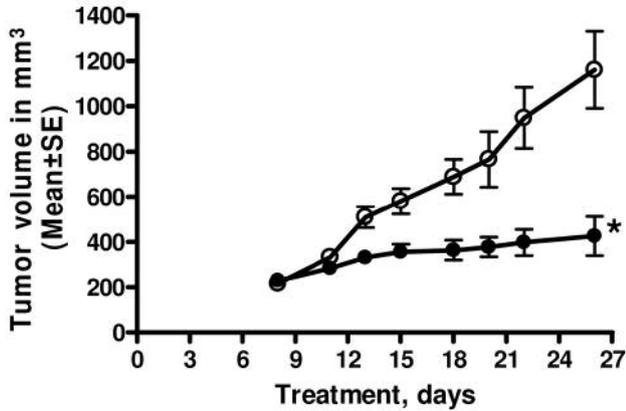


Figure 4. Effect of SD-282 on the growth of RPMI-B226 MM palpable tumors. The BNX mice were inoculated with 3×10^6 RPMI-B226 cells. On day 9, mice were treated twice daily orally with SD-282 at 90 mg/kg (closed circles) or vehicle (open circles) ($n=10$ /group). * $p < 0.01$ versus vehicle group, calculated by Student's *t*-test.

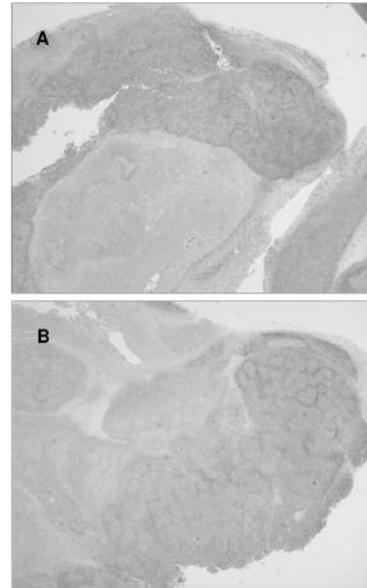


Figure 5. Effect of SD-282 on tissue necrosis in RPMI-8226 MM palpable tumors. RPMI-8226 myeloma tumors from vehicle-treated and SD-282-treated groups were formalin-fixed and paraffin-embedded. Vehicle-treated group (A) showed less tissue necrosis than did the SD-282-treated group (B).

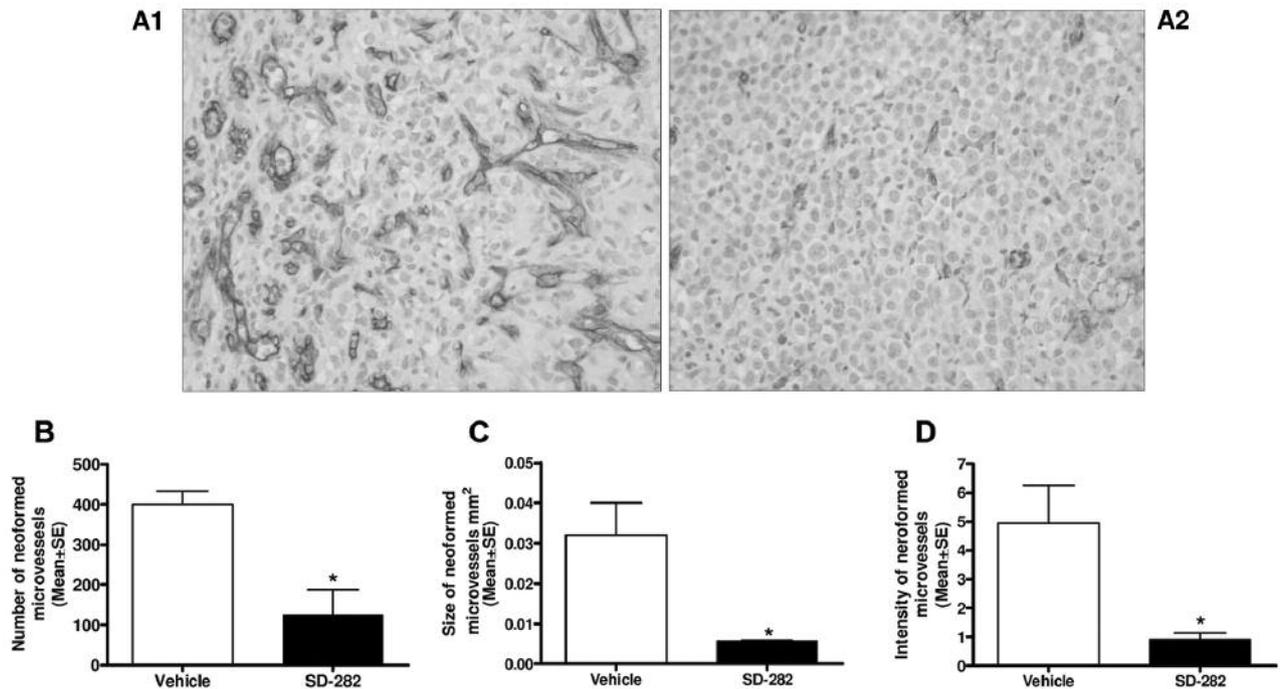


Figure 6. Effect of SD-282 on angiogenesis in RPMI-8226 MM palpable tumors. Microvessel density in tumor sections was evaluated by IHC analysis of mouse CD-34 expression within the tumor. A1, representative tumor section from a control mouse. A2, representative tumor section from a mouse treated with SD-282 at a dose of 90 mg/kg. SD-282 significantly reduced number of neo-formed micro-vessels (B; * $p < 0.01$), size of neo-formed micro-vessels (C; * $p < 0.01$), and intensity of neoformed micro-vessels (D; * $p < 0.01$), ($n=10$ /group).

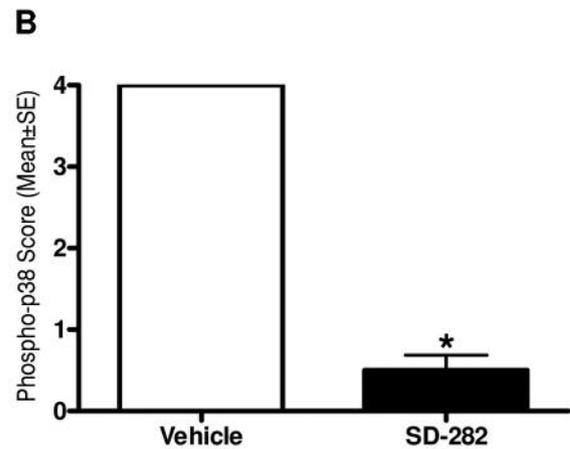
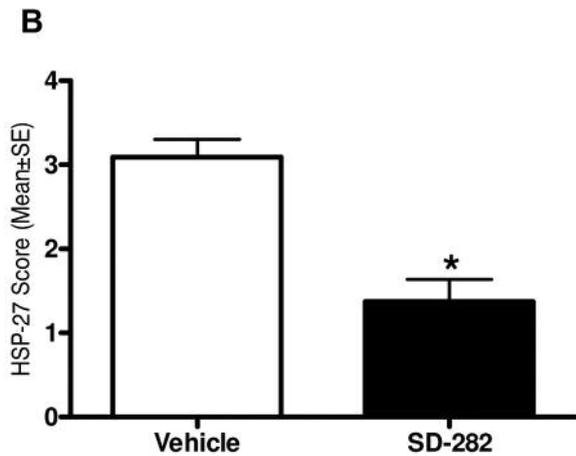
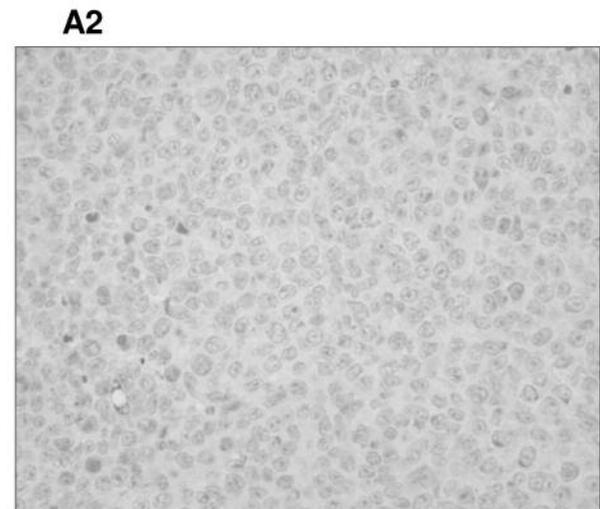
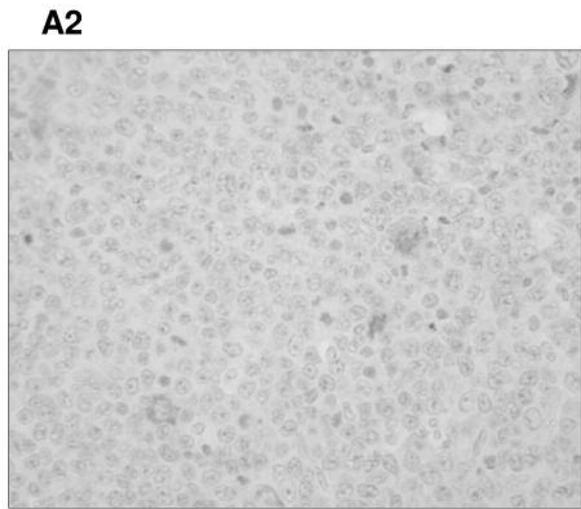
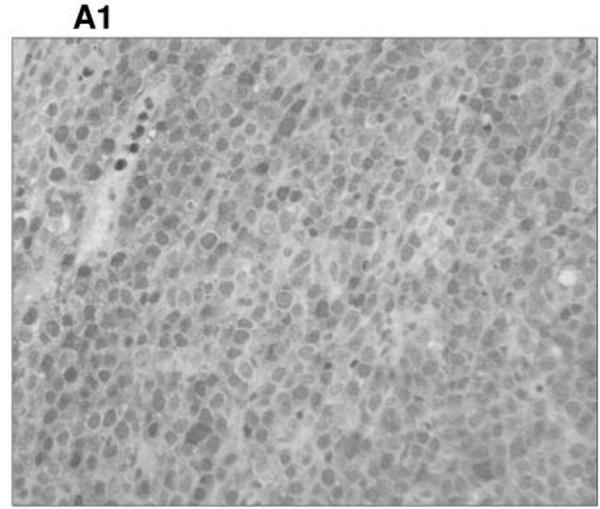
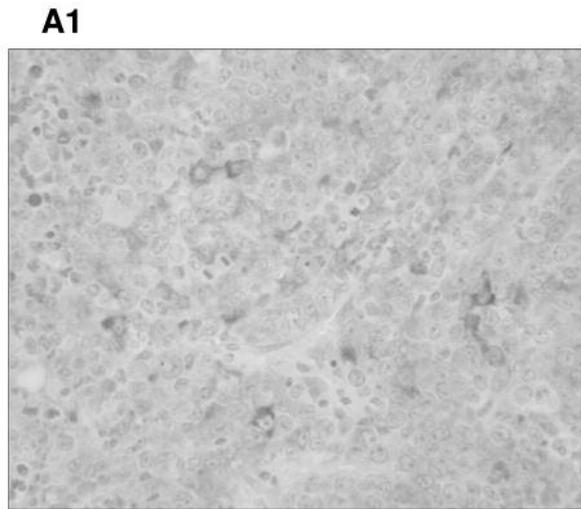


Figure 7. Effect of SD-282 on HSP-27 expression in the cytoplasm of RPMI-8226 MM palpable tumors. HSP-27 was measured by IHC analysis at the end of the study in the cytoplasm of vehicle-treated (A1) and SD-282-treated (A2) tumor cells. Treatment with SD-282 significantly decreased HSP-27 expression in the cytoplasm (B; * $p < 0.01$), (n=10/group).

Figure 8. Effect of SD-282 on phospho-p38 expression in RPMI-8226 palpable MM tumors. Activated/phosphorylated p38 MAPK expression was observed (dark staining) in stromal cells as well as in fibroblasts, neutrophils and macrophages in the vehicle-treated group (A1). Treatment with SD-282 significantly decreased phospho-p38 staining (A2, B) * $p < 0.01$), (n=10/group).

at termination. At the end of the treatment period tumor weights in grams (mean±SE) in the vehicle and SCIO-469 at 10, 30 and 90 mg/kg groups were 0.87±0.41, 0.86±0.25, 0.66±0.40 and 0.32 ± 0.04* (**p*<0.01 when compared to vehicle group), respectively.

ii) *Effect of SCIO-469 on the growth of RPMI-8226 MM pronounced tumors.* Even in the mice with pronounced tumors, SCIO-469 treatment dose-dependently reduced tumor growth as measured by tumor volume kinetics (Figure 2). SCIO-469 dose-dependently reduced the weight of the pronounced tumors at termination. At the end of the treatment period, tumor weights in grams (Mean±SE) in the vehicle and SCIO-469 at 10, 30 and 90 mg/kg groups were 2.25±0.63, 1.96±1.0, 1.47±0.90 and 0.88±0.29* (**p*<0.01 when compared to vehicle group), respectively.

iii) *Effect of SCIO-469 in combination with dexamethasone on the growth of H-929 MM palpable tumors.* The doses of SCIO-469 (30 mg/kg) alone and dexamethasone (0.5 mg/kg) alone marginally but not significantly reduced both tumor growth (as measured by volume) and tumor weights when compared to the vehicle group. Notably, tumor growth was significantly (*p*<0.01) reduced only when SCIO-469 and dexamethasone were administered in combination (Figure 3).

iv) *Effect of SD-282 on tumor growth/weight, angiogenesis, apoptosis, HSP-27 and phospho-p38 in RPMI-8226 MM palpable tumors.* As in the previous experiments with SCIO-469, SD-282 administered at 90 mg/kg twice daily significantly reduced palpable tumor growth during the course of the study (Figure 4). At the end of the treatment period, tumor weights in grams (Mean±SE) for the vehicle and SD-282 treatment groups were 1.13±0.62 and 0.28±0.29* (**p*<0.01 when compared to vehicle group), respectively. Tumor necrosis was determined to be 33% in the vehicle-treated group whereas it was 45% in the SD-282-treated group (Figure 5). The size, intensity, and number of neo-formed microvessels quantified by CD-34 IHC staining were found to be significantly reduced in the SD-282 treatment group when compared to the vehicle-treated group (Figure 6). HSP-27 staining in the cytoplasm of the tumor cells in the SD-282 treatment group was much weaker than that in the vehicle treatment group (Figure 7). The tumor cells, stromal cells, fibroblasts, neutrophils, and macrophages showed strong phospho-p38 staining in the vehicle-treated mice. In contrast, minimal phospho-p38 staining was observed in the tumors of the SD-282-treated mice (Figure 8).

Discussion

The p38 α -selective inhibitor SCIO-469 reduced human RPMI-8226 MM cell palpable and pronounced tumor growth in the murine xenograft model in a dose-dependent manner,

suggesting that SCIO-469 may be capable of reducing myeloma tumor growth in different phases of the disease. Furthermore, SCIO-469 in combination with dexamethasone significantly reduced tumor growth in the human H-929 MM xenograft tumors in the mice, suggesting the potential for combination therapy.

As expected, the SCIO-469 analog, SD-282, also reduced tumor growth and weight. SD-282 treatment was associated with reduced number, size and intensity of neo-formed microvessels which suggested that one mechanism for the reduction in tumor growth by SD-282 was the inhibition of angiogenesis. This interpretation is consistent with the observed tumor necrosis, which was severe in the inhibitor group when compared to the vehicle group. HSP-27 expression was found to be significantly higher in the vehicle-treated group than in the SD-282 treatment group. Because HSP-27 overexpression confers cellular resistance to a variety of stimuli that induce cell death through both necrosis and apoptosis, interference at this level is promising in a p38 inhibitor (2, 25). SD-282 also significantly inhibited the phosphorylation of p38 in the tumor cells. Since SD-282 and SCIO-469 inhibit p38 MAPK activity, but not phosphorylation and activation directly, these data suggested that the inhibitor successfully suppressed the production of inflammatory signals *via* the p38 MAPK signaling pathway.

In conclusion, the p38 α -selective inhibitors SCIO-469 and SD-282 reduce the activity of the p38 MAPK signaling pathway and reduce both early and advanced phase human myeloma cell growth *in vivo* in a dose-dependent manner. The combination of dexamethasone with SCIO-469 significantly inhibits tumor growth even at levels that are only marginally effective in monotherapy. In addition, this class of p38 inhibitors can reduce angiogenesis when administered early. The current study provides *in vivo* evidence supporting the hypothesis that p38 inhibitor therapy alone or in combination with dexamethasone may have efficacy in patients with multiple myeloma.

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