In vivo anti-tumor effects of the mTOR inhibitor, CCI-779, against human multiple myeloma cells in a xenograft model.

Running Head: In vivo efficacy of CCI-779 in myeloma

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Abstract

*In vitro* studies indicates therapeutic potential of mTOR inhibitors in treating multiple myeloma. To provide further support for this potential, we used the rapamycin analog, CCI-779, in a myeloma xenograft model. CCI-779, given as 10 intraperitoneal injections, induced significant, dose-dependent, anti-tumor responses against subcutaneous growth of 8226, OPM-2 and U266 cell lines. Effective doses of CCI-779 were associated with modest toxicity, only inducing transient thrombocytopenia and leukopenia. Immunohistochemical studies demonstrated the anti-tumor responses were associated with inhibition of proliferation and angiogenesis, induction of apoptosis and reduction in tumor cell size. Although CCI-779-mediated inhibition of the p70S6 kinase mTOR substrate was equal in 8226 and OPM-2 tumor nodules, OPM-2 tumor growth was considerably more sensitive to inhibition of proliferation, angiogenesis and induction of apoptosis. Furthermore, the OPM-2 tumors from treated mice were more likely to show a down-regulated expression of cyclin D1 and c-myc and upregulated p27 expression. As prior work suggested that heightened AKT activity in OPM-2 tumors might induce hypersensitivity to mTOR inhibition, we directly tested this by stably transfecting a constitutively active AKT allele into U266 cells. The *in vivo* growth of these later cells was remarkably more sensitive to CCI-779 than growth of control U266 cell.
Introduction

The PI3-K/AKT signaling pathway is important for the survival and growth of multiple myeloma (MM) cells and is an attractive target for anti-tumor therapy\(^1\)-\(^3\). An important downstream target of PI3-K/AKT is the mammalian target of rapamycin (mTOR), which mediates phosphorylation of P70S6 kinase (p70) and 4E-BP1\(^4\), proteins responsible for translation and expression of D-type cyclins and c-myc\(^5\)-\(^6\). By preventing these phosphorylation events, mTOR inhibitors downregulate such expression and induce G1 cell cycle arrest\(^7\). In addition, these drugs upregulate expression of the p27 CDK inhibitor, which may also contribute to G1 arrest\(^8\). The *in vitro* sensitivity of MM cells to the anti-tumor effects to mTOR inhibitors frequently correlates with heightened AKT activity\(^9\)-\(^11\).

Rapamycin is a classical mTOR inhibitor. As poor solubility compromised rapamycin as an intravenous agent, Wyeth-Ayerst developed a more soluble ester analog of rapamycin, CCI-779\(^12\). We have shown *in vitro* anti-MM activity of both rapamycin and CCI-779\(^9\),\(^11\),\(^13\). Exposure to these mTOR inhibitors prevents the proliferation of PTEN- and RAS-mutated myeloma cell lines as well as IL-6-stimulated proliferation of non-mutated myeloma clones. To provide further pre-clinical rationale for development of mTOR inhibitors in patients, we initiated the current study testing the effects of CCI-779 *in vivo* against human multiple myeloma tumor growth in a murine xenograft model. Our results confirm that CCI-779 is effective *in vivo* against myeloma cells and demonstrate an inhibition of proliferation, angiogenesis and induction of tumor cell apoptosis.
Materials and Methods

Myeloma cell lines and transfection: The human 8226, U266 and OPM-2 multiple myeloma cell lines were maintained as previously described\textsuperscript{2,13}. Constitutively activated myristoylated-AKT (mAkt) cDNA expression vector was purchased from Upstate (Chalottesville, VA). Transfection of U266 cells was accomplished by electroporation (230 V for 25 msec) as previously described\textsuperscript{14}. Stable transfections were selected in neomycin (350 mg/ml) and successful transfection was determined by Western blotting with antibodies specific for total and phosphorylated AKT (Ser473).

Reagents: Primary antibodies against total and phosphorylated P70 S6 kinase, STAT3, AKT, c-myc and actin were purchased from Cell Signaling (Beverly, MA). The cyclin D1 antibody and Matrigel were purchased from BD Biosciences (Palo Alto, CA). The cyclin D1 antibody was specific for D1 and did not cross-react with D2 or D3. The Ki-67 antibody was purchased from DAKO, (Carpenteria, CA). The rat anti-mouse CD34 antibody was purchased from Caltag Laboratories (Burlingame, CA). The CCI-779 was provided by Wyeth-Ayerst (Pearl River, NY).

Animals: Four-six week old male NOD/SCID mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were maintained 4/cage in pathogen-free conditions. All animal studies were conducted in accordance with protocols approved by the Animal Research Committee of the West Los Angeles Veterans Administration Medical Center.

Xenograft Model: We used the murine myeloma xenograft model of Leblanc et al\textsuperscript{15} with minor modifications. The cell lines were mixed with matrigel as previously described\textsuperscript{15} and were then injected S.C. into the left flank (200 µl/mouse containing 7.5 x 10\textsuperscript{6} 8226 cells, 3 x 10\textsuperscript{7} U266 cells or 3 x 10\textsuperscript{7} OPM-2 cells) of the mice. Tumor growth was monitored daily, and mice...
were randomized into drug treated or control groups (8-14 mice/group) when the tumor volume reached approximately 200-400 mm$^3$. The greatest length and width of the tumor was measured and the volume was calculated as $4\pi/3 \times (\text{tumor width}/2)^2 \times (\text{tumor length}/2)^2$. CCI-779 was prepared as previously described. Briefly, a 50 mg/ml stock solution was prepared in 100% EtOH. On the day of injections, the drug was diluted in 5% Tween-80, 5% polyethylene glycol-400 (Sigma, St. Louis MO) to the appropriate final concentration (final concentration of EtOH is 0.4%). The CCI-779 drug solution (200 µl) was administered IP daily X 5, followed by 2 days of no drug and then 5 additional daily injections (total of 10 injections). Mice were routinely euthanized when tumors reached >2000 mm$^3$ in volume.

**Immunohistochemistry:** At day +6 (OPM-2 and 8226) or day +13 (8226 only) some additional mice were euthanized with CO$_2$ and the tumor mass was excised. The tumor was bisected using a razor blade: one half of the tumor was immediately placed in 10% buffered formaldehyde overnight, and the other half was frozen for protein extraction. Formaldehyde fixed tumors were embedded in paraffin and cut into 5 µm-thick serial sections using standard histological procedures. Immunohistochemical staining with anti-Ki-67 or anti-CD34 primary antibody was conducted using standardized automated methods. Detection of expression used the DAKO Envision System, which uses a peroxidase-labeled synthetic polymer conjugated to goat anti-rabbit or goat anti-rat antibody with diaminobenzidine as the substrate to give a brown color. Sections were counterstained with hematoxylin/eosin. The proliferation index and was determined by assaying the area of Ki-67 staining using the MetaMorph software (see below) from 10 randomly selected fields at 20X magnification. Angiogenesis was determined by assaying the area of CD34 staining microvessels using the MetaMorph software from 10 randomly selected fields at 20X magnification.
Apoptosis was measured using the ApopNexin™ Biotin Apoptosis Detection Kit (Intergen). Briefly, sections were de-paraffinized, rehydrated, and microwaved with 30 mM sodium citrate. DNA strand breaks were labeled by conjugation with 30 µM digoxigenin-11-dUTP to the 3’-OH sites, catalyzed by 0.3 units/µl terminal deoxynucleotidyl transferase (TdT) for 60 minutes at 37°C. Samples in which TdT was omitted from the reaction were used as negative controls, and samples treated with DNase1 were used as positive controls for DNA strand breaks. The sections were counterstained with hematoxylin. The apoptotic index (AI) was determined by counting the total number of positive nuclei in ten randomly selected fields at 20X.

**Morphometric Analysis:** Morphometric analysis of cell size was performed on tissue sections with a Nikon Microphot-SA microscope (Melville, NY) equipped with plan-apochromat lenses (20X and 40X). A Diagnostic Technologies digital camera, model SPOT-RT was used to capture images with a resolution of 1520 X 1080 pixels. MetaMorph software (version 6.1) (Universal Imaging Corporation, West Chester, PA) was used to perform cellular morphometric analysis of Ki-67 stained nuclei from 4 high field images (40X). Final images for publication were prepared using Adobe Photoshop software (version 7.0).

**Western Blot Analysis:** Frozen tumors were minced with a razor blade then processed in a glass tissue homogenizer on ice. Protein was extracted in ice-cold lysis buffer [1% Triton X-100, 0.5% NP40, 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.2 mM Na3VO4, 0.2 mM NaF, and 0.2 mM phenylmethylsulfonyl fluoride]. Twenty-five micrograms of protein from each sample were boiled for 10 min in 1x SDS gel loading buffer (BioRad). Proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were blocked for 1 h in 5% nonfat dried milk, 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1%
Tween-20. The membranes were washed three times and then incubated with primary specific antibodies for 1 hr. After three more washes, membranes were incubated with 1 μg/ml horseradish peroxidase-labeled anti-mouse IgG and protein bands were detected with an enhanced chemiluminescence system.

**Statistics:** For assessment of tumor growth curves, tumor volumes were calculated as mean+/−SEM. Repeated measures analysis of variance was used to determine the statistical significance of differences between the growth of tumors by comparing tumor volumes at the start and end of treatment in the experimental groups. For other analyses, the Student t-test was used to determine significance.
Results

CCI-779 is minimally toxic to NOD/SCID mice. To assess the potential in vivo anti-myeloma effects of CCI-779, we slightly modified the murine xenograft model of Leblanc et al\textsuperscript{15}. Instead of using beige-nude-xid (BNX) mice, we used NOD/SCID mice, as our preliminary experiments showed more consistent subcutaneous myeloma tumor growth in this strain. To first test the toxicity of CCI-779 in these tumor-free mice, we injected increasing doses of the drug IP (versus vehicle control) for 5 consecutive days, rested the mice for 2 days, and then injected daily for another 5 consecutive days. The drug-treated mice (N=4/group) demonstrated some minimal weight loss in a short window of time midway through the second 5-day treatment course, although this was not statistically significant (Figure 1A). No other visible drug-induced side effects were noted. In particular, none of the mice developed dermatitis, which is a frequently reported side effect of CCI-779\textsuperscript{17}.

Examination of blood counts identified a significant (P <0.05) thrombocytopenia and leukopenia developing after the first 5 days of CCI-779 administration (Figure 1B, top panel). These cytopenias were dose-dependent and platelet counts were more sensitive to the inhibitory effect than WBC counts. Interestingly, by day +12 (Figure 1B, lower panel), following 5 additional IP injections, the platelet and WBC counts had recovered and, in some cases, overshot the baseline values. By day +18, the WBC counts had returned to normal, while the platelet counts remained slightly elevated (not shown). Mice were observed for an additional 2 weeks after cessation of CCI-779 therapy and there were no additional untoward side effects noted.

CCI-779 significantly inhibits myeloma cell growth in vivo. NOD/SCID mice were first challenged with 8226 myeloma cells (7.5 X 10\textsuperscript{6} cells/mouse) by SC injection. When the tumors were approximately 200 mm\textsuperscript{3}, the mice were randomized to receive IP injections of vehicle or
CCI-779 at 0.4 mg/kg, 4.0 mg/kg, and 20 mg/kg. Since a dose of 40 mg/kg CCI-779 produced the most toxicity in terms of inhibited WBC and platelet counts (Figure 1B), we utilized 20 mg/kg as the highest dose tested in our tumor challenged mice. We used the same treatment regimen that had tested toxicity of 5 consecutive daily injections, followed by 2 days of rest, and then 5 additional daily injections.

Figure 2 demonstrates a significant (p<0.05) anti-tumor effect of 20 mg/kg CCI-779 on 8226 tumor growth that was evident by 8-9 days after starting treatment (Figure 2, top panel). Progressive tumor growth was prevented with this dose although an actual decrease in tumor size was not observed. In contrast, 4 mg/kg and 0.4 mg/kg (the latter not shown in Figure 2) had no effect. Tumor growth following treatment with 20 mg/kg was suppressed for approximately 15 days. Subsequently, however, progressive tumor growth was renewed.

In previous studies, the presence of PTEN mutations correlated with a heightened sensitivity to mTOR inhibitors. Our previous publication suggested that was also true with myeloma cells. Therefore, we also tested the ability of CCI-779 to inhibit tumor growth in mice after challenge with the PTEN-mutated OPM-2 cell line. Using the same treatment protocol, OPM-2 growth was much more sensitive to CCI-779 than 8226 growth had been. As shown in Figure 2 (bottom panel), the highest dose of CCI-779 (20 mg/kg), induced a significant (p<0.05) reduction in tumor volume and tumors completely disappeared by day +12 of therapy. However, approximately 3-4 weeks after treatment, 75% of the tumors reappeared while 25% percent of regressed tumors never regrew (observations out to 60 days). The next lowest dose of CCI-779 (4 mg/kg) induced a significant anti-tumor effect that was less effective. The duration of this anti-tumor response lasted only approximately 10 days following termination of CCI-779 injections. In contrast, 0.4 mg/kg of CCI-779 had only a modest effect. At day +12 of treatment,
a calculated ED$_{50}$ for CCI-779 in preventing OPM-2 tumor growth was 2 mg/kg while the ED$_{50}$ against 8226 was 20 mg/kg.

**Effects of CCI-779 in vivo on proliferation, angiogenesis, apoptosis and cell size in multiple myeloma cells.** Inhibition of mTOR function induces G1 arrest$^7$. However, mTOR can also regulate cell size$^{18}$ with mTOR inhibition resulting in decreased cell volume. In addition, under some conditions$^{19}$, mTOR inhibitors can induce apoptosis. CCI-779-induced effect on either or all of these parameters could participate in the observed anti-tumor response. To investigate this issue, we performed histological studies on excised tumors.

Immunohistochemistry (IHC) for Ki-67 expression, used as an assessment of proliferation, demonstrated that CCI-779 induced a significant inhibition of cell cycle transit. Figure 3A is a quantitative assessment of area stained with Ki-67 in tumors harvested after 6 days of treatment with 20 mg/kg CCI-779 in OPM-2-challenged mice and both 6 and 13 days in 8226-challenged mice. As shown, a significant decrease in Ki-67 staining in 8226 tumors was seen at day +6 (approximately 25% reduction, p<0.05). At day +13, the decrease in Ki-67 staining was even greater, approximating 50% of control. The effect on Ki-67 staining in OPM-2 tumors by treatment with 20 mg/kg CCI-779 was even more impressive, with a decrease of approximately 40% (P<0.001) after 6 days of treatment (Figure 3A). The disappearance of OPM-2 tumors by day +13 after treatment precluded us from obtaining data on Ki-67 staining at that time point.

To assess induction of apoptosis, we used the *in situ* TUNEL assay. As shown in Figure 3B, there was a small but significant increase in apoptotic nuclei observed in 8226 tumors treated with 20 mg/kg CCI-779 at day +6 and day +13 compared to vehicle treated control tumors (T-test, P<0.05). The induction of apoptosis in OPM-2 tumors was considerably more impressive.
As shown, a marked apoptotic response was seen as early as day +6. These findings strongly suggest that in addition to a cytostatic effect, induction of apoptosis plays a role in CCI-779-dependent myeloma regression, especially in OPM-2 tumors. Figure 4 shows representative sections of TUNEL stained tumors demonstrating the effects of CCI-779 on 8226 and OPM-2 tumors.

We also tested if CCI-779 (20 mg/kg) inhibited angiogenesis in excised 8226 and OPM-2 tumors. Immunohistochemical analysis of microvessel density, as measured by in situ staining of mouse endothelial cells with CD34, revealed a significant (T-test, P<0.05) inhibition of angiogenesis by 20 mg/kg CCI-779 in both OPM-2 (a decrease of 66% at day +6) and 8226 cells (decrease of 39% at day +6 and 47% at +13) compared to control treated tumors (Figure 5A). The decrease in CD34 positive microvessel density strongly correlated with decreases in cellular proliferation and increased apoptosis of CCI-779 treated OPM-2 and 8226 cells. Figure 5B shows representative sections of CD34+ stained tumors demonstrating the effects of CCI-779 on angiogenesis in OPM-2 and 8226 tumors.

Morphometric analysis was used to assay a possible CCI-779-induced decrease in individual cell size. In 8226 tumors, there was a small but significant (P<0.05) reduction in cell size by day +6 (87.39 µm +/- 2.46 vs. 77.06 µm +/- 6.6) in the CCI-779 treated tumors compared to controls (T-test, P<0.05). However, at day +13, there was no significant difference (T-test, P>0.05). In contrast, by day +6 we observed a marked decrease in cell size in OPM-2 tumors (301 µm +/-147 vs. 60.12 µm +/- 15) (T-test, P<0.001). However, as apoptosis is associated with decreased cell size, it is unclear if this was a direct effect of mTOR inhibition or a secondary effect due to apoptosis in OPM-2 cells.
CCI-779 inhibits the P70S6 kinase cellular signaling pathway. CCI-779 specifically targets the ability of mTOR to phosphorylate downstream target molecules, in particular, P70S6 kinase. Therefore we hypothesized that CCI-779 would specifically inhibit phosphorylation of P70S6 kinase in multiple myeloma tumor cells. Western blot analysis of excised 8226 and OPM-2 tumors clearly demonstrated that CCI-779 (20 mg/kg) inhibited the phosphorylation of P70S6 kinase at threonine 389, an mTOR specific phosphorylation site in both 8226 and OPM-2 tumors (Figure 6A). In contrast, CCI-779 had little or no effect on STAT3 phosphorylation in OPM-2 cells and only a slight inhibitory effect in 8226 cells, suggesting some in vivo molecular specificity.

CCI-779-induced effects on c-myc, cyclin D1 and p27 expression. By inhibiting mTOR, both rapamycin and CCI-779 can prevent translation and expression of both D-type cyclins and c-myc5,6. These effects may account for the induction of G1 arrest and the ability to downregulate expression of these proteins can determine relative sensitivity to these drugs. In addition, mTOR inhibitors may also upregulate the expression of the p27 CDK inhibitor, which may contribute to G1 arrest8. To evaluate these effects in the current study, immunoblot assays on harvested tumor was performed for cyclin D1, c-myc and p27 protein expression (Figure 6B). In the very sensitive OPM-2 tumors, CCI-779 had a modest effect on c-myc expression only decreasing it by 50% (by densitometry) at the highest dose (20 mg/kg). Expression of cyclin D1 was much more susceptible to inhibition with remarkable decreases even at the lowest CCI-779 dose (0.4 mg/kg). Upregulated expression of the p27 CDK inhibitor was also present at the low 0.4 and 4 mg/kg doses. In contrast, CCI-779 effects on the more resistant 8226 tumors were less impressive. The drug actually increased c-myc expression in a dose-dependent fashion and only decreased cyclin D1 and upregulated p27 expression at the highest dose (20 mg/kg). Thus, the differential effects
on myc/cyclin/p27 expression mirror the relative sensitivities of these two tumors to CCI-779-induced cytoreduction.

The antibody we used to assay cyclin D1 was specific for D1 without cross reactivity to D2 or D3. This specificity was confirmed by preliminary experiments with recombinant D1 or D2 protein. When immunoblot assays were also performed using an antibody specific for cyclin D2 (specificity also confirmed with recombinant proteins), although both 8226 and OPM-2 cells robustly expressed cyclin D2 when grown in vitro, we could not detect D2 expression in 8226 or OPM-2 tumors harvested from control untreated mice.

**AKT activity regulates the in vivo sensitivity of multiple myeloma tumors to CCI-779.**

In previous experiments, the presence of PTEN mutations correlated with a heightened sensitivity to CCI-779 both in vivo and in vitro\(^{10,11,14}\). Furthermore, the above results describe a much greater effect in vivo against OPM-2 multiple myeloma cells, that contain heightened AKT activity, compared to 8226 cells, which contain quiescent AKT. To more directly test a regulatory role of AKT in sensitivity to CCI-779 in vivo, we stably transfected U266 multiple myeloma cells with a constitutively activated myristoylated-AKT construct. Western blot analysis of transfected U266 cells clearly demonstrated upregulation of phosphorylated AKT in the myr-AKT but not control cells (Figure 7A). Using the same in vivo treatment regimen previously described, growth of U266 cells overexpressing activated AKT, were much more sensitive to CCI-779 than U266 control cells. As shown in Figure 7B, CCI-779 (20 mg/kg), induced significant (p<0.05) anti-tumor effect in the myr-AKT transfected U266 cells by day +8 (tumor volume reduction of 60%) and this effect was even more pronounced by day +13 (reduction of 73%). In contrast, CCI-779 (20 mg/kg) had little or no effect on the growth of control U266 cells on day +8 (tumor volume reduction of 20%) or day +13 (reduction of 30%).
Discussion

This study demonstrates an in vivo anti-tumor effect induced by the mTOR inhibitor, CCI-779 against human multiple myeloma cells. Effective doses were minimally toxic with very transient decreases seen in platelet and, less intensely, in WBC counts. In phase I studies, thrombocytopenia was also more frequent and more severe than leukopenia\textsuperscript{12}. Interestingly, cytopenias resolved spontaneously during continued therapy between days 6 and 12 of treatment. This phenomenon is reminiscent of the experience with the mTOR inhibitor rapamycin use in renal transplant recipients where thrombocytopenia and leukopenia occur during the first 4 weeks of treatment but then resolve spontaneously\textsuperscript{19}. The doses of CCI-779 used in our mouse model were comparable to doses used in phase I-II studies (25-250 mg as a single dose), which were effective in blocking p70S6K activity downstream of mTOR in peripheral blood lymphocytes used as surrogates for tumor tissue\textsuperscript{20}. Efficacy in the myeloma xenograft model was associated with an induction of G1 arrest and apoptosis in tumor cells. These results further support the potential of mTOR inhibitors in the treatment of multiple myeloma, which has been previously suggested by our \textit{in vitro} studies\textsuperscript{9,11,13}.

The differential \textit{in vivo} sensitivities of the two tumor lines (8226 and OPM-2) we studied reflected their different sensitivities to \textit{in vitro} treatment. The 8226 cell line had an ED\textsubscript{50} of 3.0 nM for \textit{in vitro} induction of G1 arrest and the OPM-2 line has an ED\textsubscript{50} of 0.2 nM\textsuperscript{11}. The increased sensitivity of the OPM-2 cell line may be related to its PTEN-mutated state and resulting heightened AKT activity. In several other tumor models, a heightened AKT activity correlates with hypersensitivity to CCI-779 \textit{in vitro} and \textit{in vivo}\textsuperscript{14}. In support of this hypothesis, U266 cells transfected with constitutively activated myr-AKT were significantly more sensitive to the anti-tumor effects of CCI-779 than control U266 cells in our xenograft model (Figure 7B).
These findings are reminiscent of other studies in which altered AKT activity in prostate and glioblastoma cells regulates sensitivity to CCI-779 in vitro and in vivo\textsuperscript{10,14}. Although the incidence of PTEN mutation or PTEN silencing in myeloma is unknown, other molecular abnormalities may contribute to a heightened basal level of AKT activity and sensitivity to CCI-779. For example, myeloma cells expressing activating N-RAS or K-RAS mutations contain hyperactivated AKT downstream of RAS signaling and are hypersensitive to mTOR inhibitors in vitro\textsuperscript{9}. Furthermore, these mutations are relatively common in myeloma patients, occurring in up to 50\% in some studies\textsuperscript{21}. Thus, if future clinical studies are performed with CCI-779 in myeloma patients, correlations of outcome should be attempted with presence or absence of PTEN or RAS mutations and, if possible, with multiple myeloma cell AKT status. Whether or not resistant cells alter their AKT or mTOR status as an adaptation to treatment with mTOR inhibitors is also testable and future studies will be designed to ask these questions of the 75\% of OPM-2 tumors that relapse after therapy.

Of the parameters that could determine tumor size, significant CCI-779-induced alterations were demonstrated in proliferative capacity, apoptosis and angiogenesis. CCI-779 also decreased cell size to a modest extent that may have also participated in an anti-tumor response but there were clear instances where responses were seen in the absence of decreases in cell volume (i.e., at day +13 in 8226 tumors). The decrease in proliferation, assessed by Ki-67 staining, was not surprising. The mTOR inhibitor rapamycin basically induces a G1 arrest\textsuperscript{7} and prior studies by us with 8226 and OPM-2 cell lines have demonstrated an CCI-779-induced decrease G1-S phase transition following in vitro treatment\textsuperscript{11}. The G1 arrest is presumably due to a diminished D-cyclin and c-myc expression and an increased p27 CDK inhibitor expression. CCI-779-induced effects on cyclin D1, c-myc and p27 expression in 8226- versus OPM-2-
challenged mice roughly correlated with the observed sensitivity to tumor cytoreduction. In the markedly sensitive OPM-2 tumors, a strong inhibition of cyclin D1 expression and increase in p27 expression was seen even at the lowest dose of CCI-779 and modest inhibition of c-myc expression was present at the 20 mg/kg dose. In the much less sensitive 8226 tumors, cyclin D1 expression was decreased and p27 expression was increased only at the highest CCI-779 dose and c-myc expression actually increased.

In prior studies on in vitro-grown 8226 and OPM-2 cell lines, cyclin D1 expression was very modest. In contrast, cyclin D2 expression was quite significant, probably due to over-expression of the c-maf transcription factor. Using antibodies specific for cyclin D1 or D2, our studies on in vitro-grown 8226 and OPM-2 cells are similar (not shown) with significantly greater cyclin D2 expression. However, when these same antibodies are used in immunoblot assays on tumor nodules removed from untreated mice, cyclin D2 expression could not be detected. The reasons for this discrepancy are unknown. However, with little cyclin D2 expression in vivo, D1 expression may have become enhanced and primarily used as the driving force for G1-S transition, thus explaining a possible key role of CCI-779-induced D1 inhibition in the observed anti-tumor response.

In contrast to the Ki-67 results, the induction of apoptosis, was somewhat surprising, because exposure of either 8226 or OPM-2 cells to rapamycin or CCI-779 in vitro does not result in apoptosis. However, as with the Ki-67 results, the in vivo induction of apoptosis was greater in the hypersensitive OPM-2 tumors compared to 8226 tumors. One obvious difference between in vivo and in vitro conditions is the requirement for angiogenesis to maintain tumor cell viability in the former situation. Immunohistochemistry analysis demonstrated a CCI-779-dependent inhibition of angiogenesis in tumor beds in both OPM-2 and 8226 cells, possibly
inducing a hypoxic apoptotic response in treated tumors. As with inhibition of proliferation, angiogenesis in OPM-2 tumors were more sensitive to CCI-779 treatment than 8226 cells. Recent experiments have shown that the AKT/mTOR pathway regulate the expression of vascular endothelial growth factor (VEGF)\textsuperscript{26} and hypoxia inducible factor 1α (HIF-1α)\textsuperscript{27}. Therefore, it is possible that myeloma cells with heightened AKT activity are more sensitive to the CCI-779-mediated inhibition of these critical angiogenic factors. Alternatively, other factors occurring \textit{in vivo} but not \textit{in vitro} could interact with CCI-779 to result in tumor cell apoptosis. Whether this differential effect was also due to differences in AKT activity will be tested in future studies.

In summary, our results provide further support for the potential of CCI-779 in treatment of myeloma patients. The drug demonstrated a dose-dependent anti-tumor effect in a myeloma xenograft model and was relatively non-toxic at effective doses. In phase II studies of myeloma patients, it will be interesting to see if efficacy correlates with AKT status and induced cyclin/myc/p27 alterations as it appears to do in these pre-clinical studies.

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FIGURE LEGENDS

Figure 1: Minimal toxicity of CCI-779 in NOD/SCID mice. Mice (4/group) were injected IP with vehicle alone or varying doses of CCI-779 daily x 10 as described in the text. Weight shown in figure 1A as grams, mean+/-SD, n=4 (solid bars on x axis denote days of treatment). Platelet and WBC counts enumerated at day 6 (Figure 1B, upper panel) and day 12 (lower panel) of treatment and shown as percent of control (vehicle alone), mean+/-SD, n=4. Asterisks denote values significantly different (p<0.05) from control.

Figure 2: Anti-tumor effect of CCI-779. Mice (8-14/group) challenged subcutaneously with 8226 (upper panel) or OPM-2 (lower panel) cells. When tumor size reached 200 mm³, mice were randomly assigned to receive vehicle alone or varying doses of CCI-779 IP for 10 days as described in the text. Results are tumor size, mean+/-SEM. Solid bars on x axis denote days of IP treatment.

Figure 3A: Ki-67 staining of tumors: OPM-2- or 8226-challenged mice were treated with vehicle alone or 20 mg/kg CCI-779, tumors were harvested at days +6 and +13 (8226 mice) or day +6 (OPM-2 mice) and sections stained for Ki-67. Results represent area of microscopic field (20x magnification) stained positively assessed as described in Materials & Methods. Data are mean+/-SD, n=10 fields, for each group. Asterisks denote significant differences (p<0.05) between control and CCI-779-treated mice.

Figure 3B: TUNEL staining of tumors: Mice challenged and treated similar to that in Figure 3A. Tumor sections stained by TUNEL kit to identify apoptosis. Results are number of TUNEL-stained apoptotic nuclei/microscopic field (20x magnification), mean+/-SD, n=10 fields for each group. Asterisks denote significant differences (p<0.05) between control and CCI-779-treated mice.
Figure 4: CCI-779 induces myeloma cell apoptosis. Representative slides of TUNEL-stained sections for 8226- and OPM-2-challenged mice treated with vehicle or 20 mg/kg of CCI-779. Magnification=40x. Arrows show several TUNEL-positive apoptotic nuclei.

Figure 5A: Anti-angiogenic effects of CCI-779. Mice challenged and treated as in Figure 3 and the angiogenesis assayed as described in Materials & Methods. Results represent CD34+ area of microscopic field (20x magnification) stained positively assessed as described in Materials & Methods. Data are mean+/−SD, n=10 fields, for each group. Asterisks denote significant differences (p<0.05) between control and CCI-779-treated mice.

Figure 5B: Representative slides of CD34-stained sections for 8226- and OPM-2-challenged mice treated with vehicle or 20 mg/kg of CCI-779 at day +8. Magnification=40x. Arrows show several microvessels.

Figure 6A: CCI-779 inhibits myeloma cell p70S6kinase phosphorylation in vivo. After 5 days of vehicle alone or CCI-779 treatment (20 mg/kg) in 8226- or OPM-2-challenged mice, tumor nodules were harvested and extracted protein immunoblotted for expression of total p70S6kinase (p70), phosphorylated p70, total STAT-3 or phosphorylated STAT-3. This experiment was repeated two additional times with identical results.

Figure 6B: Effects of CCI-779 on expression of cyclin D1 and c-myc. OPM-2 or 8226-challenged mice were treated with vehicle alone or varying doses of CCI-779 (shown above blots as mg/kg) for 5 days, after which tumor nodules were harvested and extracted protein immunoblotted for expression of c-myc, cyclin D1 and actin. This experiment was repeated once with identical results.

Figure 7A: AKT activity in myristoylated-AKT transfected U266 cells. Expression of total and phosphorylated AKT (ser473) was determined in U266 cells transfected with myristoylated-
AKT (myr-AKT) or empty vector (EV) control cells as described in the Materials and Methods section.

**Figure 7B: AKT activity regulates tumor sensitivity to CCI-779.** Mice (8/group) challenged subcutaneously with 3 X10^7 myr-AKT transfected U266 or empty-vector transfected U266 cells as described in Figure 2. When tumor size reached 200 mm^3, mice were randomly assigned to receive vehicle alone or 20 mg/kg CCI-779 IP for 10 days as described in the text. Results indicate the mean volume (mm^3) +/- SEM of CCI-779 treated tumors compared to vehicle control tumors assayed on day +8 and +13 of treatment.
Figure 1.A

![Graph showing weight (grams) over days for different doses of a substance. The x-axis represents days from 0 to 21, and the y-axis represents weight from 20 to 35 grams. The graph includes lines for control, 0.4 mg/kg, 4 mg/kg, 20 mg/kg, and 40 mg/kg, with error bars indicating variability.]
Figure 1B.
Figure 2.
3A. % Ki-67 Positive Area

3B. Apoptotic Index
4.

Control

Day 6

Day 13

OPM-2

Day 6

CCI-779

A

B

C

D

E

F

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5A.

![Graph showing microvesel density (as % of total area) for different groups: 8226 Day6 Control, 8226 Day6 CCI, 8226 Day13 Control, 8226 Day13 CCI, OPM-2 Day6 Control, OPM-2 Day6 CCI. The treatments are labeled with asterisks indicating statistical significance.]

5B.

![Images showing microvesel density under different conditions: Control, CCI-778 (20 mg/kg). Arrows indicate areas of interest.]

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6A.

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In vivo anti-tumor effects of the mTOR inhibitor, CCI-779, against human multiple myeloma cells in a xenograft model

Patrick Frost, Farhad Moatomed, Bao Hoang, Yijiang Shi, Joseph Gera, Huajun Yan, Philip Frost, Jay Gibbons and Alan Lichtenstein