Combination Mammalian Target of Rapamycin Inhibitor Rapamycin and HSP90 Inhibitor 17-Allylamino-17-Demethoxygeldanamycin Has Synergistic Activity in Multiple Myeloma

Lanie K. Francis,1 Yazan Alsayed,1 Xavier Leleu,2 Xiaoying Jia,2 Ujjal K. Singh,1 Judith Anderson,1 Michael Timm,3 Hai Ngo,2 Ganwei Lu,1 Alissa Huston,1 Lori A. Ehrlich,1 Elizabeth Dimmock,2 Suzanne Lentzsch,1 Teru Hideshima,2 G. David Roodman,1 Kenneth C. Anderson,2 and Irene M. Ghobrial2

Abstract

Purpose: The phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (mTOR) pathway and the heat shock protein family are up-regulated in multiple myeloma and are both regulators of the cyclin D/retinoblastoma pathway, a critical pathway in multiple myeloma. Inhibitors of mTOR and HSP90 protein have shown in vitro and in vivo single-agent activity in multiple myeloma. Our objective was to determine the effects of the mTOR inhibitor rapamycin and the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) on multiple myeloma cells.

Experimental Design: Multiple myeloma cell lines were incubated with rapamycin (0.1-100 nmol/L) and 17-AAG (100-600 nmol/L) alone and in combination.

Results: In this study, we showed that the combination of rapamycin and 17-AAG synergistically inhibited proliferation, induced apoptosis and cell cycle arrest, induced cleavage of poly(ADP-ribose) polymerase and caspase-8/caspase-9, and dysregulated signaling in the phosphatidylinositol 3-kinase/AKT/mTOR and cyclin D1/retinoblastoma pathways. In addition, we showed that both 17-AAG and rapamycin inhibited angiogenesis and osteoclast formation, indicating that these agents target not only multiple myeloma cells but also the bone marrow microenvironment.

Conclusions: These studies provide the basis for potential clinical evaluation of this combination for multiple myeloma patients.

Advances in the understanding of the molecular pathogenesis of multiple myeloma have revolutionized its therapy, although much remains to be elucidated (1, 2). Targeted agents, such as thalidomide and proteasome inhibitors, provide exciting therapeutic options (2, 3). However, only about 30% to 35% of patients respond to these therapies as single agents (4, 5). Therefore, there is a strong rationale for the combination of novel therapies that target signaling pathways that are activated in multiple myeloma. Almost all multiple myeloma tumors dysregulate at least one of the cyclin D genes (2, 6, 7). Gene expression analysis in multiple myeloma has shown that the expression level of cyclin D1, D2, or D3 mRNA is distinctly higher in multiple myeloma cells than in normal plasma cells (2, 6). The critical role of cyclin D dysregulation suggests that there is a therapeutic niche for targeting these molecules through their various regulators. The phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway is a key regulator of apoptosis, cell cycle regulation, and tumor proliferation in multiple myeloma (10, 12). AKT induces the accumulation of cellular cyclin D1 by preventing the degradation of cyclin D1 by the proteasome (13, 14). The mTOR is a downstream component of the PI3K/AKT pathway that forms a molecular complex with other binding proteins leading to the phosphorylation of p70S6K and 4EBP-1 (15). The latter leads to activation of cyclin D1/cyclin-dependent kinase (CDK) 4 (Fig. 1; ref. 14). Rapamycin, a mTOR inhibitor, blocks the tumor cell cycle at the G1 checkpoint (16). RAD001 and CCI-779 are rapamycin analogues and have shown in vitro and in vivo activity against multiple myeloma (17, 18).

Heat shock proteins are named for their increased synthesis after heat shock (11, 19). HSP90 is a chaperone protein that...
plays an important role in the refolding of proteins exposed to environmental stress and the stabilization and survival of certain signaling proteins (19). HSP90 client proteins are kinases or transcription factors involved in signal transduction and include AKT, p53, Bcr-Abl, Raf-1, and ErbB2 (20). Inhibition of HSP90 induces cell cycle arrest (21). This arrest is accompanied by dephosphorylation of Rb and rapid down-regulation of cyclin D- and cyclin E-associated kinase activities with loss of expression of cyclins D1, D3, and E, as well as the associated CDKs, CDK4 and CDK6 (Fig. 1A; ref. 21). 17-Allylamino-17-demethoxygeldanamycin (17-AAG; Kosan Biosciences, Hayward, CA) binds to the ATP-binding pocket of HSP90 and prevents the interaction between the chaperone and its target. 17-AAG has shown cytotoxic activity against multiple myeloma cells, and administration of 17-AAG in a severe combined immunodeficient/nonobese diabetic mouse model of multiple myeloma shows in vivo antitumor activity (11).

Phase I clinical trials using 17-AAG and other HSP90 inhibitors in patients with relapsed/refractory multiple myeloma were recently reported and showed manageable toxicity (11, 22).

The interactions between multiple myeloma cells and the bone marrow microenvironment regulate the growth and survival of multiple myeloma cells and play a critical role in angiogenesis and multiple myeloma bone disease (23, 24). Increased angiogenesis is a striking characteristic of multiple myeloma and has prognostic value in these patients (24). Bone destruction is a hallmark of multiple myeloma, with 70% to 80% of patients showing bone involvement (23). Previous studies have shown that mTOR and HSP90 inhibitors exert antiangiogenic effects and that mTOR signaling is critical for osteoclast survival (25–27).

Given that both 17-AAG and rapamycin induce cell cycle arrest and affect cyclin D-dependent proteins, we hypothesized that the combination of rapamycin and 17-AAG would lead to...
synergistic inhibition of multiple myeloma growth and survival. In this study, we show that the combination of rapamycin and 17-AAG synergistically inhibits proliferation, induces apoptosis and cell cycle arrest, and dysregulates cyclin D1/Rb-dependent signaling. In addition, we show that both 17-AAG and rapamycin inhibit angiogenesis and osteoclast formation. These studies provide the basis for clinical trials to determine the in vivo effect of these novel agents not only on multiple myeloma cells but also on the bone marrow microenvironment.

**Materials and Methods**

**Cell lines and primary cells.** Dexamethasone-sensitive human multiple myeloma cell line MM.1S was kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). KSAS/I human multiple myeloma cell line was kindly provided by Dr. Diane Jelinek (Mayo Clinic, Rochester, MN). U266 human multiple myeloma cell line was purchased from the American Type Culture Collection (Manassas, VA), and the OPM2 cell line was kindly provided by Dr. Alan Lichtenstein (University of California, Los Angeles, CA). All multiple myeloma cell lines were cultured in RPMI 1640 (Sigma Chemical, St. Louis, MO) containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Informed consent was obtained from all patients in accordance with the Declaration of Helsinki protocol. Approval of these studies was obtained by the University of Pittsburgh and Dana-Farber Cancer Institute Institutional Review Boards. Bone marrow aspirates were subjected to Ficoll-Paque gradient centrifugation (Amerham, Piscataway, NJ), and mononuclear cells were separated. Mononuclear cells were suspended in RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

**Bone marrow specimens** were obtained from patients with multiple myeloma. Mononuclear cells separated by Ficoll-Paque density sedimentation were used to establish long-term bone marrow cultures, as in prior studies (28). When an adherent cell monolayer had sedimented, the residual bindingsites on the filters were blocked by incubating with 5% non-fat dried milk in wash buffer (0.5% Triton X-100 or NP40, 500 mM NaCl, 2% FBS, 10 mM sodium orthovanadate, 1 mg/mL sodium pyrophosphate, 1 mg/mL sodium fluoride, 1 mg/mL EDTA, 50 mM HEPES, 1.5 mg/mL magnesium chloride, 10% glycerol, 1% phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin). Lysates were analyzed by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA), and the residual binding sites on the filters were blocked by incubating with TBST (10 mM NaCl, 0.5% Tween 20) and 5% milk for 1 to 3 hours at room temperature or overnight at 4°C.

The filters were subsequently incubated with phosphorylated AKT (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphorylated ribosomal S6 (pS6), cyclin D1, phosphorylated Rb, HSP27, HSP70, caspase-8, caspase-9, and poly(ADP-ribose) polymerase (PARP) antibodies (Cell Signaling, Beverly, MA) and developed using an enhanced chemiluminescence kit following the manufacturer’s instructions (Pierce, Rockford, IL). Blots were stripped and reprobed with anti-actin antibody (Sigma Chemical) or anti-tubulin (Cell Signaling) to ensure equivalent protein loading. Different time points were chosen to determine the effect of the agents on phosphorylated proteins (2 hours up to 16 hours) and total proteins (16-48 hours).

**AKT kinase assay.** AKT kinase assay kit (Cell Signaling) was used as described previously (28). Cells were cultured in the presence or absence of rapamycin or 17-AAG or the combination for 48 hours at 37°C. The cells were subsequently lysed in 1× lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM B-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/mL leupeptin]. Lysates were immunoprecipitated with immobilized AKT primary antibody (Santa Cruz Biotechnology) and incubated with gentle rocking overnight at 4°C. Cell lysate/immobilized antibodies were microcentrifuged at 14,000 x g for 30 seconds at 4°C. Pellets were washed twice with 1× cell lysis buffer and twice with 1× kinase buffer [25 mM Tris (pH 7.5), 5 mM MgCl2, 2 mM B-glycerophosphate, 2 mM NaCl].
Table 1. CI of the combination of rapamycin and 17-AAG, indicating very strong synergism according to the Calcusyn software

<table>
<thead>
<tr>
<th>Rapamycin (nmol/L)</th>
<th>17-AAG (nmol/L)</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>600</td>
<td>0.056</td>
</tr>
<tr>
<td>30</td>
<td>600</td>
<td>0.02</td>
</tr>
<tr>
<td>100</td>
<td>600</td>
<td>0.046</td>
</tr>
</tbody>
</table>

NOTE: CI < 0.1 indicates very strong synergism; CI = 0.1 to 0.3 indicates strong synergism; and CI = 0.3 to 0.85 indicates synergism.

DTT, 0.1 mmol/L sodium orthovanadate, 10 mmol/L MgCl₂]. Pellets were then suspended in 50 μL of 1× kinase buffer supplemented with 1 μL of 10 mmol/L ATP and 1 μg glycogen synthase kinase-3 (GSK-3) fusion protein and incubated for 30 minutes at 30°C. Reaction was terminated with 25 μL 3× SDS sample buffer. Samples were heated to 95°C to 100°C for 2 to 5 minutes, run on SDS-PAGE, and electrotransferred to nitrocellulose membrane. Kinase activity was detected by immunoblotting with phosphorylated GSK-3α/β (pGSK-3α/β; Ser21/9) antibody (CellSignaling).

Angiogenesis assay. The AngioKit (TCS Cellworks, Buckingham, United Kingdom) is composed of human endothelial cells cocultured with human fibroblasts and myoblasts in a 24-well plate containing optimized medium supplied by the manufacturer. The endothelial cells proliferate and then migrate through the matrix to form tubular structures. By the end of 2 weeks, they merge to form a network of anastamosing tubules closely resembling a capillary bed. Each 24-well plate has 6 control wells and 18 test wells. Wells were treated with vascular endothelial growth factor (positive control) and two were treated with suramin or both. Two control wells were treated with vascular endothelial growth factor (positive control) and two were treated with suramin

(negative control), in which there is near total inhibition of angiogenesis. The optimized medium and test samples were replaced on days 4, 7, and 9 after initial treatment. On day 11, the residual medium was aspirated, and cultures were fixed and stained with antibodies to CD31 to detect vessel formation. The degree of tube formation was evaluated by light microscopy and quantitated using computerized image analysis (Angiosys, TCS Cellworks).

Osteoclast assay. To test the effect of rapamycin, 17-AAG, and the combination thereof on myeloma bone destruction, we used the in vitro human osteoclast formation model as described previously (30, 31). In brief, nonadherent healthy human marrow mononuclear cells (1 × 10⁶/100 μL) were plated in 96-well plates in the presence or absence of DMSO or rapamycin, 17-AAG, or the combination. Receptor activator of nuclear factor-κB ligand (100 ng/mL) and macrophage colony-stimulating factor (20 ng/mL) were added to all wells. Negative control wells of medium only or macrophage colony-stimulating factor only were added. Cultures were maintained in an atmosphere of 5% CO₂ and air at 37°C for 3 weeks. The cultures were fed twice weekly by replacing half the medium with an equal volume of fresh medium containing the drugs of interest. After 3 weeks of culture, cells were fixed with 1% formaldehyde PBS, and the number of osteoclast-like multinucleated cells (more than three nuclei) that cross-reacted with the 23c6 monoclonal antibody (PharMingen, San Diego, CA), which identifies osteoclast-like cells, was scored.

Statistical analysis. Results were reported as the mean ± SD for typical experiments done in three replicate samples and compared by the Student’s t test. Results were considered significantly different for Ps < 0.05. All experiments were done at least thrice to ensure reproducibility of the results. For the synergistic activity, data were analyzed using Calcusyn software ( Biosoft, Ferguson, MO) to determine if the combination of rapamycin and 17-AAG was additive or synergistic. An isobologram is a graph that indicates the equipotent combinations of various doses and can be used to show additivity, synergism, or antagonism. Results from viability assays (MTT) were expressed as fraction of cells killed by the individual drugs or the combination in drug-treated cells versus untreated cells. The

![Fig. 2. A, DNA synthesis thymidine uptake assay at 48 hours showing the effect of rapamycin, 17-AAG, and the combination on CD138⁺ plasma cells obtained from a patient with multiple myeloma. Rapamycin induced 15% inhibition, 17-AAG induced 52% inhibition, and the combination induced 65% inhibition compared with control. B, MTT growth inhibition assay at 48 hours in normal peripheral blood mononuclear cells from two healthy donors. Single-agent rapamycin (20 nmol/L), 17-AAG (800 nmol/L), and the combination showed minimal inhibition of growth, with the combination inducing 12% and 25% inhibition compared with control (CTRL), respectively. C, MTT growth inhibition assay at 48 hours of MM.1S treated with rapamycin, 17-AAG, and the combination in the presence of IL-6 (10 ng/mL) or IGF-I (50 ng/mL). 17-AAG and the combination of rapamycin and 17-AAG overcome the protective effects of IL-6 and IGF-I.](https://www.aacrjournals.org/doi/fig/...
The Chou-Talalay method, the basis for this program, calculates a combination index (CI) to indicate additive or synergistic effects. The following equation is used: $CI = \frac{(D_1)}{(D_{x1})} + \frac{(D_2)}{(D_{x2})} + \frac{(D_1)(D_2)}{(D_{x1})(D_{x2})}$, in which $(D_1)$ and $(D_2)$ are the doses for drugs 1 and 2, having $x$ effect in combination. $(D_{x1})$ and $(D_{x2})$ are the drug doses for drugs 1 and 2 having the same $x$ effect when used alone. When $CI = 1$, effects are additive. When $CI < 1.0$, effects are synergistic. $CI < 0.1$ indicates very strong synergism as defined by the CalcuSyn manual.

**Results**

*Effects of rapamycin, 17-AAG, and the combination on growth inhibition of multiple myeloma cell lines.* The effects of rapamycin (1-100 nmol/L) and 17-AAG (100-600 nmol/L), either alone or in combination, on growth inhibition of multiple myeloma cell lines (MM.1S, U266, KAS6/1, and OPM2) were determined using the MTT colorimetric assay at
24 and 48 hours. As shown in Fig. 1B, rapamycin (10-100 nmol/L) induced growth inhibition in MM.1S, with 20 nmol/L inducing 40% growth inhibition. There was no dose response with higher doses than 20 nmol/L. All doses were statistically significant compared with control (P < 0.05). The effect of 17-AAG was dose dependent in MM.1S with 600 nmol/L inducing 35% growth inhibition (P = 0.03). Inhibition of 50% to 60% was obtained with 17-AAG (1,000 nmol/L; data not shown). However, given that the dose equivalent to 17-AAG (1,000 nmol/L) induced toxicity in patients in phase I trials (32), further experiments were conducted using 17-AAG up to 600 nmol/L only. The combination of rapamycin and 17-AAG significantly inhibited the growth of multiple myeloma cell lines to a greater extent compared with the same doses of rapamycin or 17-AAG alone [P = 0.02 compared with single-agent 17-AAG (600 nmol/L); P < 0.05 compared with the respective doses of single-agent rapamycin]. Similar results were obtained with other cell lines, including U266, OPM2, RPMI, and KAS6/1 cell lines as shown in Fig. 1C with U266. The addition of rapamycin 24 hours before 17-AAG or vice versa did not increase cytotoxicity in multiple myeloma cell lines compared with the combination of both agents simultaneously (data not shown).

The interaction between rapamycin and 17-AAG was analyzed using CalcuSyn software program to determine whether this combination had additive or synergistic effects.

Fig. 4. A, immunoblotting for pS6 and PARP using MM.1S cells treated with rapamycin, 17-AAG, and the combination for 6 hours. Rapamycin (1-20 nmol/L) induces complete abrogation of pS6 with the lowest tested dose. 17-AAG inhibited pS6 in a dose-dependent fashion. The combination of rapamycin (20 nmol/L) and 17-AAG (600 nmol/L) showed complete abrogation of pS6, similar to the effect of single-agent rapamycin. Rapamycin induced minimal cleavage of PARP, whereas 17-AAG induced a dose-dependent cleavage of PARP. The combination induced an effect higher than that of each agent alone. Equal loading was confirmed by immunoblotting for tubulin. FL, full length; CL, cleaved form. B, immunoblotting for pS6 and PARP using MM.1S treated with rapamycin (20 nmol/L), 17-AAG (600 nmol/L), and the combination for 2, 4, and 6 hours. The effect of rapamycin occurred on pS6 as early as 2 hours of treatment. 17-AAG inhibited pS6 at 6 hours. PARP cleavage occurred in a time-dependent fashion. C, immunoblotting for caspase-8 and caspase-9 using MM.1S treated with rapamycin (1-20 nmol/L), 17-AAG (300-600 nmol/L), and the combination for 12 hours. Rapamycin induced minimal cleavage of caspase-8 and caspase-9, whereas 17-AAG induced a dose-dependent cleavage of caspase-8/caspase-9. The combination induced an effect higher than that of each agent alone. D, AKT kinase assay using MM.1S cells treated with rapamycin, 17-AAG, and the combination for 48 hours. Rapamycin (1-20 nmol/L) did not affect AKT kinase activity, as shown by pGSK-3β level, an AKT kinase substrate. 17-AAG (600 nmol/L) inhibited pGSK-3β. The combination of the two agents had a similar effect to single-agent 17-AAG. E, immunoblotting for HSP70 and HSP27 using MM.1S cells treated with rapamycin, 17-AAG, and the combination for 48 hours. Rapamycin (1-50 nmol/L) did not affect HSP70 and HSP27, whereas 17-AAG (600 nmol/L) up-regulated HSP70 and down-regulated HSP27. The combination had similar effects to 17-AAG. Equal loading was confirmed by immunoblotting for β-actin. F, immunoblotting for cyclin D1 and phosphorylated Rb (p-Rb) using OPM2 cells treated with rapamycin, 17-AAG, and the combination for 16 hours. Rapamycin, 17-AAG, and the combination inhibited cyclin D1 and phosphorylated Rb. G, immunoblotting for cyclin D1 using MM.1S cells treated with rapamycin, 17-AAG, and the combination for 16 hours. Cyclin D1 was not affected by rapamycin, 17-AAG, or the combination. H, immunoblotting for phosphorylated Rb (pRb) using MM.1S cells treated with rapamycin, 17-AAG, and the combination for 16 hours. Rapamycin and the combination of rapamycin and 17-AAG induced down-regulation of phosphorylated Rb 17-AAG alone minimally inhibited phosphorylated Rb.
on multiple myeloma cell growth. To calculate CI, we generated isobolograms of varying concentrations of rapamycin with 17-AAG in MM.1S cells. Figure 1D and Table 1 show the dose-effect curve of rapamycin, 17-AAG, and the combination. At doses ranging from 20 to 50 nmol/L rapamycin combined with 600 nmol/L 17-AAG, CI ranged from 0.02 to 0.056, suggesting that this combination was very strongly synergistic.

We further confirmed the effect of rapamycin and 17-AAG on samples from three multiple myeloma patients. As shown in Fig. 2A using DNA synthesis assay, the combination of 20 nmol/L rapamycin and 600 nmol/L 17-AAG induced a significant inhibitory effect on proliferation of primary CD138+ cells. The effect of the combination was more than the effect of each agent alone. In contrast, the combination of 20 nmol/L rapamycin and 600 nmol/L 17-AAG did not trigger cytotoxicity in peripheral blood mononuclear cells from two normal volunteers (Fig. 2B). These results show that the combination of rapamycin and 17-AAG triggers significant cytotoxicity in multiple myeloma cell lines and patient cells, without toxicity in normal peripheral blood mononuclear cells.

We next examined whether these drugs could overcome the growth and survival advantages conferred on multiple myeloma cells by interleukin-6 (IL-6) and insulin-like growth factor-I (IGF-I). Multiple myeloma cells were cultured with increasing doses of rapamycin, 17-AAG, and a combination of these agents in the presence of IL-6 (10 ng/mL) or IGF-I (50 ng/mL). As shown in Fig. 2C, the combination of 17-AAG (600 nmol/L) and rapamycin (20-100 nmol/L) showed a significant inhibitory effect on the growth and survival of multiple myeloma cell lines in the presence of IL-6 or IGF-I.
Effect of rapamycin, 17-AAG, and combination therapy on apoptosis. To analyze whether treatment with rapamycin, 17-AAG, or the combination of the two had an effect on apoptosis of multiple myeloma cells, we did Annexin V/PI flow cytometry on MM.1S and U266 cell lines. As shown in Fig. 3A, single-agent rapamycin did not induce apoptosis in U266 cell line, consistent with its cytostatic effect on multiple myeloma cell lines (33). 17-AAG (600 nmol/L) induced 40% apoptosis at 48 hours. Notably, the combination of rapamycin (20-50 nmol/L) and 17-AAG (600 nmol/L) completely abrogated osteoclast formation to 0% (P = 0.007 compared with single-agent rapamycin; P = 0.07 compared with 17-AAG).

Effect of rapamycin, 17-AAG, and combination therapy on cell cycle analysis. We then did cell cycle analysis of multiple myeloma cells treated with rapamycin, 17-AAG, and a combination of the two agents at 24 and 48 hours in MM.1S and U266 cell lines. As shown in Fig. 3B, 17-AAG induced G2/M arrest at 24 hours and G1 arrest at 48 hours, consistent with its effect in other cell lines (34). Rapamycin (20-100 nmol/L) induced G1 arrest at 48 hours. The combination of 17-AAG (600 nmol/L) and rapamycin showed significant apoptosis at 48 hours precluding analysis of cell cycle.

Effect of rapamycin and 17-AAG on growth signaling in multiple myeloma cell lines. To investigate the potential mechanisms of synergy involved in combination therapy, we studied proteins involved in apoptosis, PI3K/mTOR, and HSP90 pathways by immunoblotting using MM.1S and OPM2 multiple myeloma cells treated with rapamycin (0.1-100 nmol/L), 17-AAG (100-1,000 nmol/L), and a combination of these agents in a time- and dose-dependent fashion. Rapamycin (1-20 nmol/L) completely abrogated pS6, downstream of mTOR (Fig. 4A). Doses as low as 0.1 nmol/L rapamycin induced complete abrogation of pS6 (data not shown). Figure 4A shows that 17-AAG (300-600 nmol/L) inhibited pS6 below the baseline level in a dose-dependent fashion but not to the same extent as rapamycin. The effect of rapamycin on pS6 occurred as early as 2 hours of treatment, whereas the effect of 17-AAG occurred later at 6 hours of treatment (Fig. 4B).

To investigate the mechanism of synergy of rapamycin and 17-AAG on apoptosis, we determined the effect of rapamycin, 17-AAG, and the combination of cleavage of PARP and caspase-8 and caspase-9. As shown in Fig. 4A-C, rapamycin induced minimal cleavage of PARP and caspases, consistent with its cytostatic effect. 17-AAG induced a dose-dependent cleavage of PARP and caspase-8/caspase-9, whereas the combination of rapamycin and 17-AAG induced a higher degree of PARP and caspases cleavage compared with the effect observed with each agent alone.
To further identify the site of interaction of 17-AAG with the PI3K pathway, we investigated the effect of 17-AAG on AKT, upstream of mTOR. Previous studies have shown that AKT is a client protein of HSP90 (35). We therefore examined the effect of 17-AAG on AKT. AKT kinase assays were done on MM.1S cells treated with rapamycin (1-200 nmol/L), 17-AAG (600 nmol/L), and the combination at 48 hours. Figure 4D shows inhibition of pGSK-3α/β with single-agent 17-AAG (600 nmol/L) but not with rapamycin (1-20 nmol/L). The combination of 17-AAG and rapamycin inhibited pGSK-3α/β to the same extent as single-agent 17-AAG. These data indicate that 17-AAG inhibits the PI3K pathway at the level of AKT.

We next examined the effect of rapamycin, 17-AAG, and the combination on the HSP90 client proteins. As shown in Fig. 4E and consistent with previous reports, HSP70 expression was up-regulated in response to 17-AAG (32). Rapamycin did not affect the level of HSP70. Similarly, HSP27 was down-regulated in response to 17-AAG but not with rapamycin alone. To further investigate the mechanism of synergy of 17-AAG with rapamycin, we examined the effect of these agents on cyclin D1/Rb. In OPM2, but not MM.1S cells, single agents rapamycin and 17-AAG and the combination inhibited cyclin D1 protein (Fig. 4F and G). These data are consistent with previous reports showing inhibition of cyclin D1 in response to rapamycin only in cell lines with constitutively increased AKT activity, such as OPM2 (9). We then did immunoblotting for phosphorylated Rb, downstream of cyclin D1, to investigate whether G1-related proteins downstream of cyclin D1 were inhibited in response to rapamycin and 17-AAG in MM.1S. Figure 4H shows that phosphorylated Rb was inhibited in response to single agents rapamycin and 17-AAG and the combination in MM.1S cells. This effect occurred in both MM.1S and OPM2, as shown in Fig. 4E and H.

**Effect of rapamycin, 17-AAG, and the combination on angiogenesis and osteoclast formation.** The bone marrow microenvironment plays a crucial role in the proliferation and resistance of multiple myeloma. Therefore, we investigated the effect of rapamycin and 17-AAG on the bone marrow microenvironment, specifically angiogenesis and osteoclast formation. As shown in Fig. 5A, rapamycin induced a marked decrease in angiogenesis (less than the negative control suramin), even at the lowest level tested (0.01 nmol/L). 17-AAG showed inhibition of angiogenesis below the level of suramin at 100 nmol/L and completely abrogated angiogenesis at 500 nmol/L (Fig. 5B).

To test the effect of rapamycin, 17-AAG, and the combination on myeloma bone destruction, we used the in vitro human osteoclast formation model. As shown in Fig. 5C and D, rapamycin (20-100 nmol/L) inhibited osteoclast formation in a dose-dependent fashion with 20 nmol/L inhibiting osteoclast formation down to 81% of control and 100 nmol/L to 23% of control (P = 0.01). 17-AAG (300-600 nmol/L) inhibited osteoclast formation in a dose-dependent fashion with 600 nmol/L inhibiting osteoclast formation to 37% (P = 0.01). The combination of rapamycin (20 nmol/L) and 17-AAG (600 nmol/L) completely abrogated osteoclast formation to 0% (P = 0.007 compared with single-agent rapamycin; P = 0.07 compared with 17-AAG). These results indicate that both rapamycin and 17-AAG inhibit angiogenesis and osteoclast formation in vitro.

**Discussion**

Combinations of novel agents that target multiple signaling pathways in multiple myeloma are required to overcome resistance to treatment and to achieve higher response and survival rates in patients with multiple myeloma. Previous studies have shown that the combination of rapamycin with other agents, such as CC-5013 or dexamethasone, leads to a synergistic effect on multiple myeloma cells. We chose this combination for several reasons: both agents target G1 regulatory proteins, such as cyclin D1, which is commonly dysregulated in multiple myeloma tumors (6); both agents interact at the PI3K pathway at the level of AKT, a pathway known to be activated in multiple myeloma (11); both PI3K and HSP90 pathways are critical for multiple myeloma cell survival and resistance to apoptosis (11, 36); and rapamycin and 17-AAG have shown single-agent activity in preclinical studies in multiple myeloma (11, 36).

In this study, we have shown that the combination of rapamycin and 17-AAG, tested at pharmacologically achievable doses, resulted in a strong synergistic effect on multiple myeloma cell growth in vitro. The combination led to growth inhibition of multiple myeloma cells and induction of apoptosis in all multiple myeloma cell lines tested. Increased sensitivity to mTOR inhibitors has been shown in multiple myeloma cells having elevated levels of AKT kinase activity, whereas cells containing lower AKT activity were relatively resistant (36). Our data indicate that, in multiple myeloma cell lines sensitive or resistant to rapamycin, the combination of 17-AAG and rapamycin resulted in a synergistic antiproliferative activity, indicating that 17-AAG may overcome resistance to rapamycin in multiple myeloma. In addition, the combination of rapamycin and 17-AAG overcame the protective effects of IL-6 and IGF-I. Interestingly, there was no disparity in growth inhibition between low doses (10-20 nmol/L) and higher doses (100 nmol/L) of rapamycin on the multiple myeloma cell lines tested. This indicates that low doses of rapamycin analogues can be as effective as high doses in clinical trials. We are currently testing the effects of low dose RAD001, a rapamycin analogue, in a phase II clinical trial of patients with relapsed/refractory multiple myeloma.

We showed that the combination of rapamycin and 17-AAG induced a significant effect on apoptosis-related proteins, such as cleavage of PARP and caspase-8 and caspase-9, indicating a mechanism of synergy, and an effect on both intrinsic and extrinsic pathways of apoptosis. We then delineated downstream signaling cascades targeted by rapamycin and 17-AAG. Single-agent rapamycin induced a significant inhibition of pS6 in all multiple myeloma cell lines tested even with the lowest tested dose and as early as 2 hours of treatment. These results occurred in cell lines sensitive or relatively less sensitive to rapamycin, indicating that inhibition of S6 phosphorylation should not be used as a surrogate marker of response to this agent in vivo. We further investigated the effects of these agents on proteins in the PI3K pathway and G1 cell cycle regulatory proteins. 17-AAG induced inhibition of pS6 to a lesser degree than rapamycin, indicating that it interacts with the PI3K pathway.
pathways upstream of mTOR. We then showed that 17-AAG inhibited AKT activity, consistent with previous reports that AKT is one of the chaperone proteins of HSP90 (20). In addition, we showed that cyclin D1 and/or phosphorylated Rb were inhibited in multiple myeloma cell lines treated with rapamycin and 17-AAG, indicating another possible mechanism of synergy.

Previous studies have shown that low AKT activity in multiple myeloma cell lines induced resistance to rapamycin by allowing continued cap-independent protein synthesis of cyclin D1 (37). In this study, we showed that cyclin D1 was down-regulated in response to rapamycin in OPM2 cell line but not in MM.1S, indicating that cyclin D1 may be a useful marker of response to rapamycin in multiple myeloma. Interestingly, phosphorylated Rb, another G1 regulatory protein downstream of cyclin D1, was inhibited in response to rapamycin and the combination of the two agents in both MM.1S and OPM2. Further studies are merited to investigate the role of other cyclins and CDKs in the inhibition of phosphorylated Rb in cell lines with lower AKT activity. These data suggest mechanisms for synergy of rapamycin and 17-AAG through targeting of multiple proteins of the PI3K/AKT/mTOR pathway and G1 regulatory proteins.

Finally, we showed that the combination of rapamycin and 17-AAG inhibits not only growth and proliferation in multiple myeloma cells but also proliferation of angiogenesis and osteoclast formation, indicating a potentially inhibitory effect on the bone marrow microenvironment in multiple myeloma. We showed that rapamycin at doses as low as 0.01 nmol/L inhibits angiogenesis. 17-AAG induced complete inhibition of angiogenesis at 600 nmol/L. In addition, rapamycin (20 nmol/L) and 17-AAG (600 nmol/L) induced inhibition of osteoclasts, with the combination completely abrogating osteoclast formation. These data indicate that this combination may be useful in preventing further bone destruction in patients with multiple myeloma.

In summary, we show that the combination of rapamycin and 17-AAG is synergistic in multiple myeloma cells in vitro and inhibits angiogenesis and osteoclast formation. This study provides the basis for clinical evaluation of this combination in patients with multiple myeloma.

References
Combination Mammalian Target of Rapamycin Inhibitor Rapamycin and HSP90 Inhibitor 17-Allylamino-17-Demethoxygeldanamycin Has Synergistic Activity in Multiple Myeloma

Lanie K. Francis, Yazan Alsayed, Xavier Leleu, et al.


Updated version Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/22/6826

Cited articles This article cites 36 articles, 12 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/22/6826.full.html#ref-list-1

Citing articles This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/12/22/6826.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.