Targeting Akt and Heat Shock Protein 90 Produces Synergistic Multiple Myeloma Cell Cytotoxicity in the Bone Marrow Microenvironment

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Abstract

Purpose: We hypothesized that targeting both Akt and heat shock protein (HSP) 90 would induce cytotoxic activity against multiple myeloma (MM) cells and target the bone marrow (BM) microenvironment to inhibit angiogenesis, osteoclast formation, as well as migration and adhesion of MM cells.

Experimental Design: MM cell lines were incubated with perifosine (5 and 10 μmol/L) and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG; 50 and 100 nmol/L) alone and in combination.

Results: The combination of Akt inhibitor perifosine and HSP90 inhibitor 17-DMAG was synergistic in inducing MM cell cytotoxicity, evidenced by inhibition of DNA synthesis and induction of apoptosis. In addition, perifosine and 17-DMAG almost completely inhibited osteoclast formation: perifosine interfered with both early and late stages of osteoclast progenitor development, whereas 17-DMAG targeted only early stages. We next showed that combined therapy overcomes tumor growth and resistance induced by BM stromal cells and endothelial cells as well as the proliferative effect of exogenous interleukin-6, insulin-like growth factor-I, and vascular endothelial growth factor. Moreover, the combination also induced apoptosis and growth inhibition in endothelial cells and inhibited angiogenesis. Finally, we showed that the two agents prevented migration of MM cells toward stromal-derived factor-1 and vascular endothelial growth factor, which are present in the BM milieu, and also prevented adhesion of MM cells to fibronectin.

Conclusions: This study provides the preclinical framework for treatment protocols targeting both the Akt and HSP pathways in MM.

Multiple myeloma (MM) represents a severely debilitating and fatal neoplastic disease of B-cell lineage and is the second most common hematologic malignancy with an annual incidence of ~4/100,000 individuals (1). Despite the number of therapies available for treatment, it remains an incurable disease with an average survival of 3 to 5 years following diagnosis (2, 3). The phosphatidylinositol 3-kinase (PI3K)/Akt pathway acts as a critical regulator of apoptosis, cell cycle regulation, and tumor proliferation in MM (4). This pathway plays an important role in both cell survival and apoptosis and is therefore a promising potential area for targeted therapy (5). PI3K activates phosphatidylinositol-dependent kinase 1 (6), which in turn activates the serine-threonine kinase Akt, a mediator of MM proliferation (7, 8). Major MM growth factors, such as interleukin-6 (IL-6) and insulin-like growth factor-I (IGF-I), also activate the PI3K pathway (9, 10). Perifosine is a member of a new class of antitumor drugs, which potently inhibits Akt (5). Inhibition of the PI3K/Akt pathway at several levels using PI3K inhibitors or Akt inhibitors, such as perifosine, triggers both in vitro and in vivo MM cytotoxicity (11, 12). Therefore, the PI3K/Akt pathway represents an important target for novel treatments in MM.

Heat shock protein (HSP) 90 is a family of chaperone proteins involved in the chaperoning and refolding of proteins, which have been destabilized by stress (13). It is also required for the normal stability of a variety of signaling...
proteins, such as kinases or transcription factors including Akt; conversely, HSP90 disruption leads to degradation of these key proteins, thereby affecting cell signaling (14). The HSP90 inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) binds to the HSP90 protein and inhibits its function. 17-DMAG is similar in structure and function to 17-allylamino-17-demethoxygeldanamycin, the first-in-class HSP90 inhibitor that showed significant activity against MM cells both in vitro and in vivo (15, 16).

The interactions between MM cells and the bone marrow (BM) microenvironment regulate the growth and survival of MM cells and play a critical role in angiogenesis and MM bone disease. Increased angiogenesis is a striking characteristic of MM and has prognostic value in these patients. Bone destruction is a hallmark of MM, with 70% to 80% of patients manifesting bone involvement. In addition, vascular endothelial growth factor (VEGF) induces not only angiogenesis but also migration and adhesion of MM cells in the BM microenvironment. We and others have previously shown that the PI3K pathway regulates migration in MM (17, 18). In addition, the PI3K pathway also regulates VEGF secretion in MM (18).

We here determined that targeting Akt (perifosine) and HSP90 (17-DMAG) pathways induces cytotoxicity against MM cells in the BM microenvironment as well as inhibits angiogenesis, osteoclast formation, MM cell migration, and adhesion. These preclinical studies provide the framework for combined clinical protocols to improve patient outcome in MM.

**Materials and Methods**

**Cell lines.** Dexamethasone-sensitive human MM cell line MM.1S was kindly provided by Dr. S. Rosen (Northwestern University, Chicago, IL). OPM2 cell line was kindly provided by Dr. A. Lichtenstein (University of California, Los Angeles, CA). U266 and RPMI8226 human MM cell lines were purchased from the American Type Culture Collection. All MM cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical), 2 μmol/L L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). The human umbilical vascular endothelial cell (HUVEC) line (Cambrex) was cultured in EGM-2 MV medium (Cambrex) reconstituted according to the manufacturer.

All primary samples were obtained after approval from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients according to the declaration of Helsinki protocol. All samples were isolated from BM by Ficoll-Hypaque density sedimentation followed by CD138+ microbead selection (Miltenyi Biotec). Peripheral blood mononuclear cells were obtained from healthy volunteers.

**Drugs and inhibitors.** Perifosine was provided by Keryx Biopharmaceuticals and 17-DMAG was provided by the National Cancer Institute. IL-6, IGF-1, and VEGF were purchased at R&D Systems.

**Growth inhibition assay and DNA synthesis.** The inhibitory effect of perifosine on MM cell growth with either perifosine, 17-DMAG, or the combination was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International) dye absorbance, as described (19). Proliferation was measured by DNA synthesis, as described (19). DNA synthesis was measured by the [3H]thymidine uptake (Perkin-Elmer).

**Flow cytometric analysis.** Cell cycle analysis was profiled by flow cytometry using propidium iodide staining (5 μg/ml; Sigma Chemical) after 12 and 24 h of culture with either perifosine, 17-DMAG, or the combination. Apoptosis was quantitated using Apo2.7 flow cytometric analysis (Beckman Coulter, Inc.), as described (19).

**Immunoblotting.** MM cells were harvested and lysed using lysis buffer (Cell Signaling Technology), as previously described (19). Whole-cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories). The antibodies used for immunoblotting included the following: anti-phosphorylated Akt (Ser\(^{743}\)), anti-phosphorylated Akt (Thr\(^{468}\)), phosphorylated Akt, anti-phosphorylated extracellular signal-regulated kinase (ERK; Thr\(^{202}/\)Tyr\(^{204}\)), anti-ERK1/2, anti-phosphorylated glycogen synthase kinase (GSK) 3α/β (Ser\(^{21}/\)78), anti-phosphorylated S6 ribosomal (Ser\(^{240}/244}\)), anti-phosphorylated p38 (Thr\(^{180}/\)Tyr\(^{182}\)), anti-phosphorylated stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK; Thr\(^{183}/\)Tyr\(^{185}\)), anti-SAPK/JNK1/2, anti-HSP90, anti-phosphorylated HSP27, anti-HSP27, anti-caspase-8, anti-caspase-9, anti-caspase-3, anti-Mcl-1, and anti-poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology) as well as anti-β-actin (Sigma Chemical) and anti-tubulin (Santa Cruz Biotechnology).

**In vitro Akt kinase assay and Akt communoprecipitation assay.** In vitro Akt kinase assay (Cell Signaling Technology) was done following the manufacturer’s procedure. Briefly, MM.1S and HUVECs were cultured with either perifosine, 17-DMAG, or the combination. Lysates were immunoprecipitated with immobilized Akt primary antibody. Kinase activity was detected by immunoblotting with phosphorylated GSK3α/β (Ser\(^{21}/\)78) antibody (Cell Signaling Technology).

**Osteoclast assay.** The in vitro human osteoclast assay was used to evaluate the effects of perifosine and 17-DMAG on the bone destruction/resorption associated with MM tumor cells, as described (20). Nonadherent human marrow mononuclear cells were plated with either perifosine, 17-DMAG, or the combination. Plates were treated with the drug combinations at the time of initial setup or after 1 or 2 weeks in culture. Receptor activator of nuclear factor-κB ligand (50 ng/ml) and macrophage colony-stimulating factor (10 ng/ml) were added to all wells. Following 3 weeks of culture, cells were fixed in 1% formaldehyde PBS, and the number of 23c6 (PharMingen) multinucleated cells was counted.

**Colony-forming cell assay.** Colony-forming cell assays were done using BM mononuclear fraction cultured in commercially available methylcellulose for human colony-forming cell assays (MethoCult, StemCell Technologies) and treated with either perifosine, 17-DMAG, or the combination. Plates were treated with the drug combinations at the time of initial setup or after 1 or 2 weeks in culture. Receptor activator of nuclear factor-κB ligand (50 ng/ml) and macrophage colony-stimulating factor (10 ng/ml) were added to all wells. Following 3 weeks of culture, cells were fixed in 1% formaldehyde PBS, and the number of 23c6 (PharMingen) multinucleated cells was counted.

**Morphogenesis assay on Matrigel.** HUVECs were layered onto the Matrigel surface (Becton Dickinson) polymerized in EGM-2 MV reconstituted medium and treated with either perifosine, 17-DMAG, or the combination. Cell growth and tridimensional organization were observed using a reverted phase-contrast light microscope, starting at 6 h, as published (21).

**Statistical analysis.** The synergistic effect [combination index (CI) < 0.8] of the combination of perifosine and 17-DMAG was analyzed using CalcuSyn (Biosoft), a software program based on the Chou-Talalay method, as described (19). An isobologram is a graph that indicates affected fraction and CI.

**Results**

The combination of perifosine and 17-DMAG blocks proliferation and induces cytotoxicity of MM cells. The effects of single-agent perifosine, 17-DMAG, and the combination of agents were assessed on the growth of MM cell lines with high Akt activity (OPM2) and lower Akt activity (MM.1S, RPMI8226,
and U266; ref. 22). The increasing doses of perifosine (5 and 10 μmol/L), 17-DMAG (50 and 100 nmol/L), and their combination induced dose-dependent increase in cytotoxicity in the MM.1S cells (Fig. 1A). For example, perifosine (10 μmol/L, 48 h) induced 30% cytotoxicity that was synergistically enhanced to 53% (CI, 0.61) and 73% (CI, 0.42) when combined with 17-DMAG at 50 and 100 nmol/L, respectively. The combination of perifosine and 17-DMAG induced synergistic cytotoxicity (Fig. 1B; Table 1). Similar results were shown in RPMI8226 and U266 MM cell lines (data not shown). In contrast, the OPM2 cell line (with high Akt activity) was very sensitive to perifosine, with an IC_{50} at 5 μmol/L, but was not synergistic with 17-DMAG (Fig. 1C).

We next confirmed the activity of perifosine, 17-DMAG, and the combination in MM patient samples. Perifosine and 17-DMAG induced significant cytotoxicity in CD138+ primary MM cells, evidenced by increased growth inhibition with the combination of agents versus either single agent (P = 0.038; Fig. 1D). In contrast, Fig. 2A shows a lack of a significant cytotoxic effect of perifosine, 17-DMAG, or the combination of agents on peripheral blood mononuclear cells (Fig. 2A).

To determine the effect of sequential therapy with perifosine or 17-DMAG, we treated MM.1S cells with 17-DMAG (50 nmol/L) for 24 h followed by addition of perifosine (5 and 10 μmol/L) for another 24 h or vice versa. Cytotoxicity induced by the combination was significantly higher when MM.1S cells were pretreated with 50 nmol/L 17-DMAG (P = 0.01) versus perifosine and was similar to treatment with the two agents for 48 h (Fig. 2B). Moreover, MM.1S cell cytotoxicity was greatly enhanced when cells were treated for 24 h with 50 nmol/L 17-DMAG alone and then washed before treatment with 10 μmol/L perifosine for another 24 h (data not shown). Similar results were observed at 72 h using the same combinations.

Table 1. Affected fractions and combination indices with perifosine and 17-DMAG, alone and in combination, on MM.1S at 48 h

<table>
<thead>
<tr>
<th>No.</th>
<th>Perifosine (μmol/L)</th>
<th>17-DMAG (nmol/L)</th>
<th>FA</th>
<th>CI</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>5</td>
<td>50</td>
<td>0.33</td>
<td>0.867</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>100</td>
<td>0.46</td>
<td>0.596</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>50</td>
<td>0.53</td>
<td>0.617</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>100</td>
<td>0.66</td>
<td>0.422</td>
</tr>
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Abbreviation: FA, affected fraction.
Combined perifosine and 17-DMAG induces apoptosis and cell cycle arrest through JNK mitogen-activated protein kinase and caspase-dependent mechanisms. We next characterized the molecular mechanisms whereby this combination induces cytotoxicity in MM cells. We first showed that perifosine and 17-DMAG induced significant apoptosis, evidenced by propidium iodide and Apo2.7 staining and flow cytometric analysis. Perifosine induced dose-dependent apoptosis in MM.1S with 10 μmol/L inducing 25% apoptosis at 24 h, which increased to 40% and 50% with 50 and 100 nmol/L 17-DMAG, respectively (Fig. 2C). Perifosine (5 μmol/L) also induced G2-M arrest while increasing apoptosis, as shown by an increase in sub-G2-G1 cells. Perifosine (5 μmol/L) induced 12% sub-G2-G1 cells at 24 h, whereas 50 nmol/L 17-DMAG did not induce significant sub-G2-G1 cells but enhanced the effect of perifosine on G2-M arrest (63.4%; data not shown).

To determine the mechanism of induction of apoptosis by this combination, we investigated the effect of perifosine, 17-DMAG, and the combination on MM.1S cells using immunoblotting. Perifosine induced caspase-8, caspase-9, caspase-3, and PARP cleavage in a dose-dependent fashion (Fig. 2D), with caspase activation and PARP cleavage at 10 h. 17-DMAG triggered minimal induction of apoptosis in MM.1S cells. Interestingly, whereas perifosine-induced caspase activation was via the intrinsic apoptotic pathway, 17-DMAG–induced apoptosis, although minimal, was via the extrinsic pathway. Importantly, the combination significantly increased apoptosis via both pathways. Furthermore, perifosine and 17-DMAG decreased expression of the antiapoptotic molecule Mcl-1 in a dose-dependent manner. Previous studies have shown in MM cells that SAPK/JNK1/2 plays an important role in apoptosis in MM.1S cells (23), and therefore, we examined the effect of both agents on JNK phosphorylation. Perifosine (5 and 10 μmol/L, 6 h) induced phosphorylation of JNK1/2 in MM.1S cells in a dose-dependent fashion (Fig. 2D). In contrast, 17-DMAG alone did not induce activation of JNK mitogen-activated protein kinase (MAPK) pathway.

Effects of perifosine, 17-DMAG, and the combination on signaling in MM cell lines. To further investigate the mechanism of synergy between perifosine and 17-DMAG, we next
examined PI3K/Akt and HSP90 signaling in MM.1S cells treated with perifosine (5 and 10 μmol/L), 17-DMAG (50 and 100 nmol/L), and the combination for 6 h using immunoblotting and immunoprecipitation. Akt and HSP90 coimmunoprecipitate in MM cells, indicating direct interaction of these two pathways (data not shown). Perifosine completely abrogated phosphorylation of Akt (Ser^{173}) and downstream target proteins, such as phosphorylation of GSK3α/β and ribosomal S6 (Fig. 3A). 17-DMAG (50-100 nmol/L) induced slight dose-dependent decreased phosphorylation of the same proteins, indicating that 17-DMAG targets the PI3K/Akt pathway. We then confirmed the effect of perifosine on Akt activity using an in vitro Akt kinase assay. Perifosine alone and with 17-DMAG, but not 17-DMAG, inhibited phosphorylation of GSK3α/β fusion protein (Fig. 3B).

Interestingly, 17-DMAG and perifosine had differential effects on MAPK and HSP90 pathways (Fig. 3C). 17-DMAG inhibited MAPK proteins: 17-DMAG inhibited p38 MAPK phosphorylation, whereas perifosine had no effect. Similarly, 17-DMAG completely inhibited ERK1/2 MAPK protein phosphorylation, whereas perifosine induced activation of this protein. We next showed that 17-DMAG inhibited phosphorylation of HSP27 and increased expression of HSP27 and HSP70, indicating inhibition of HSP90 signaling, as previously observed (24, 25). Perifosine did not affect HSP27 or HSP70 (Fig. 3D). Interestingly, although either agent alone did not affect the level of HSP90, the combination decreased HSP90 protein level using whole-cell lysate (Fig. 3D) and after immunoprecipitation of Akt (data not shown).

**Perifosine, 17-DMAG, and the combination inhibit osteoclastogenesis.** MM has significant effects on the BM microenvironment, particularly in promoting osteoclast activation and subsequent bone destruction (26). Therefore, we sought to investigate whether perifosine, 17-DMAG, and the combination inhibited early or late event in osteoclast formation, and we conducted time course experiments. In a 21-day culture system, human BM cells were treated with perifosine, 17-DMAG, or the combination for all 3 weeks, the last 2 weeks, or only the last week of culture. The addition of perifosine (5 and 10 μmol/L), 17-DMAG (50 nmol/L), and the combination almost completely inhibited osteoclast formation even at low doses of perifosine (5 μmol/L; Fig. 4A). Inhibition of osteoclast formation of BM cells was significantly lower when 17-DMAG was added during the last week (60% inhibition) in comparison with 100% inhibition with 3 weeks of treatment. However, perifosine (5 and 10 μmol/L) was able to overcome osteoclast formation even when added only in the last week (100% inhibition), indicating that perifosine interferes with the early and late stages of differentiation and proliferation of osteoclast progenitors, whereas 17-DMAG targets only early stages of osteoclast development.

**17-DMAG, but not perifosine, inhibits development of the precursors of osteoclast, CFU-GMs.** We next examined the effects of 17-DMAG (50 nmol/L), perifosine (5 and 10 μmol/L), and the combination on the differentiation of CFU-GMs, which are precursors for osteoclast (27). Either drug alone or the combination was added to the colony cultures at plating and incubated for 15 days. 17-DMAG significantly decreased...
the number of CFU-GM (mean, 2 ± 0.5 colonies per well) in comparison with control (mean, 20 ± 3), whereas perifosine had no effect on CFU-GM (mean, 18 ± 3; P = 0.01; Fig. 4B).

The combination of perifosine and 17-DMAG overcomes resistance induced by the BM microenvironment. Because the BM microenvironment confers growth and drug resistance in MM cells, we next studied the effect of perifosine and 17-DMAG on MM cells in the presence of BMSCs. MM.1S cells were cultured with perifosine (5-20 μmol/L) and 17-DMAG (50 nmol/L) in the presence or absence of BMSCs. Adherence of MM.1S cells to BMSCs triggered ~2.0-fold (P < 0.01) increase in [3H]thymidine uptake at 48 h. Perifosine and 17-DMAG inhibited MM cell growth in the presence of BMSCs in a dose-dependent fashion, whereas the combination of the two agents significantly increased this effect (P < 0.001), confirming that this combination retains significant anti-tumor activity even in the presence of the BM milieu (Fig. 4C). In addition, because IL-6 and IGF-I are known growth factors for MM cells and induce activation of Akt (28, 29), we determined whether the combination of perifosine and 17-DMAG overcomes the proliferative effect induced by IL-6 and IGF-I. IL-6 (50 ng/mL) and IGF-I (100 ng/mL) induced proliferation of MM cells, which was blocked by perifosine and 17-DMAG and significantly decreased by the combination (P = 0.013; Fig. 4D).

The combination of perifosine and 17-DMAG inhibits survival of endothelial cells. The MTT colorimetric assay was next used to assess cytotoxicity and thymidine uptake to assess proliferation of endothelial cells at 48 h. We observed dose-dependent increase in cytotoxicity with either perifosine (5 and 10 μmol/L), 17-DMAG (50 and 100 nmol/L), and their combination. 17-DMAG (100 nmol/L) induced 36% cytotoxicity, which was synergistically enhanced to 58% (CI, 0.51) and 71% (CI, 0.53) with perifosine (5 and 10 μmol/L), respectively, using the MTT assay (Fig. 5A; Table 2). Similar results were observed using the thymidine uptake assay to study inhibition of HUVEC growth by perifosine, 17-DMAG, and the combination.

We next characterized the molecular mechanisms whereby this combination induces cytotoxicity in HUVECs. To confirm the importance of Akt and HSP90 pathways in the survival of endothelial cells, we examined the effect of perifosine and 17-DMAG on these signaling pathways. Perifosine (5 and 10 μmol/L) and 17-DMAG (50 nmol/L) inhibited Akt kinase activity by blocking phosphorylation of GSK3α/β, the combination inducing a greater decrease than either agent alone (Fig. 5B). Similarly, perifosine, and to a lesser extent 17-DMAG, inhibited phosphorylation of S6 ribosomal protein (Ser240/244).

Fig. 4. A, osteoclast formation assay. Peripheral blood mononuclear cells were cultured for 3 wk with macrophage colony-stimulating factor (M-CSF) + receptor activator of nuclear factor-κB ligand (50 ng/mL). Following 3 wk of culture, the number of 23c6 multinucleated cells was counted. Perifosine (5 and 10 μmol/L) and 17-DMAG (50 nmol/L) were added at the time of initial setup or after 1 or 2 wk in culture. Medium, M-GSF (10 ng/mL), and macrophage colony-stimulating factor + receptor activator of nuclear factor-κB ligand (50 ng/mL) without drug treatment were used as controls. B, colony-forming cell assay. Nonadherent mononuclear cells were cultured in methylcellulose semisolid medium in the presence or absence of perifosine (5 and 10 μmol/L) and 17-DMAG (50 nmol/L) for 15 d. CFU-GM was then counted. Growth factors and coculture with BM microenvironment cells do not protect MM cells against the combined perifosine-induced and 17-DMAG – induced cytotoxicity. C, MM.1S cells were cultured with the combination of perifosine (5-20 μmol/L) and 17-DMAG (50 nmol/L) for 48 h in the presence or absence of BMSCs. Cell proliferation was assessed using the [3H]thymidine uptake assay. D, MM.1S cells were cultured with perifosine (5 and 10 μmol/L) and 17-DMAG (50 nmol/L) in the presence or absence of IL-6 (50 ng/mL) or IGF-I (100 ng/mL) for 48 h. Cytotoxicity was assessed by the MTT assay. Columns, mean of triplicate experiments; bars, SD.
a downstream target protein of Akt, at 6 h (Fig. 5C). In addition, 17-DMAG (50 nmol/L) induced up-regulation of HSP27 and HSP70 expression, and down-regulation of HSP27 phosphorylation without changing the level of HSP90, consistent with previous reports; in contrast, perifosine did not significantly affect HPS90 signaling (Fig. 5C).

Furthermore, we examined the effect of perifosine, 17-DMAG, and the combination on induction of apoptosis in endothelial cells. Perifosine (5 and 10 μmol/L) and 17-DMAG (50 and 100 nmol/L) induced a dose-dependent increase in apoptosis, as shown by Apo2.7 staining and flow cytometry analysis in HUVECs at 48 h (Fig. 5D, left). Perifosine (5 and 10 μmol/L) induced 15% and 26% apoptosis in HUVECs that was enhanced to 28% and 68%, respectively, with 50 nmol/L 17-DMAG. 17-DMAG alone (100 nmol/L) induced 25% apoptosis at 48 h in HUVECs. To determine the mechanism of perifosine and 17-DMAG combination-induced apoptosis, we investigated the effect of each agent and the combination on HUVECs using immunoblotting. The combination significantly triggered caspase-8, caspase-9, caspase-3, and PARP cleavage in a dose-dependent fashion, with caspase activation and PARP cleavage at 10 h. It also induced a decrease in expression of the antiapoptotic Mcl-1 protein (Fig. 5D, right).

Perifosine and 17-DMAG inhibit neoangiogenesis. We next investigated whether perifosine and 17-DMAG could directly inhibit in vitro capillary formation on Matrigel using HUVECs. After 6 h, HUVECs spread throughout the Matrigel surface and aligned to form branching, anastomosing, and thick tubes with multicentric junctions, which gave rise to a closely knit network of capillary-like structures (30). Perifosine and 17-DMAG triggered a dose-dependent inhibition of capillary formation by HUVECs, as evidenced by either isolated or aggregated spherical cells in small clumps, with few elongated cells and no anastomosed tubes. Perifosine (5 and 10 μmol/L), 17-DMAG (50 nmol/L), and the combination significantly inhibited the network branches and capillary formation ($P = 0.001$; Fig. 6A). The effect of 17-DMAG on capillary formation was more pronounced than perifosine.

Perifosine and 17-DMAG overcome the protective effect of endothelial cells on MM cells. To further define the mechanism...
Table 2. Affected fractions and combination indices with perifosine and 17-DMAG against MM.1S cells at 48 h

<table>
<thead>
<tr>
<th>No.</th>
<th>Perifosine (μmol/L)</th>
<th>17-DMAG (nmol/L)</th>
<th>FA</th>
<th>CI</th>
</tr>
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<tr>
<td>1</td>
<td>5</td>
<td>50</td>
<td>0.44</td>
<td>0.783</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>100</td>
<td>0.56</td>
<td>0.519</td>
</tr>
<tr>
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<td>10</td>
<td>50</td>
<td>0.52</td>
<td>0.951</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>100</td>
<td>0.69</td>
<td>0.537</td>
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</table>

Perifosine and 17-DMAG inhibit MM cell migration and adhesion. The BM microenvironment also regulates the homing of MM cells and their adhesion to the stromal cells and osteoclasts and osteoblasts (17, 33). Several chemokines and cytokines regulate migration and adhesion in MM, including stromal-derived factor-1 and VEGF (31, 34, 35). In addition, adhesion is highly regulated by fibronectin, a VLA-4 ligand (36). Previous studies have shown that the PI3K pathway is important for adhesion and migration signaling in MM (34). Therefore, we next sought to explore the effect of perifosine and 17-DMAG on migration and adhesion of MM cells in the presence of stromal-derived factor-1 and VEGF. Stromal-derived factor-1 (30 nmol/L) and VEGF (10 ng/mL) induced significant migration of MM.1S cells, with 2.5-fold increase in migration compared with control (Fig. 6C). Perifosine (10 μmol/L) and 17-DMAG (50 nmol/L) treatments for 2 h significantly inhibited (2.5-fold inhibition) migration in response to these cytokines. Similarly, we examined the effect of perifosine and 17-DMAG on the adhesion of MM cells to fibronectin in the presence or absence of VEGF. Adhesion of MM cells to fibronectin was increased in the presence of VEGF (10 ng/mL) compared with bovine serum albumin control (Fig. 6D). Pretreatment of MM cells with perifosine (10 μmol/L) and 17-DMAG (50 nmol/L) for 1 h significantly inhibited adhesion to fibronectin even in the presence of VEGF.

Together, these data indicate that perifosine and 17-DMAG not only target MM cell but also regulate the BM microenvironment, including angiogenesis, osteoclast formation, as well as the migration and adhesion of MM cells within the BM milieu.

Discussion

Despite advances in understanding the molecular pathogenesis of MM and promising new therapies, almost all patients eventually relapse with resistant disease. There is therefore a strong rationale for combining novel therapies that target intrinsic molecular pathways mediating MM cell resistance. In addition, the interaction between MM cells and the BM microenvironment regulates the growth and resistance of MM cells and plays a critical role in angiogenesis and MM bone disease (31, 37, 38). The PI3K and HSP90 pathways are important regulators of the survival of MM cells (9, 39, 40). PI3K catalyzes the synthesis of a second messenger, resulting in the activation of phosphatidylinositol-dependent kinase 1, which in turn activates the serine-threonine kinase Akt. Akt is a mediator of tumor expansion in MM (6, 9). The role of Akt in the survival and growth of osteoclasts and endothelial cells has not been previously elucidated; however, prior studies using mammalian target of rapamycin inhibitors, which act downstream of Akt, have shown that this pathway is an important regulator of osteoclastogenesis and angiogenesis (15, 41, 42). In addition, we and others have shown that the PI3K pathway regulates homing, migration, and adhesion of MM cells (17, 34). Previous studies using the novel Akt inhibitor perifosine have shown significant cytotoxicity in MM cells (23); however, the effect of this clinically available oral Akt inhibitor on the BM microenvironment (endothelial cells, osteoclasts, and osteoclasts precursors) and trafficking and adhesion of MM cells in the BM has not been previously examined. HSP90, a client protein, includes kinases or transcription factors involved in signal transduction, including Akt, p53, Bcr-Abl, Raf-1, and ErbB2. We have shown that HSP90 inhibitors have in vitro and in vivo cytotoxicity against MM cells and have shown clinical activity of HSP90 inhibitors in MM with 71% responding patients in an interim analysis of the phase I study in MM patients (15, 40, 43–45). However, the role of HSP90 in regulation of MM trafficking, adhesion, and migration of MM cells in the BM microenvironment has not been previously examined.

In this study, we showed that Akt and HSP90 coimmunoprecipitate in MM cells, indicating direct interaction of those two pathways. We showed that the combination of the Akt inhibitor perifosine and the HSP90 inhibitor 17-DMAG synergistically induces apoptosis and inhibits MM cell growth and proliferation. Interestingly, the sequence of the two agents indicated that the use of 17-DMAG before perifosine or the use of both agents simultaneously induces higher cytotoxicity compared with using perifosine before the addition of 17-DMAG, suggesting that 17-DMAG sensitizes MM cells to the perifosine-induced cytotoxicity. Perifosine and 17-DMAG inhibited Akt activity and downstream signaling pathways, including phosphorylated S6 kinase. In addition, the combination inhibited HSP90 signaling, including activation of HSP70 and inhibition of HSP27 phosphorylation. Interestingly, 17-DMAG inhibited the ERK MAPK and p38 MAPK pathways, whereas perifosine induced these cascades, suggesting that 17-DMAG may overcome the resistance conferred by ERK activation in MM. Finally,
the combination induced apoptosis mediated by both the intrinsic and extrinsic pathways and JNK activation without affecting normal peripheral blood mononuclear cells, suggesting a favorable therapeutic index.

Because the BM microenvironment induces proliferation and resistance of MM cells, we focused in this study on the effect of these two agents on the BM microenvironment. We specifically focused on angiogenesis and osteoclast formation, along with the trafficking of MM cells to BM. We first showed that perifosine and 17-DMAG prevent the growth of osteoclast and osteoclast precursors. In a 21-day culture system, perifosine (5 and 10 μmol/L), 17-DMAG (50 nmol/L), and the combination almost completely inhibited osteoclast formation even at low doses of perifosine (5 μmol/L). Inhibition of osteoclast formation was significantly lower after 1 week of 17-DMAG treatment (60% inhibition) in comparison with 3 weeks of treatment (100% inhibition). Importantly, perifosine (5-10 μmol/L) by 1 week was able to overcome osteoclast formation (100% inhibition), indicating that perifosine interferes with the early and late stages of differentiation and proliferation of osteoclast progenitors, whereas 17-DMAG interferes only with the early stages of osteoclast development. We showed that 17-DMAG inhibits the development of osteoclast precursors and CFU-GM, whereas perifosine had no effect on the growth of osteoclast precursors, supporting this view.

We then investigated the role of these agents on endothelial cells. We first showed that the combination overcomes resistance induced by stromal cells and endothelial cells when cocultured with MM cells. The combination was also able to overcome the proliferative effect of exogenous IL-6, IGF-I, and VEGF on MM cells. Moreover, the combination induced apoptosis and growth inhibition in endothelial cells through inhibition of Akt activity, HSP90 downstream signaling, as well as caspase and PARP cleavage. We then showed that the combination inhibited new vessel formation in the Matrigel assay. Finally, we showed that the two agents also prevented migration of MM cells toward chemokines and cytokines that are present in the BM milieu, including stromal-derived factor-1 and VEGF, and also prevented adhesion of MM cells to fibronectin, present within the BM microenvironment. These data indicate that targeting Akt and HSP90 inhibits trafficking and adhesion of MM cells to the BM.

In summary, we have shown that 17-DMAG greatly sensitized MM tumor cells to perifosine-induced cytotoxicity. The combination overcomes drug resistance-induced MM cells by the BM milieu as well as inhibits osteoclastogenesis and angiogenesis. These studies provide the basis for clinical trials using these agents to target Akt and HSP90 to overcome drug resistance and improve patient outcome in MM.

**Fig. 6.** A, morphogenesis assay on Matrigel. HUVECs were cultured on polymerized Matrigel in EGM-2 MV medium with either perifosine (5 and 10 μmol/L), 17-DMAG (50 nmol/L), or the combination for 4 to 12 h. Cell growth and tridimensional organization were observed using a reverted phase-contrast light microscope. Network branches were enumerated using a high-power phase-contrast light microscope. B, MM.1S cells were cultured with either perifosine (5 and 10 μmol/L), 17-DMAG (50 nmol/L), or the combination for 48 h in the presence or absence of HUVECs. Cell proliferation was assessed using the [3H]thymidine uptake assay. In vitro adhesion and migration of MM.1S and HUVECs. C, Transwell migration assay showing inhibition of migration of MM.1S cells by perifosine (10 μmol/L), 17-DMAG (50 nmol/L), and the combination for 4 h in the presence of SDF-1 (30 nmol/L) or VEGF (30 ng/mL) was placed in the lower chambers and migration induced compared with control (Ctrl) without the addition of cytokine. Inhibition of migration was observed with perifosine, 17-DMAG, and the combination. D, adhesion assay of MM.1S cells. MM.1S cells showed increased adhesion in fibronectin-coated wells (control) compared with bovine serum albumin (BSA)-coated wells. Adhesion to fibronectin in the presence of VEGF (10 ng/mL) was decreased with perifosine (10 μmol/L), 17-DMAG (50 nmol/L), and the combined treatment for 1 h. Columns, mean of triplicate experiments; bars, SD.
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