Dual Inhibition of Akt/Mammalian Target of Rapamycin Pathway by Nanoparticle Albumin-Bound–Rapamycin and Perifosine Induces Antitumor Activity in Multiple Myeloma

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Abstract

The phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway mediates multiple myeloma (MM) cell proliferation, survival, and development of drug resistance, underscoring the role of mTOR inhibitors, such as rapamycin, with potential anti-MM activity. However, recent data show a positive feedback loop from mTOR/S6K1 to Akt, whereby Akt activation confers resistance to mTOR inhibitors. We confirmed that suppression of mTOR signaling in MM cells by rapamycin was associated with upregulation of Akt phosphorylation. We hypothesized that inhibiting this positive feedback by a potent Akt inhibitor perifosine would augment rapamycin-induced cytotoxicity in MM cells. Perifosine inhibited rapamycin-induced phosphorylated Akt, resulting in enhanced cytotoxicity in MM.1S cells even in the presence of interleukin-6, insulin-like growth factor-I, or bone marrow stromal cells. Moreover, rapamycin-induced autophagy in MM.1S MM cells, as evidenced by electron microscopy and immunocytochemistry, was augmented by perifosine. Combination therapy increased apoptosis detected by Annexin V/propidium iodide analysis and caspase/poly(ADP-ribose) polymerase cleavage. Importantly, in vivo antitumor activity and prolongation of survival in a MM mouse xenograft model after treatment was enhanced with combination of nanoparticle albumin-bound–rapamycin and perifosine. Utilizing the in silico predictive analysis, we confirmed our experimental findings of this drug combination on PI3K, Akt, mTOR kinases, and the caspases. Our data suggest that mutual suppression of the PI3K/Akt/mTOR pathway by rapamycin and perifosine combination induces synergistic MM cell cytotoxicity, providing the rationale for clinical trials in patients with relapsed/refractory MM. Mol Cancer Ther; 9(4); 963–75. ©2010 AACR.

Introduction

Multiple myeloma (MM) is a bone marrow (BM) cancer driven by the interaction between clonal plasma cells and the BM microenvironment (1, 2). Among the major pathways mediating cytokine-induced MM cell growth and survival, phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) kinase cascade plays a cardinal role in cell proliferation, survival, and development of drug resistance (3–5). Cytokine-induced activation of Akt results in various downstream antiapoptotic effects via BCL2-associated agonist of cell death and forkhead transcription factor phosphorylation and inhibition of the catalytic subunit of caspase-9. Besides its direct antiapoptotic effects, phosphorylated Akt (p-Akt) promotes growth and survival via phosphorylation of GSK-3β and mTOR. Moreover, Akt-induced activation of mTOR enables mRNA translation through the activation of P70S6 kinase and the inhibition of 4E-BP1, a translational repressor of mRNAs. Consequently Akt, which is constitutively activated in MM patient cells and correlates with advanced stage and poor prognosis (6), represents a rational target for novel therapeutics.

Identifying mTOR as a key kinase downstream of Akt led to the prediction that rapamycin, a universal inhibitor of mTORC1-dependent S6K1 phosphorylation, may be useful in the treatment of MM (7–9). In vitro and in vivo preclinical studies have shown anti-MM activity of rapamycin and its analogues (CCI-779 and RAD001; refs. 10–14). First generation mTOR inhibitors, when used as single agents, have shown only modest efficacy in clinical trials (15–17), resulting in attempts to define...
mechanisms underlying rapamycin resistance. A growing body of evidence supports the hypothesis that resistance to rapamycin results from a strong positive feedback loop from mTOR/S6K1 to Akt, resulting in Akt activation (18–20). Indeed immunohistochemical analysis of paired tissue biopsies, before and after treatment with rapamycin derivatives, revealed that nonresponders frequently developed increased p-Akt, supporting the view that increased intratumoral phosphorylation of Akt mediates rapamycin resistance (21, 22).

The low response rate observed in many tumor types to rapamycin derivatives led to two strategies to overcome rapamycin resistance. First, the implementation of nanoparticle albumin-bound (nab) technology to augment rapamycin delivery to tumor tissue (23, 24). Second, combination strategies such as rapamycin with lenalidomide, with the ability to overcome the protective effects of growth factors in the tumor milieu, are in use (10).

Given that mTOR inhibitors induce PDK1/Akt activity in MM cells (25), we have examined the utility of adding an Akt inhibitor to overcome mTOR resistance and have also taken the advantage of nanoparticle technology with nab-rapamycin. To date, the best-characterized and most developed clinical inhibitor of Akt is the novel alkylphospholipid perifosine (26, 27). We first confirmed that suppression of mTOR signaling by rapamycin was associated with upregulation of Akt activation. We therefore inquired whether perifosine could (a) inhibit rapamycin-induced p-Akt, (b) augment rapamycin-induced cytotoxicity in vitro, and (c) translate into enhanced in vivo antitumor activity when used with the nab-based rapamycin (ABI-009). Our data suggest that rapamycin-induced cytotoxicity was predominantly triggered as a consequence of autophagy in MM cells. The combination of rapamycin and perifosine resulted in two cell death–inducing events: autophagy and apoptosis. Furthermore, the combination of nab-rapamycin and perifosine resulted in significant antitumor activity in an in vivo human MM cell xenograft murine model. Finally, using systems biology approach (28, 29), we confirmed our experimental findings regarding the biological effects of this drug combination. These studies therefore provide the preclinical rationale for combination clinical trials in patients with MM.

Materials and Methods

Cell culture and reagents

**MM-derived cell lines.** Dexamethasone-sensitive (MM.1S) MM cell line was provided by Dr. Steven Rosen (Northwestern University). The INA-6 cell line was kindly provided by Dr. Martin Gramatzki (University of Erlangen-Nuernberg). OPM1 cell line was provided by Dr. P. Leif Bergsagel (Mayo Clinic). All MM cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical), 2 mol/L l-glutamine, 100 units/mL penicillin, and 100 g/mL streptomycin (Life Technologies). Generation of BM stroma cells (BMSC) from BM specimens from MM patients obtained after appropriate institutional review board–approved informed consent has been previously described (10). Once confluent, the cells were trypsinized and passaged as needed. BMSCs were incubated in 96-well culture plates (~5,000 to 10,000 BMSCs/well) for 24 h; MM.1S cells were then added to the wells (2 × 10,000 per well) and incubated with media alone, rapamycin, perifosine, or combination for 48 h at 37°C at the specified concentrations.

**Rapamycin.** Rapamycin was obtained from Calbiochem. **Perifosine.** Perifosine (NSC 639966), a synthetic substituted heterocyclic alkylphospholipid, was provided by Keryx Biopharmaceuticals. **nab-Rapamycin.** nab-Rapamycin (ABI-009) was provided by Abraxis Bioscience LLC. **Akti-1/2.** Akti-1/2 was procured from Calbiochem.

Cell viability and proliferation assays

**Colorimetric assay.** Colorimetric assays were done to assay drug activity. Forty-eight–hour cultures were pulsed with 10 μL of 5 mg/mL MTT (Chemicon International, Inc.) to each well, followed by incubation at 37°C for 4 h, and addition of 100 μL isopropanol with 0.04 HCl. Absorbance readings at a wavelength of 570 nm (with correction using readings at 630 nm) were taken on a spectrophotometer (Molecular Devices Corp.).

**Proliferation assay.** DNA synthesis was measured by tritiated thymidine uptake (3H-TdR) (Perkin-Elmer) as previously described (10). Briefly, MM.1S cells (2–3 × 10,000 per well) were incubated in 96-well culture plates alone or in coculture with BMSCs, recombinant interleukin-6 (IL-6; 10 ng/mL), or insulin-like growth factor-1 (IGF-I; 25 ng/mL) in the presence of media or varying concentrations of rapamycin, perifosine, or combination for 48 h at 37°C.

Immuno blotting

M cells were harvested, and whole-cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad Laboratories), as described previously (10). The antibodies used for immunoblotting included anti-p-Akt (Ser172), anti-Akt, anti-phosphorylated P70S6K (p-P70S6K), anti-P70S6K, anti-glyceraldehyde-3-phosphate dehydrogenase, anti-caspase-8, anti-caspase-3, anti-caspase-9, anti-poly (ADP-ribose) polymerase, and anti-tubulin (Cell Signaling Technology, Inc.).

Detection of apoptosis by annexin V/propidium iodide staining

Detection of early apoptotic cells was done with the Annexin V/propidium iodide (PI) detection kit (Immunotech/Beckman Coulter). Briefly, 10⁶ MM cells were exposed for 24 to 48 h to rapamycin (10 nmol/L), perifosine (5 μmol/L), or combination, washed, and then incubated in the dark at room temperature with Annexin V/FITC and PI for 15 min. Annexin V⁺ PI⁻ apoptotic cells
were enumerated using the Epics flow cytometer. Cells that were Annexin V/FITC1 positive (with translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane) and PI negative (with intact cellular membrane) were considered as early apoptotic cells, whereas positivity for both Annexin V/FITC1 and PI was associated with late apoptosis or necrosis.

**Immunocytochemical detection of LC3**

MM.1S cells were cultured in the presence of media, 10 nmol/L rapamycin, 5 umol/L perifosine, or combination for 3 h at 37°C, and cytopsins were prepared. Cells were fixed in 4% paraformaldehyde. The anti-LC3 polyclonal antibody (MBL I.C.) was diluted with PBS at 1:100 and incubated with cells overnight at 4°C. FITC-conjugated anti-rabbit IgG at 1:100 dilutions was added for 1 h at 4°C, and then 4',6-diamidino-2-phenylindole containing mounting medium and coverslips were added promptly. Samples were observed by fluorescence microscopy and were digitally photographed.

**Electron microscopy**

MM.1S cells were cultured in the presence of media or 10 nmol/L rapamycin, 5 umol/L perifosine, or combination for 3 and 16 h at 37°C. Cells were collected and fixed with 2.0% paraformaldehyde/2.5% EM grade glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) at 37°C. After fixation, samples were placed in 2% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer (pH 7.4), dehydrated in a graded series of ethyl alcohol, and embedded in resin. Ultrathin sections were cut and placed on formvar-coated slot copper grids. Sections were then counterstained with uranyl acetate and lead citrate and viewed with a TecnaiTM G2 Spirit Bio TWIN electron microscope. Digital images were acquired with an AMT 2k CCD camera (direct magnification, 1,400× and 6,800×).

**In silico study**

In *silico* study was done using the iC-PHYS Oncology Technology (Cellworks Group, Inc.; refs. 30, 31). The iC-PHYS Oncology platform consists of a dynamic representation of the signaling pathways underlying tumor physiology at the biomolecular level. All the key relevant proteins and associated gene and mRNA transcripts with regard to tumor-related signaling are comprehensively included in the system with their relationship quantitatively represented. The modeling of time-dependent changes in the fluxes of the constituent pathways has been carried out using modified ordinary differential
equations and mass action kinetics. The state of the system was set to simulate late tumor stage. The drug concentrations used in the model is assumed to be post ADME (absorption, distribution, metabolism, and excretion). The bottom layer is the computational backplane that enables the system to be dynamic and computes all the mathematics in the middle layer. The oncology platform is ported to iC-PHYS and is simulated so that all the molecules attain the control steady-state values (~by $1 \times 10^9$ s), following which the triggers are introduced into the system. This leads to a phase of disease progression, and the model stabilizes at steady disease levels by $2 \times 10^3$ s.

In initial conditions, the model simulated the kinetic interactions of the PI3K/Akt/mTor interactome based on proteomic data characterizing the pathophysiology of late-stage cancer disease. Rapamycin (10 nmol/L; Ki, 1e−2 nmol/L), perifosine (5 μmol/L; Ki, 3.79e−1 μmol/L), and their combination were tested on the system to observe the consequent effects on mTOR, p-Akt, and caspase levels.

**MM xenograft murine model**

The *in vivo* anti-MM activity of both single-agent nab-rapamycin and perifosine and the combination of nab-rapamycin and perifosine treatment was evaluated in CB-17 severe combined immunodeficient (SCID) mice obtained from Charles River Laboratories. Housed and monitored in the Animal Research Facility at the Dana-Farber Cancer Institute, mice were subjected to animal studies according to the protocols approved by the Animal Ethics Committee. Forty male mice (5–6 wk old) were irradiated (2 Gy, 200 rad) using cesium 137 ($^{137}$Cs) irradiator source; 24 h after irradiation 2.5 x $10^6$ MM.1S cells suspended in 100 μL of RPMI medium were inoculated s.c. When tumors were measurable, mice were randomly assigned into cohorts (10 mice per cohort) receiving nab-rapamycin (10 mg/kg by tail vein injections on days 1, 3, and 5 weekly for 4 wk), perifosine (125 mg/kg p.o. by gavage weekly), or both, or control vehicles. Tumor sections were stained with anti-p-Akt (Ser473), cleaved caspase-3 antibody (Cell Signaling Technology, Inc.), or LC3 antibody APG8a N-term (Abgent, Inc.) or were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining.

**Immunohistochemical staining**

Immunohistochemical staining was done using the standard avidin-biotin complex-peroxidase method on formalin-fixed, paraffin-embedded tissue sections of tumor excised from xenografts following 1 wk of treatment with either nab-rapamycin (10 mg/kg by tail vein thrice weekly), perifosine (125 mg/kg p.o. by gavage weekly), both, or control vehicles. Tumor sections were stained with anti-p-Akt (Ser473), cleaved caspase-3 antibody (Cell Signaling Technology, Inc.), or LC3 antibody APG8a N-term (Abgent, Inc.) or were subjected to terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining.

**Statistical analysis**

All *in vitro* experiments were done in triplicate and repeated at least thrice; a representative experiment was selected for Figs. 1D, 2B, and 2D. Statistical significances of differences were determined using Student’s *t* test, with minimal level of significance at *P* < 0.05. All statistical analysis of the *in vivo* data was determined using GraphPad prism software (GraphPad Software, Inc.). Synergism was determined by using the Chou-Talalay method (32).

**Results**

**Rapamycin induces p-Akt in MM cells, whereas perifosine inhibits p-Akt**

To confirm the effects of rapamycin signaling on MM cells, MM.1S cells were exposed to increasing concentrations of rapamycin for 2 hours. Rapamycin treatment resulted in a dose-dependent decrease of p-P70S6K. This was accompanied by an increase in phosphorylation of Akt at Ser473, starting at doses as low as 1 nmol/L. Inhibition of p-P70S6K and activation of p-Akt were observed as early as 30 min after exposure of MM cells to rapamycin, indicating that suppression of p-P70S6K

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**Table 1. Rapamycin and perifosine combination index (CI) values based on the Chou-Talalay method**

<table>
<thead>
<tr>
<th>Combination</th>
<th>Rapamycin (nmol/L)</th>
<th>Perifosine (μmol/L)</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>25</td>
<td>0.165</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>5.0</td>
<td>0.468</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>2.5</td>
<td>0.386</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>5.0</td>
<td>0.468</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>2.5</td>
<td>0.352</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>5.0</td>
<td>0.446</td>
</tr>
</tbody>
</table>

NOTE: MM.1S cells were cultured at varying concentrations of rapamycin (1, 5, and 10 nmol/L) with perifosine (2.5 and 5 μmol/L) and cytotoxicity evaluated by MTT
and activation of Akt are early, concurrent, and lasting effects induced by rapamycin in MM.1S cells (Fig. 1A).

We next examined the effects of perifosine on mTOR/Akt signaling in MM cells. MM.1S cells were cultured for 2 hours in the presence of increasing doses of perifosine (2.5–5 μmol/L). Because perifosine was able to completely inhibit Akt phosphorylation at 5 μmol/L, we next did a time course (0.5–5 hours) to examine the effects of perifosine (5 μmol/L) on Akt and P70S6K phosphorylation. Our data (Fig. 1B) shows that perifosine inhibited Akt without exhibiting evident effects on P70S6K phosphorylation in a dose-dependent and time-dependent manner.

We next incubated MM.1S cells with rapamycin (10 nmol/L), perifosine (5 μmol/L), or the combination for the specified times to study effects on cell signaling and cytotoxicity. As shown in Fig. 1C, rapamycin treatment resulted in increased p-Akt, which was overcome by the combination as early as 6 hours, associated with enhanced cytotoxicity at 48 hours (Fig. 1D).

To determine whether rapamycin effects were cell line specific, we tested other MM cell lines. Our data show activation of Akt in OPM1, OPM2, and U266 MM cells in the presence of rapamycin (10 nmol/L) at 6 hours. Similar to MM.1S cells, the combination of rapamycin and perifosine abrogated Akt phosphorylation in OPM1, OPM2, and U266 cells and resulted in enhanced cytotoxicity with the combination treatment in all three MM cell lines (Supplementary Data). Furthermore,
Figure 3. Rapamycin-mediated autophagy resulted in apoptosis when combined with perifosine. A, electronmicroscopy (direct magnification, 4,800×) of representative MM.1S cells cultured for 3 h in the presence or absence of rapamycin (10 nmol/L), perifosine (5 μmol/L), or their combination (N, nucleus; AV, autophagic vacuole). Note the abundance of membranous vacuoles in rapamycin and perifosine combination–treated cells.

B, immunofluorescence analysis for LC3 localization in MM.1S cells grown in the absence or presence of single drugs or their combination for 3 h. The control and perifosine-treated cells exhibited less intense diffuse distribution of LC3-associated green fluorescence compared with rapamycin-treated and combination-treated cells. Although rapamycin-treated and combination-treated cells displayed increased LC3 immunostaining, the punctate pattern was more evident when cells were treated with rapamycin in combination with perifosine. C, representative transmission electron micrographs [direct magnification, 4,800× (top) and 1400× (bottom)] depicting ultrastructures of MM.1S cells cultured in media or treated with rapamycin (10 nmol/L), perifosine (5 μmol/L), or their combination for 16 h. Whereas rapamycin-treated cells expressed multiple membranous vesicles (autophagy) with no evidence of nuclear (N) pyknosis, the perifosine-treated cells manifested morphologic features of apoptosis (chromatin condensation, C). Cells treated with the combination of rapamycin and perifosine developed features of both autophagy and apoptosis.
a 48-hour coculture of MM.1S cells with rapamycin 
(10 nmol/L) and the selective Akt kinase inhibitor 
Akti-1/2 (1.25 μmol/L) potentiated rapamycin-induced 
cytotoxicity (Supplementary Data), confirming the inhibition. 

Using Chou-Talalay method, we examined possible 
additive or synergistic antiproliferative effects of rapamycin 
and perifosine following 48 hours of treatment in 
MM.1S cells. Doses below IC_{50} for rapamycin (1–10 
nmol/L) and perifosine (2–5 μmol/L) were used for 
combination studies. Combination index (CI) ranged 
from 0.468 to 0.165, suggesting synergistic growth 
inhibitory activity (Table 1).

**Rapamycin and perifosine overcome the growth 
and survival advantage conferred by IL-6, IGF-I, 
and BMSCs in MM.1S cells**

Because of the critical role played by BMSCs and cytokines 
such as IL-6 and IGF-I on the growth and survival 
of MM cells and their effect on the PI3K/Akt pathway in 
the context of drug resistance, we examined the effects of 
rapamycin and perifosine combination in the presence of 
cytokines and stroma. As shown in Fig. 2A, IL-6 trig-
gerated Akt phosphorylation, which was inhibited when 
rapamycin and perifosine were combined. The suppres-
sion of p-Akt by rapamycin and perifosine after IGF-I

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**Figure 3 Continued.** D, fluorescence-activated cell sorting analysis for Annexin V/PI. MM.1S cells were cultured in the presence or absence of rapamycin and perifosine or their combination for 24 and 48 h. Annexin V/PI staining was then done to assess apoptosis/necrosis. Annexin V labeling (bottom right quadrants) represents the population undergoing apoptosis. Annexin V and PI double labelings (top right quadrants) represent cells that have already died by apoptosis. Live cells are represented in the bottom left quadrants. Increasing percentages of Annexin V–positive and PI-positive cells with increasing exposure to rapamycin perifosine combination treatment is shown in the top right quadrants. E, MM.1S cells were cultured for 24 h in control media, rapamycin (10 nmol/L), perifosine (5 μmol/L), or rapamycin and perifosine combination. The cells were harvested, lysed, and subjected to Western blotting using anti-caspase-8, anti-caspase-3, anti-caspase-9, anti-poly(ADP-ribose) polymerase antibodies, and anti-tubulin.
stimulation was not as robust, suggesting that once activated IGF-I signaling strongly upregulates Akt activity and there might be other signaling circuits contributing to p-Akt phosphorylation. However, when combined, rapamycin and perifosine increased the cytotoxicity in IL-6–stimulated and IGF-I–stimulated MM.1S cells (Fig. 2B).

Similarly, the combination was studied in the context of BMSCs. Adherence of MM.1S cells to BMSCs triggered upregulation of p-Akt; the combination blocked this effect, resulting in p-Akt downregulation (Fig. 2C). Furthermore, the proliferative advantage conferred by BMSCs was overcome by the combination, as shown by [3H]thymidine uptake and confirmed by CI of 0.986.

**Rapamycin-induced autophagy resulted in apoptosis when combined with perifosine**

Because an increasing number of studies indicate that inhibition of mTOR results in induction of autophagy, we examined whether rapamycin treatment triggers autophagy in MM.1S cells. Because our data show rapamycin-induced downregulation of p-P70S6K as early as 30 min suggesting rapid mTOR inhibition, we first determined whether rapamycin treatment triggered early autophagy. Second, because of p-Akt’s ability to disinhibit mTOR (33), we hypothesized that inhibition of rapamycin-induced p-Akt activity by the combination of rapamycin and perifosine might facilitate initiation of autophagy. MM.1S cells were exposed to rapamycin (10 nmol/L), perifosine (5 μmol/L), the combination, or media alone for 3 hours, and ultrastructural morphology of the cells were analyzed by electron microscopy. As seen in Fig. 3A, rapamycin-treated cells exhibited morphologic changes characteristic of autophagy with presence of single-membrane and double-membrane limiting vesicles sequestering the cytosolic material, which were not evident in perifosine-treated cells. These were more abundant when rapamycin and perifosine were combined. These microscopic observations suggested that rapamycin results in autophagy in MM.1S cells at early time points and that rapamycin-induced autophagy was enhanced when rapamycin and perifosine were combined.

To confirm rapamycin-induced autophagy and gain insights into the extent of increased autophagy triggered by the combination, we examined the effect of these drugs on localization of LC3, which serves as a marker of autophagy. We tested the effect of 3-hour treatment with rapamycin, perifosine, or both on localization of LC3 in MM.1S cells by immunofluorescence microscopy (Fig. 3B). Untreated control cells exhibited diffuse distribution of LC3-associated green fluorescence, whereas rapamycin-treated MM.1S cells displayed a punctate pattern of LC3 immunostaining with increased fluorescence indicating colocalization with autophagosomes. Perifosine-treated cells expressed less intense and mostly perinuclear staining, whereas the combination showed more focal LC3 green fluorescence predominantly in conglomerates, which suggests maturation of autophagic vacuoles.

Although autophagy is a response to various anticancer therapies, the extent to which autophagy contributes to cell death, known as type 2 or autophagic cell death, remains unclear. Shown in Fig. 3C are morphologic changes in MM.1S cells induced after 16 hours of treatment with rapamycin, perifosine, or the combination. Whereas untreated cells had normal nuclear and cytoplasmic morphology, rapamycin-treated cells developed typical features of autophagy with centrally condensed nuclear chromatin and numerous membranous vesicles. Higher magnification revealed double or multiple membrane boundaries surrounding cytoplasmic material and alternating with electron dense vesicles. Conversely, perifosine-treated cells manifested morphologic characteristics of apoptosis, with nuclear condensation (pyknosis) and fragmentation (karyorhexis), cell shrinkage, plasma membrane blebbing, and vacuolization. Rapamycin and perifosine cotreatment resulted in morphologic features of both autophagy and apoptosis, with evidence of double-membrane autophagolysosomes containing cytoplasmic fragments and disintegrated organelles typical of autophagy as well as condensation and margination of chromatin characteristic of apoptosis.

Given that rapamycin-perifosine cotreatment induced both apoptosis and autophagy features in MM.1S cells, we investigated the effect of this combination on apoptosis. As shown in Fig. 3D and E, although rapamycin induced caspase-8 cleavage, it did not result in significant apoptosis of MM cells at 24 or 48 hours. However perifosine resulted in apoptosis and necrosis of 30% of MM cells at 48 hours. The combination resulted in enhanced caspase-dependent apoptosis, manifested by increased caspase-3, caspase-8, caspase-9, and poly(ADP-ribose) polymerase cleavage (Fig. 3E).

Because the combination of rapamycin and perifosine was able to activate both autophagy and apoptosis in MM cells, we next investigated whether these cell death-associated phenomena were interconnected and defined their role in rapamycin and perifosine combination–induced programmed MM cell death. We evaluated whether blocking of either apoptosis or autophagy would compromise rapamycin and perifosine combination–induced cytotoxicity by assessing viability of MM.1S cells in the presence or absence of z-VAD-fmk or 3-MA pretreatment (Supplementary Data). Neither blockade of autophagy nor inhibition of apoptosis rescued MM cells from death induced by the combination, suggesting that cell death resulted once either mechanism was initiated.

**In silico rapamycin and perifosine combination study confirms the Akt/mTOR kinase downregulation and activated caspases**

For a more comprehensive understanding of the cellular mechanisms underlying the synergy of this combination, we proceeded with in silico tumor cell modeling. The objective was to analyze the predictive effects of the mTOR inhibitor rapamycin and the AKT inhibitor perifosine on the key kinases upregulated in cancer and on other major end points for cancer phenotypes of proliferation, survival, and tumor microenvironment. The
in silico study was done on the iC-PHYS Oncology platform. Various clinically important markers were observed, and their levels were quantitatively compared under conditions of untreated control, rapamycin alone (10 nmol/L), perifosine alone (5 μmol/L), or the combination.

The key marker values are presented as the percentage difference between control versus each drug alone or the combination (Fig. 4). The in silico study confirmed that rapamycin-induced mTOR/ATP inhibition associates with upregulated p-Akt. As expected, perifosine alone reduced Akt activity but did not have any effect on mTOR kinase level. Meanwhile, the combination decreased both Akt and mTOR kinases (Fig. 4A). Rapamycin alone had no effect on caspase activation, whereas perifosine, as expected, increased the activity of caspase-3, caspase-6, and caspase-9, and the combination ultimately resulted in cumulative signaling effects (Fig. 4B).

Effects of nab-Rapamycin and perifosine alone or in combination on MM tumor growth in vivo

We finally sought to establish whether our in vitro observations would translate to anti-MM activity in vivo using our MM murine xenograft model. Due to the poor water solubility of rapamycin, we studied nab-rapamycin as a promising candidate for our in vivo MM studies. We first evaluated the toxicity and anti-MM activity of nab-rapamycin treatment for 4 weeks in our MM xenografts SCID mouse model. Both i.v. daily (30 mg/kg/d) and 3× weekly (20 and 40 mg/kg/d) administration of nab-rapamycin resulted in significant inhibition of MM tumor growth and increased the survival of animals (data not shown). To investigate whether combined treatment with nab-rapamycin and perifosine would augment the anti-MM activity of each agent alone, MM tumor-bearing SCID mice were treated for 4 weeks with nab-rapamycin (10 mg/kg/d; n = 10 mice) via tail vein injections on days 1, 3, and 5 for 4 weeks, perifosine (125 mg/kg; n = 10 mice) via oral gavage on day 5 for 4 weeks, or combination (n = 10 mice) with nab-rapamycin on days 1, 3, and 5 and perifosine given on day 5 for 4 weeks). Combined treatment with nab-rapamycin and perifosine significantly inhibited the growth of MM cell xenografts compared with administration of solvent alone (Fig. 5A). Although each drug as a single agent inhibited tumor expansion...
(P < 0.05), combined nab-rapamycin and perifosine induced tumor growth arrest assessed by tumor growth inhibition index of 90% at the end of treatment.

Moreover, at 5 weeks follow-up after completion of nab-rapamycin or perifosine treatment, tumors started to regrow as early as 2 weeks. In contrast, all mice treated with the combination had smaller tumors, suggesting that therapeutic effects were maintained even after treatment was terminated.

Toxicity observed with the combination of nab-rapamycin and perifosine was evidenced by 20% weight loss at day 12 after initiation of treatment, which reversed after completion of treatment (Fig. 5B).

The control and treated animals were maintained for their natural life span or sacrificed in the presence of a very large (tumor volume, ≥1,000 mm³) or ulcerated tumor. A significant survival advantage was observed when nab-rapamycin was combined with perifosine, as shown in Fig. 5C. At day 61 after the beginning of treatment, only 10% of the animals survived in the control group versus 40% in each single-drug–treated groups; in contrast, 80% of the animals were alive in the combination-treated mice. Moreover, 80% of mice in the combination-treated arm were still alive at day 75 following treatment initiation. There were no survivors in the control or monotherapy cohorts.

Given the therapeutic efficacy of nab-rapamycin and perifosine combination in our in vivo MM model, we next examined the associated histologic events. Four mice were subjected to a similar in vivo study; mice were sacrificed, and tumors were collected after 1 week of treatment. As seen in Fig. 6A, nab-rapamycin induced p-Akt in tumor tissue, which was inhibited when nab-rapamycin was combined to perifosine. LC3 immunohistochemical staining identified distinct patterns: LC-3 diffuse cytoplasmic expression (basal) in vehicle-treated and nab-rapamycin–treated tumors versus patchy distribution staining in perifosine-treated tumor (Fig. 6B). Interestingly, the combination-treated tumor showed increased LC3 staining in both diffuse and patchy patterns, along with more cleaved caspase-3 and TUNEL-positive cells (Fig. 6C and D). These findings therefore support our in vitro data showing amplification of both autophagy and apoptosis.

**Discussion**

There is growing interest in targeting the PI3K/Akt/mTOR signaling cascade because of its critical role in the development of drug resistance. Indeed, the discovery that rapamycin specifically blocks mTOR suggested its potential in cancer therapy. However, the cytoreduction and G1 arrest triggered by rapamycin in vitro did not translate into significant single-agent clinical antitumor activity, highlighting the need for studying combination and alternative strategies.

Several studies done on various cancer types (lung cancer, glioma, and colorectal carcinoma) including MM have characterized the molecular mechanisms of reduced sensitivity to rapamycin. Specifically, rapamycin-induced
activation of Akt follows the disinhibition of IGF-I/insulin receptor substrate-1 (IRS-1) signaling subsequent to downregulation of p-P70S6K (20, 25). Additionally, a recent study in rhabdomyosarcoma cell lines and xenografts suggested that mTOR/S6K1 inhibition-mediated feedback activation of Akt can also occur via an IRS-1-independent mechanism (18) due to the ability of rapamycin-insensitive mTORC2 (complexed with Rictor).

Figure 6. Ex vivo immunohistochemistry staining for p-Akt, LC3, TUNEL, and cleaved caspase-3 in MM xenografts. A, p-Akt was detected by immunohistochemistry using paraffin-embedded sections of tumor excised from MM mouse xenografts. Increased staining for p-AKT was observed in the nab-rapamycin, and this was inhibited in perifosine and combination-treated tumors relative to untreated (control) or perifosine-treated mice. B, LC3 protein was detected by immunohistochemistry using paraffin-embedded sections of tumor excised from MM mouse xenografts. A more pronounced staining for LC3 was observed in the nab-rapamycin and perifosine combination–treated case relative to untreated (control), nab-rapamycin–treated, or perifosine–treated mice. C and D, representative photomicrographs of TUNEL and cleaved caspase-3 immunohistochemical staining of both tumors excised from MM xenograft mice treated with control vehicle, nab-rapamycin, perifosine, or both nab-rapamycin and perifosine. Greater caspase-3 and TUNEL staining was observed in tumor from combination-treated relative to control and single agent–treated mice.
to directly phosphorylate and activate Akt at Ser473, thus providing a level of additional positive feedback to the pathway.

Because mTOR may function both upstream and downstream of Akt, an agent directly targeting Akt rather than targeting its precursors, such as IGF-1/IRS-1 and P13K, would more likely overcome reduced sensitivity to rapamycin. After confirming that suppression of mTORC1 signaling by rapamycin in MM cells was associated with upregulation of Akt phosphorylation and that inhibition of p-P70S6K and activation of Akt, occurred as concurrent, early, and lasting effects; we used the Akt inhibitor perifosine for the direct inactivation of rapamycin-induced Akt. Consistent with previous data, perifosine resulted in inhibition of constitutive phosphorylation of Akt. Importantly, because the lowest dose (5 μmol/L) at which perifosine exhibited strong p-Akt inhibition had minor effect on P70S6K phosphorylation status, we show that combining rapamycin with perifosine results in inhibition of rapamycin-induced Akt without influencing rapamycin-mediated mTORC1 signaling, thereby enhancing rapamycin-mediated cytotoxicity.

Because rapamycin does not cause apoptosis in MM at low concentrations and a growing body of evidence indicates that rapamycin-induced antitumor effect is likely mediated through autophagy, we studied autophagy in MM cells to elucidate the mechanism of rapamycin-induced anti-MM activity. Essential for maintaining cell autonomous survival in normal growing conditions, autophagy is necessarily self-limited; various intracellular and extracellular stimuli enhance autophagic cell death (type II programmed cell death) if the stress is sustained. Through inhibition of mTOR, which suppresses autophagy, rapamycin activates the autophagic process. The observations that inhibition of autophagy by small interfering RNA directed against the autophagy-related gene beclin 1 abrogates rapamycin-induced cytotoxicity and that silencing of mTOR with small interfering RNA increases the inhibitory effect of rapamycin by stimulating autophagy (34) suggest that rapamycin-induced autophagy is primarily an antitumor rather than a cell protective effect. However, whether mTOR inhibitors promote autophagy and autophagic cell death in MM was previously unknown. Moreover, recent data have suggested that proautophagic rapamycin activity may prevent apoptosis (35). Here we have shown that autophagy occurs in MM cells shortly after rapamycin treatment, correlating with the inhibition of mTOR as an early-dose and low-dose response to rapamycin. Because the extent of autophagy increased in a dose-dependent and time-dependent manner with no notable apoptosis, as assessed by Annexin V/PI analysis, we suggest that rapamycin’s cytotoxic effect on MM cells is mainly mediated via autophagy rather than apoptosis. Because activated Akt has been shown to inhibit mTOR and suppress autophagy, we augmented rapamycin-induced autophagy by perifosine inactivation of Akt.

Data from several studies point out that autophagy and apoptosis may be interconnected in some settings and even simultaneously regulated by the same trigger resulting in different cellular outcomes. Akt/mTOR is one of the few converging molecular links in both autophagy and apoptosis signaling. Our data suggest that rapamycin-induced autophagy in MM cells results in apoptosis when combined with perifosine. However, neither alternative nor concomitant inhibition of apoptosis and autophagy rescued MM cell when rapamycin and perifosine were combined, suggesting a more complex signaling interaction underlying the synergistic effects of this promising anticancer drug combination.

To this end, we used the in silico predictive modeling system based on mathematical analysis of cellular networks provided by a systems biology approach. Multiscale in silico study of the predicted biology of rapamycin and perifosine combined effects on the tumor cell confirmed and complemented our in vitro experimental findings.

Although mTOR inhibitors, such as rapamycin analogues CCI-779, RAD001, and AP23573, have shown preclinical promise, their roles as single agents in phases 2 and 3 studies have resulted in only modest responses. Preclinical studies of nab-rapamycin (ABI-009) in breast and colon cancer in in vivo models showed antitumor activity, suggesting potential clinical utility. Moreover nab-rapamycin was well tolerated (no observed hypercholesterolemia and hypertriglyceridemia) overcoming the limitations posed by the poor water solubility of rapamycin (36). Specifically the binding of water-insoluble rapamycin to nanoparticle albumin permits albumin-mediated transcytosis of rapamycin by microvessel endothelial cells, and the SPARC-albumin interaction may further increase accumulation of albumin-bound drug in the tumor. Whereas the role of SPARC in MM is not fully understood, there is evidence that SPARC is upregulated in extramedullary tumor growth of MM (37, 38). Additionally, nab-rapamycin recently showed promising data in phase I clinical trials in patients with advanced nonhematologic malignancies, prompting us to test nab-rapamycin in our studies.

We examined the effects of nab-rapamycin with the Akt inhibitor perifosine in vivo in our MM murine xenograft models, hypothesizing that anti-MM therapeutic effects would be enhanced both by dual inhibition of the Akt/mTOR pathway and also due to lower doses and better tolerability of nab-rapamycin. Our in vivo results showed that combination treatment led to statistically significant MM tumor growth inhibition and increased survival in mice. Collectively our data suggest that mutual suppression of the PI3K/Akt/mTOR pathway by rapamycin and perifosine cotreatment induces both autophagy and apoptosis resulting in synergistic cytotoxicity in MM, providing the rationale for combination clinical trials in patients with MM.
Disclosure of Potential Conflicts of Interest

N. Raje has a research grant from AstraZeneca and is a consultant to Amgen, Celgene, and Novartis. No other potential conflicts of interest were disclosed.

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