Mechanism by Which Mammalian Target of Rapamycin Inhibitors Sensitize Multiple Myeloma Cells to Dexamethasone-Induced Apoptosis

Huajun Yan, Patrick Frost, Yijiang Shi, Bao Hoang, Sanjai Sharma, Myrna Fisher, Joseph Gera, and Alan Lichtenstein

Departments of Medicine and Pathology, Greater Los Angeles VA Healthcare Center, University of California at Los Angeles School of Medicine and Jonsson Comprehensive Cancer Center, Los Angeles, California

Abstract
Mammalian target of rapamycin (mTOR) inhibitors curtail cap-dependent translation. However, they can also induce post-translational modifications of proteins. We assessed both effects to understand the mechanism by which mTOR inhibitors like rapamycin sensitize multiple myeloma cells to dexamethasone-induced apoptosis. Sensitization was achieved in multiple myeloma cells irrespective of their PTEN or p53 status, enhanced by activation of AKT, and associated with stimulation of both intrinsic and extrinsic pathways of apoptosis. The sensitizing effect was not due to post-translational modifications of the RAFTK kinase, Jun kinase, p38 mitogen-activated protein kinase, or BAD. Sensitization was also not associated with a rapamycin-mediated increase in glucocorticoid receptor reporter expression. However, when cap-dependent translation was prevented by transfection with a mutant 4E-BP1 construct, which is resistant to mTOR-induced phosphorylation, cells responded to dexamethasone with enhanced apoptosis, mirroring the effect of coexposure to rapamycin. Thus, sensitization is mediated by inhibition of cap-dependent translation. A high-throughput screening for translational efficiency identified several antiapoptotic proteins whose translation was inhibited by rapamycin. Immunoblot assay confirmed rapamycin-induced down-regulated expressions of XIAP, CIAP1, HSP-27, and BAG-3, which may play a role in the sensitization to apoptosis. Studies in a xenograft model showed synergistic in vivo antmyeloma effects when dexamethasone was combined with the mTOR inhibitor CCI-779. Synergistic effects were associated with an enhanced multiple myeloma cell apoptosis in vivo. This study supports the strategy of combining dexamethasone with mTOR inhibitors in multiple myeloma and identifies a mechanism by which the synergistic effect is achieved.

Introduction
Mammalian target of rapamycin (mTOR) inhibitors have shown significant potential in multiple myeloma (1–3). These drugs target the mTOR kinase whose immediate downstream substrates are the p70S6 kinase and 4E-BP1 translational repressor (reviewed in ref. 4). mTOR-dependent phosphorylation of p70 and 4E-BP1 is critical for cap-dependent translation of cell cycle proteins (5, 6). Thus, mTOR inhibitors induce G1 arrest (4).

Although cell cycle arrest might temporarily halt myeloma progression, affected clones could regrow if tumor cells have not been killed. Thus, mTOR inhibitors would, theoretically, be more effective if they were used to induce apoptosis. Interestingly, mTOR activity can regulate apoptotic death in some situations (reviewed in ref. 7). In fact, when combined with dexamethasone (3), mTOR inhibitors can induce a remarkable apoptotic response in multiple myeloma cells.

To date, the mechanism by which mTOR inhibitors potentiate multiple myeloma cell apoptosis is unknown. Although the best known effect of mTOR inhibition is depression of cap-dependent translation, mTOR inhibition can also affect other signal pathways by inducing post-translational modifications. These pathways can potentially regulate apoptosis and stress responses (8, 9), growth factor responses (10), and glucocorticoid receptor function (11, 12). Thus, these latter effects of mTOR inhibition are also potential explanations for the enhancement of dexamethasone-induced multiple myeloma cell apoptosis.

In this report, we confirm that the mTOR inhibitor rapamycin sensitizes multiple myeloma cells to dexamethasone-induced apoptosis, that sensitization is independent of p53 and PTEN mutational status, and that AKT activity enhances the apoptotic response. We then rule out the possibility that sensitization is mediated by effects on RAFTK kinase, c-Jun NH2-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), the BCL-2 family member protein BAD, and the glucocorticoid receptor. In contrast, when cap-dependent translation was prevented by transfection of multiple myeloma cells with a mutant 4E-BP1, which is resistant to mTOR-induced phosphorylation, transfected cells responded to dexamethasone with significantly enhanced apoptosis, mirroring the effect of coexposure to rapamycin. These results indicate that dephosphorylation of 4E-BP1 with attendant decrease in free eIF-4E levels and cap-dependent translation mediates the proapoptotic effect of rapamycin. Screening for translational efficiency of apoptosis-regulating transcripts showed that rapamycin selectively decreased translation of antiapoptotic proteins, but translation of many proapoptotic proteins was maintained. Further experiments in a xenograft model showed that the enhanced apoptotic effect of combining mTOR inhibitors with dexamethasone might result in enhanced antitumor effects in vivo.

Materials and Methods
Reagents, cell lines, and cell treatments. The multiple myeloma lines were maintained as described previously (1, 2). All antibodies were...
cells were incubated with a 1:100 dilution of rabbit anti-4E-BP1 antibody to confirm expression of the 4E-BP1 mutant transgene, the fixed and washed anti-caspase-3 antibody for 30 minutes and analyzed by flow cytometry. To permeabilize cells were then incubated with a 1:5 dilution of PE-conjugated monoclonal antibody to detect the myc tag of the expressed construct.

Gradient was monitored via UV absorbance at 260 nm. Polysome-associated polysomal RNA to monosomal RNA signal intensities after rapamycin was done as described previously (13). Briefly, cells were lysed in cycloheximide, and following removal of nuclei and mitochondria, polysomes was done as described previously (13). Polysomal RNA was extracted and precipitated. The polysome profile of the mRNA moved from the monosomal to polysomal fractions was extracted and precipitated. The polysome profile of the gene contains serine/threonine-to-alanine mutations at five separate sites, thus preventing serine/threonine phosphorylation. The vector expressing the dominant-negative p70S6 kinase containing a myc tag, termed pRK5-mTORi, was a kind gift from Dr. Richard Pearson (Peter MacCallum Cancer Institute, Melbourne, Australia). Myeloma cells were cotransfected with 1.5 μg of these constructs along with 0.5 μg pEGFP-C2. For each mutant construct, an empty vector was also transfected with the enhanced green fluorescent protein (EGFP)—expressing plasmid. After 24 hours, the cells were treated with or without dexamethasone and cultured for an additional 36 hours. To identify apoptotic cells, we used a phycoerythrin (PE)—conjugated antibody specific for activated caspase-3 (BD Biosciences). For staining, 10^6 cells were washed with PBS and fixed and permeabilized with 0.5 μL cytofix/cytoperm solution (BD Biosciences). The cells were then incubated with a 1:5 dilution of PE-conjugated monoclonal anti-caspase-3 antibody for 30 minutes and analyzed by flow cytometry. To confirm expression of the 4E-BP1 mutant transgene, the fixed and washed cells were incubated with a 1:100 dilution of rabbit anti-4E-BP1 antibody (Cell Signaling) for 30 minutes on ice. The cells were washed and incubated with a 1:100 dilution of PE-conjugated F(ab')2 donkey anti-rabbit IgG (The Jackson Laboratory, Bar Harbor, ME) in 20% fetal bovine serum for 1 hour on ice and analyzed by flow cytometry. To confirm expression of the p70 dominant-negative transgene, we used a rabbit anti-myc polyclonal antibody to detect the myc tag of the expressed construct.

Polysome and microarray analysis. Extraction and display of polyosomes was done as described previously (13). Briefly, cells were lysed in cycloheximide, and following removal of nuclei and mitochondria, supernatants were layered onto 15% to 50% sucrose gradients and spun at 38,000 rpm for 2 hours at 4°C in a SW40 rotor (Beckman Instruments, San Antonio, TX). Centrifuged gradients were fractionated into eleven 1 mL fractions using an ISCO density gradient fractionator. RNA from individual fractions was extracted and precipitated. The polysome profile of the gradient was monitored via UV absorbance at 260 nm. Polysome-associated RNA and monosome-associated RNA was separately pooled. RNA was then used to generate labeled cDNA and probes as described previously (13). The resulting cDNA probe (10 μg) was hybridized to each GEArray-Apoptosis membrane (SuperArray Bioscience, Frederick, MD) and the hybridization signal was detected with Chemiluminescent Detection kit (SuperArray Bioscience). The relative RNA content of each lane was analyzed utilizing GEArray analyzer and ScanAlayze software. The change in translational state induced by rapamycin for a given mRNA was defined as the ratio of polysomal RNA to monosomal RNA signal intensities under control conditions (i.e., no rapamycin). A value of >1 indicates that the mRNA moved from the monosomal to polysomal fractions on rapamycin treatment. If the ratio was <1, the value was inverted and a negative number was used to indicate a change in the reverse direction, indicating a shift for a given mRNA from polysomal to monosomal fractions.

**Xenograft model.** As described previously (14), OPM-2 myeloma cells were mixed with Matrigel (BD Biosciences) and then injected s.c. (3 × 10^6 per mouse) into NOID/SCID mice. Mice were randomized to different treatment groups when the tumor volume reached ~200 to 400 mm^3^.

Tumor volume was calculated as described previously (14). CCI-779 (a generous gift from Wyeth, Inc., Pearl River, NY) was given i.p. each day for 5 days followed by 2 days of no drug and then five additional daily injections (total of 10 injections). Dexamethasone was also injected i.p. each day for the same 10 days at a dose of 9 μg/mouse.

**Immunohistochemistry.** After 4 days of treatment, some mice were sacrificed and tumor was processed for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining as described previously (14). The apoptotic index was determined by counting the total number of positive nuclei in 10 randomly selected fields at ×20 magnification.

**Statistics.** t test was used to determine significance of differences between groups.

**Results.** Rapamycin enhances dexamethasone-induced multiple myeloma cell apoptosis. By itself, rapamycin, used at 1 or 10 nmol/L, had no significant effect on apoptosis of the OPM-2, 8226, or MM1.S multiple myeloma cell lines (Fig. 1A), although, by cell cycle analysis, this concentration effectively induced G1 arrest. Dexamethasone, used alone at 1 μmol/L, had a minimal apoptotic effect on OPM-2 cells and a moderate effect on 8226 cells (27 ± 5% apoptosis, mean ± SD, n = 3). As expected, based on previous reports (15), MM1.S cells were more sensitive to dexamethasone used alone with >80% death when used at 1 μmol/L (data not shown). Figure 1A shows that the effects of dexamethasone on MM1.S cells were significant even when used at 0.01 μmol/L (52 ± 6%, mean ± SD). Although rapamycin had no effect by itself, when combined with dexamethasone, a remarkable increase in multiple myeloma cell death ensued in all three cell lines. In immunoblot experiments not shown, cleavage of caspase-3, caspase-8, and caspase-9 was not induced by rapamycin alone, was minimal in OPM-2 and 8226 multiple myeloma cells exposed to dexamethasone alone, but was marked when the two agents were used in combination. Thus, the enhanced apoptosis seen when rapamycin is added to dexamethasone is associated with activation of both intrinsic and extrinsic death cascades. These studies confirm that rapamycin can enhance dexamethasone-induced apoptosis in relatively dexamethasone-sensitive multiple myeloma cells (i.e., MM1.S) as well as relatively resistant cells (i.e., OPM-2). PTEN status also seems unrelated, as OPM-2 cells are PTEN null and 8226 expresses wild-type PTEN (1). In addition, as MM1.S cells contain wild-type p53 (16) and 8226 cells are p53 null (17), it seems that the enhanced apoptotic effect is also independent of p53 status.

In dose-response experiments, the ability of combined treatment to induce apoptosis was more related to the concentration of dexamethasone used rather than rapamycin (Fig. 1B). As shown, although the degree of apoptosis was directly correlated with dexamethasone concentration, a threshold concentration of rapamycin between 0.01 and 1 nmol/L seemed sufficient to allow enhanced apoptosis. This is consistent with expected optimal mTOR inhibition induced by rapamycin when concentrations are >0.1 nmol/L.

Sequential exposure to the two drugs induced comparable synergistic multiple myeloma cell apoptosis. When OPM-2 cells purchased from Cell Signaling, Inc. (Beverly, MA). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.**
were exposed to dexamethasone \( (10^{-6} \text{ mol/L}) \) plus rapamycin \( (10 \text{ nmol/L}) \) concurrently, apoptosis rose from 23 ± 3% (dexamethasone alone) to 83 ± 5%. In the same experiment, when OPM-2 cells were first exposed to rapamycin for 6 hours, washed, and then incubated with dexamethasone for 48 hours, apoptosis was 70 ± 4%. When the sequence was reversed (dexamethasone for 6 hours followed by rapamycin for 48 hours), apoptosis was 80 ± 6%.

A similar synergistic induction of multiple myeloma cell apoptosis was seen when both agents were used to treat three primary multiple myeloma specimens isolated from patient bone marrow. Dexamethasone alone at 1 µmol/L induced 24 ± 6% apoptosis (mean ± SD, \( n = 3 \) after 48-hour treatment) and rapamycin at 100 nmol/L induced 14 ± 4% apoptosis. When the two drugs were combined, 65 ± 6% apoptosis resulted (mean ± SD).

Cell lines with heightened AKT kinase activity are more sensitive to G1 arrest induced by mTOR inhibitors (18, 19). To test if AKT activity also could regulate sensitivity to apoptosis, we investigated U266 multiple myeloma cells stably transfected with an activated AKT allele or empty vector (14). Both cell lines were resistant to any significant degree of apoptosis induced by either rapamycin at 100 nmol/L or dexamethasone at \( 10^{-8} \text{ to } 10^{-6} \text{ mol/L} \) when used alone (Fig. 1C). However, a significant degree of apoptosis was induced when both drugs were used against AKT-transfected U266 cells (black columns), whereas such enhanced apoptosis was not seen in the empty vector control cell line. Thus, in similar fashion to the regulatory role of AKT in responsiveness to G1 arrest (18, 19), enhanced AKT activity results in a greater degree of apoptosis following combination treatment with rapamycin plus dexamethasone.

Enhanced apoptosis is not due to effects on RAFTK, BAD, JNK/p38 MAPK, or glucocorticoid receptors. Previous work (20) showed that dexamethasone-induced apoptosis of multiple myeloma cells was mediated via activation of RAFTK. Thus, we asked whether dexamethasone-induced RAFTK activation was significantly increased by coexposure to rapamycin. Consistent with prior reports (20), tyrosine phosphorylation of RAFTK was induced in OPM-2 cells at 3 hours (Fig. 2A) by dexamethasone. However, no RAFTK activation was induced by rapamycin nor was there any increase in activation when rapamycin was added to dexamethasone. Similar results were detected in 8226 and MM1.5 multiple myeloma cells. These data rule out a possible effect of rapamycin on RAFTK as an explanation for enhanced apoptosis.

Another potential mechanism by which rapamycin could sensitize to apoptosis is through effects on BAD. An mTOR-dependent activation of the p70S6 kinase results in p70-induced phosphorylation of BAD on Ser 136 (9). Ser 136 phosphorylation probably as a result of the PTEN-null state of this cell line (1) and resulting up-regulated activity of AKT, which can also phosphor-

### Figure 1. Synergistic apoptosis in varying multiple myeloma cell lines and at varying concentrations of drugs. A. OPM-2, 8226, and MM1.5 cell lines cultured for 48 hours in medium (C), rapamycin alone (R), dexamethasone alone (D), or combination of both (R/D). Rapamycin concentration was 100 nmol/L for all lines. Dexamethasone concentration was 1 µmol/L for OPM-2 and 8226 and 0.01 µmol/L for MM1.5 cells. Columns, mean % apoptosis of three independent experiments; bars, SD. The degree of apoptosis induced by the drug combination in all three cell lines was significantly greater \( (P < 0.05) \) than the sum of that induced by rapamycin and dexamethasone when used alone. B. OPM-2 cells were treated without or with increasing concentrations of rapamycin (RAP), dexamethasone (DEX), or the combination of both drugs. Rapamycin used at 100 nmol/L had no inhibitory effect on constitutive BAD phosphorylation. Constitutive BAD Ser \( ^{136} \) phosphorylation in OPM-2 cells was detected under serum-free conditions (Fig. 2B) probabil as a result of the P70S6 kinase results in p70-induced phosphorylation of BAD on Ser \( ^{136} \) (9). Ser \( ^{136} \) phosphorylation probably as a result of the PTEN-null state of this cell line (1) and resulting up-regulated activity of AKT, which can also phosphor-

<table>
<thead>
<tr>
<th>Drug Concentration (µmol/L)</th>
<th>OPM-2</th>
<th>8226</th>
<th>MM1.5</th>
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<tr>
<td>0.01</td>
<td>23</td>
<td>82</td>
<td>64</td>
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<tr>
<td>0.1</td>
<td>37</td>
<td>98</td>
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<td>1.0</td>
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**Note:** All data are expressed as mean ± SD of three independent experiments.
JNK (22, 23) or p38 stress-activated protein kinases (SAPK; ref. 24). Although dexamethasone, when used alone, does not induce activation of SAPKs (23), it was possible that, when combined with rapamycin, activation does occur and participates in heightened apoptosis. Furthermore, activation of JNK can mediate rapamycin-induced apoptosis in p53-null cells (8). Both p38 and JNK activity were modestly activated by rapamycin in OPM-2 cells (Fig. 2C) and this activation was inhibited by specific p38 and JNK inhibitors, which completely ablated kinase activation when used at 10 μmol/L. However, identical concentrations of these inhibitors had no effect on the enhanced apoptosis induced by combining rapamycin with dexamethasone (Fig. 2C, right). Similar results were seen with the 8226 multiple myeloma cell line (data not shown).

Thus, it is clear that activation of SAPK cascades by rapamycin was not critical to the drug’s ability to enhance multiple myeloma cell death.

Rapamycin can also enhance glucocorticoid receptor function by its ability to alter the ratio of FKBPS1 to FKBPS2 immunophillin within the glucocorticoid receptor heterocomplex (11, 12, 25, 26). Thus, to address this possible effect in dexamethasone-induced apoptosis, we did glucocorticoid receptor–induced reporter expression assays in multiple myeloma cells. The firefly luciferase-expressing reporter plasmid was transfected into the OPM-2 cell line, cells were treated with dexamethasone with or without the addition of rapamycin, and reporter expression was assayed. As expected, addition of dexamethasone at 10^−8 to 10^−6 mol/L induced a huge amount of reporter expression relative to cells not stimulated with dexamethasone (100- to 160-fold; Fig. 3A, white columns). However, coaddition of rapamycin, at a concentration known to result in enhancement of dexamethasone-induced apoptosis, had no effect on reporter expression (Fig. 3A, black columns). A similar absence of effect induced by rapamycin on reporter expression was seen in 8226 cells (data not shown).

Because rapamycin might prevent an enhanced luciferase reporter expression of protein due to its ability to curtail translation, we repeated the reporter expression experiment shown in Fig. 3A but assayed luciferase mRNA instead of protein. As shown in Fig. 3B, a significant induction of luciferase reporter RNA was seen following dexamethasone stimulation but addition of rapamycin either as a 3-hour pretreatment or as a coexposure had no effect on luciferase RNA expression. These results rule out the possibility that rapamycin enhances dexamethasone-mediated apoptosis via modulation of glucocorticoid receptor–induced gene expression.

**Genetic prevention of 4E-BP1 phosphorylation but not p70S6 kinase phosphorylation enhances dexamethasone-induced apoptosis.** mTOR regulates translation via two separate parallel pathways: via activation of p70 or by phosphorylation of 4E-BP1. To test whether inhibition of one or both of these pathways mediated the ability of rapamycin to sensitize to apoptosis, we used a transient transfection system where cells were transfected with a dominant-negative p70 construct or a 4E-BP1 mutant, which is incapable of undergoing mTOR-mediated phosphorylation. The p70 dominant negative is a kinase-inactive mutant shown to prevent endogenous p70 activation (27). The 4E-BP1 mutant columns). However, coaddition of rapamycin, at a concentration known to result in enhancement of dexamethasone-induced apoptosis, had no effect on reporter expression (Fig. 3A, black columns). A similar absence of effect induced by rapamycin on reporter expression was seen in 8226 cells (data not shown).

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contains serine/threonine-to-alanine mutations at five (S/T)P sites, thus preventing serine/threonine phosphorylation and preventing the release of eIF-4E from 4E-BP1 (28). Each of these ectopically expressed genes would theoretically mimic the effects of rapamycin on either 4E-BP1 or p70, allowing the presence of huge amounts of nonphosphorylatable 4E-BP1 or prevention of p70S6 kinase activity. Our vectors were cotransfected with an EGFP construct, which allowed identification of transfected cells. EGFP-expressing transfected cells were first tested for transgene expression by two-color flow cytometry. The transfection efficiency was 10% to 15% in repeated experiments assayed by determination of green fluorescence. As shown in Fig. 4A, when gating on EGFP-expressing cells, transfection with the 4E-BP1 mutant resulted in an ~10-fold enhancement of 4E-BP1 expression when compared with the empty vector control (mean channel fluorescence of 4,958 versus 510) and transfection of the p70 dominant-negative construct resulted in an ~50-fold enhancement of p70 expression (mean channel fluorescence of 980 versus 20.6).

In subsequent experiments, multiple myeloma cells were transiently cotransfected with the mutant constructs plus EGFP construct followed by treatment with drugs and two-color flow cytometry to determine the percentage of cells undergoing apoptosis. Apoptosis was determined in EGFP-gated cells by a PE-conjugated antibody specific for activated caspase-3. As shown in Fig. 4B, empty vector transfected OPM-2 myeloma cells showed a small but significant amount of apoptosis when exposed to dexamethasone alone (18 ± 3%, mean ± SD of three experiments), but rapamycin was ineffective when used alone. As shown previously with untransfected cells, empty vector transfected cells were capable of a significant enhancement (P < 0.05) of apoptosis when dexamethasone was combined with rapamycin (Fig. 4B, D/R; 62 ± 5%, mean ± SD). When cells were transfected with the

Figure 3. Rapamycin does not increase glucocorticoid receptor transactivation. A, the reporter plasmid was transfected into OPM-2 cells and cells were treated with or without dexamethasone (concentration in mol/L) and with or without rapamycin (100 nmol/L) for 3 hours when firefly luciferase activity was assessed. A Renilla luciferase gene-containing plasmid was used in cotransfections to control for transfection efficiency. Data are relative expression levels induced by dexamethasone when compared with identical cells not stimulated with dexamethasone. White columns, dexamethasone alone; black columns, dexamethasone + rapamycin. Columns, mean of three experiments; bars, SD. B, OPM-2 cells were transfected with the reporter plasmid and cells were treated in medium for 3 hours (C), exposed to 1 μmol/L dexamethasone for 3 hours (D), exposed to dexamethasone for 3 hours after a 3-hour pretreatment with 100 nmol/L rapamycin (D+R), or concurrently exposed to dexamethasone + rapamycin for 3 hours (D+P). RT-PCR assay was then done for expression of luciferase RNA (LUC) or control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA. Experiment was repeated once with identical results.

Figure 4. A nonphosphorylatable 4E-BP1 mutant sensitizes to dexamethasone-induced apoptosis. A, OPM-2 cells were transiently transfected with the mutant 4E-BP1 (4E-BP1m) or its empty vector or the dominant-negative p70 construct (P70dn) or its empty vector. Two-color flow cytometric analysis was then done for EGFP expression (green fluorescence, X axis) or transgene expression (red fluorescence, Y axis). Mutant 4E-BP1 expression was identified with an antibody that recognizes 4E-BP1 and dominant-negative p70 expression was identified with an antibody to the myc tag of the construct. B, OPM-2 cells were transiently transfected with the nonphosphorylatable mutant 4E-BP1 or its appropriate empty vector or the dominant-negative p70 or its empty vector control. Twenty-four hours later, cells were treated in medium (control) or with 1 μmol/L, dexamethasone, 100 nmol/L rapamycin, or the combination of the two drugs, after which transfected cells were isolated by gating on EGFP fluorescence and the percentage of EGFP-positive cells stained with an antibody to activated caspase-3 was identified. Columns, mean % apoptosis of three separate experiments; bars, SD.
4E-BP1 mutant, a remarkable enhancement of apoptosis induced by dexamethasone alone was shown when compared with cells transfected with empty vector (58 ± 6% compared with 18 ± 3%, mean ± SD of three separate experiments; P < 0.05). The levels of dexamethasone-induced apoptosis in 4E-BP1 mutant-transfected cells were comparable with that seen in both empty vector transfected and 4E-BP1-transfected cells treated with dexamethasone plus rapamycin. In contrast, ectopic expression of the dominant-negative p70 (Fig. 4B, P70dn) had no enhancing effect on dexamethasone-induced apoptosis. These results indicate that preventing phosphorylation of 4E-BP1 markedly sensitizes to dexamethasone-induced apoptosis of myeloma cells, strongly supporting the notion that the enhancing effect of rapamycin is mediated via dephosphorylation of 4E-BP1. In experiments not shown, the peptide elongation inhibitor cycloheximide could not sensitize to dexamethasone-induced apoptosis. This indicates that rapamycin does not enhance apoptosis through a generalized inhibition of mRNA translation but points to selective effects on translation of key proteins.

Assessment of translational state of apoptosis-regulating proteins. To investigate which proteins may be selectively affected by rapamycin, we used a high-throughput methodology (13), whereby microarray analysis of mRNA translational state can be assessed. This technique is based on the observation that well-translated transcripts are typically associated with polysomes, whereas poorly translated mRNAs are monosomal. OPM-2 cells were treated with or without rapamycin (100 nmol/L) for 18 hours and extracts were prepared for polysome analysis after sucrose gradient separation. Polysomal material and monosomal material (monitored by UV absorbance at 260 nm) was separately pooled and the associated RNAs were extracted and reverse transcribed separately to generate labeled probes and hybridized to a specific “Apoptosis” microarray (OHS-012 Oligo GEArray Human Apoptosis Microarray), which interrogates 112 key genes involved in apoptosis. The mRNA translational state for a given transcript was defined as the ratio of polysomal to monosomal signal intensity. The differences between these ratios in rapamycin-treated versus untreated cells gave an estimate of change in translational state (see Materials and Methods).

Transcripts of 52 genes from the 112 genes assessed (46%) were reproducibly detected. We identified a subset of 8 mRNAs whose translation was significantly inhibited (>2.5-fold inhibition) and 8 others whose translational state significantly increased (>2.5-fold increase) on exposure to rapamycin (Fig. 5A). Of the eight genes whose translation was significantly increased, six are considered proapoptotic (Fas ligand, p53-binding protein 2, BIM, p53, DR5, and hara-kiri), whereas two are antiapoptotic (NOL3 and RIPK1). Of the eight genes whose translation was significantly decreased, five provide an antiapoptotic influence (BAG-3, XIAP, CIAP1, TNFRSF6B, and BCL-2) and three are proapoptotic (BAX, BOK, and CRADD).

In addition to translational state, a measure of total steady-state level of any mRNA could be obtained from these microarrays by summing the signal intensities of the polysomal and monosomal fractions. By this analysis, of the proteins listed in Fig. 5A, only p53 and the p53-binding protein 2 were significantly altered. Up-regulated levels of steady-state mRNA were seen for both p53 (2.8-fold increase with rapamycin treatment) and p53-binding protein 2 (3.5-fold increase).

Of particular note are the top three genes whose translation was the most depressed by rapamycin, all of which are important antiapoptotic genes: BAG-3, XIAP, and CIAP1. BAG-3 is a BAG-1-related protein that modulates apoptosis through its interaction with BCL-2 (29) and can regulate viability of chronic lymphocytic leukemia cells (30). XIAP and CIAP1 are well-known antiapoptosis proteins that act by inactivating caspases (reviewed in ref. 31). Two other antiapoptosis proteins whose translation was more modestly decreased by rapamycin were BCL-2 and TNFRSF6B, the latter is a tumor necrosis factor decoy receptor. Two proapoptotic proteins with modestly decreased translation were BAX and BOK (32). To further support the potential importance of rapamycin-induced decreased translation of at least the most severely affected antiapoptotic proteins in the high-throughput screen, we did immunoblot assays. As shown in Fig. 5B and C, rapamycin caused a significant decrease in protein levels of BAG-3, XIAP, and CIAP1 in multiple myeloma cells, whereas dexamethasone used alone had little effect. The inhibition of expression was even more impressive when cells were exposed to rapamycin plus dexamethasone.

The expression of two additional proteins were examined, XBP-1 and HSP-27, which were not interrogated on the screening array but had been shown previously as regulating dexamethasone-induced apoptosis in multiple myeloma cells (33, 34). As shown in Fig. 5B, there was no significant effect of rapamycin on XBP-1.

Figure 5. Effect of rapamycin on translation and expression of critical antiapoptotic genes in OPM-2 cells. A, list of genes whose translational efficiency was altered >2.5-fold by treatment with rapamycin. Numbers, fold increase or decrease in translational state. B, Immunoblot assay of selected proteins after treatment of OPM-2 cells without or with rapamycin (100 nmol/L), dexamethasone (10–6 mol/L) or combination of both (R+D). C, Immunoblot assay for expression of HSP-27 in OPM-2 cells treated with increasing concentrations of rapamycin (in nmol/L), dexamethasone (in mol/L), or combination of both.
expression, but as shown in Fig. 5C, expression of HSP-27 was significantly inhibited. Dexamethasone, when used alone, also inhibited HSP-27 expression and the combination of both drugs completely ablated expression.

Several of these alterations in antiapoptotic proteins may play a role in a re-setting of the death threshold, such that a stimulus-like dexamethasone now results in a greater degree of apoptosis. On the other hand, several potentially important proapoptotic proteins showed up-regulated translation on exposure to rapamycin, including hara-kiri, DR5, Fas ligand, p53, p53-binding protein 2, and BIM.

**Synergistic antitumor effect in vivo due to combined treatment with dexamethasone and CCI-779.** To test if the enhanced myeloma cell apoptotic response could be extended in vivo, we used a xenograft model in which we have shown previously moderate efficacy of mTOR inhibitors. We used CCI-779 as an mTOR inhibitor in vivo, as it is a more soluble ester analogue of rapamycin and is currently in clinical trials. Our previous work in this model (14) showed that, when CCI-779 is used at 20 mg/kg (10 i.p. injections) in mice harboring growing s.c. OPM-2 tumors, tumor nodules rapidly decreased in size and then completely disappeared. However, after 3 to 4 weeks, 70% (7 of 10) of the tumors reappeared and displayed progressive growth. We thus injected new mice with the same s.c. challenge of OPM-2 cells and combined the 20 mg/kg dose of CCI-779 with dexamethasone. When tumors reached 200 to 300 mm³, treatment was initiated with vehicle alone (control), dexamethasone alone (9 μg/mouse per day × 10), CCI-779 alone (20 mg/kg per day × 10), or the combination of the two drugs. As shown in Fig. 6 (top), dexamethasone used alone at this dose had no discernible antitumor effects. In contrast, CCI-779 used alone displayed similar tumor regression as we have shown previously (14). S.c. nodules in all eight mice completely disappeared for ~2 weeks. However, tumors in six of eight mice reappeared with ensuing growth kinetics that were as rapid as seen in nontreated mice. In mice receiving dexamethasone combined with CCI-779, however, a greater long-term antitumor effect was seen. Only two of those eight challenged mice showed regrowth of tumor and the growth rate was less rapid than the regrowing tumors from mice treated only with CCI-779 (Fig. 6, top).

To test whether an enhanced apoptotic response in vivo accompanied the above antitumor response, some treated mice were sacrificed at day 4 after initiation of treatment and tumors were stained by the TUNEL technique. As shown in Fig. 6, both dexamethasone and CCI-779, when used alone, induced a significant but modest apoptotic response in tumors with apoptotic indices of 15 ± 4 and 26 ± 5, respectively (compared with 4 ± 2 in untreated control mice, mean ± SD, n = 10 microscopic fields for each group). However, as shown in Fig. 6, mice receiving combination therapy showed a marked apoptotic response.

**Discussion**

The results of this study support the notion that rapamycin sensitizes multiple myeloma cells to dexamethasone-induced apoptosis through its ability to induce dephosphorylation of 4E-BP1. An unphosphorylated 4E-BP1 would sequester the eIF-4E initiation factor and prevent cap-dependent translation of selected proteins. These effects on cap-dependent translation could theoretically alter the balance between proapoptotic and antiapoptotic proteins, thus enhancing the ability of a dexamethasone-induced signal to reach a threshold for cell death. The fact that an unphosphorylatable 4E-BP1 can also enhance dexamethasone-induced apoptosis does not exclude other potential rapamycin-dependent effects that could participate in sensitization. However,
we were able to rule out several potential mechanisms by which rapamycin sensitizes to apoptosis, which have been shown previously or suggested in other models. Rapamycin had no effect on RAF/RTK kinase activation, BAD phosphorylation, JNK or p38 MAPKs, or glucocorticoid receptor function, ruling out these possible mediators of a proapoptotic effect.

The ability of the transfected nonphosphorylatable 4E-BP1 to similarly sensitize multiple myeloma cells to dexamethasone-induced apoptosis supports the contention that the sensitizing effect of rapamycin is due to inhibition of 4E-BP1 phosphorylation, sequestering of eIF-4E, and inhibition of cap-dependent translation of selected proteins, which is similar to previous studies (35–37). To address this, we did a high-throughput screening analysis for translational efficiencies of a series of apoptosis-regulating proteins, testing their altered translation by rapamycin. Translation of several antiapoptotic proteins were significantly inhibited by rapamycin, most notably BAG-3, XIAP, and CIAP1. Additional proteins not assayed by our screen were investigated via immunoblot assay because they have been shown previously as important regulators of dexamethasone-induced multiple myeloma cell apoptosis. Specifically, cellular levels of XBP-1 and BCL-XL, which can curtail dexamethasone-induced apoptosis, were not affected by rapamycin. However, the mTOR inhibitor significantly prevented expression of HSP-27, a known viability protector in dexamethasone-treated multiple myeloma cells (34) and this may have also played a role in the sensitization effect. A previous study (38) has also shown the ability of rapamycin to suppress protein levels of HSP-27. Thus, the rapamycin-induced down-regulation of BAG-3, XIAP, CIAP1, and HSP-27 may play a role in the sensitization. If a threshold exists where apoptosis will occur when a proapoptotic load exceeds an antiapoptotic buffering capacity, it may be impossible to ascribe the sensitization to dexamethasone-induced apoptosis to effects on one specific antiapoptotic protein. As described by Lowe et al. (39), if apoptotic thresholds exist, the contributions of individual proteins are not additive and elimination of any individual component could shift the entire system below the threshold.

In contrast to inhibited translation/expression of antiapoptotic proteins, translation of many proapoptotic proteins was maintained or enhanced. Increased translation of these proapoptotic proteins may also play a role in the sensitization caused by rapamycin. Expression of these proapoptotic proteins during rapamycin exposure is likely due to cap-independent translation mediated internally by internal ribosome entry sites (IRES) found in the 5'-untranslated regions of selected transcripts. The rapamycin-induced stress may, in fact, stimulate IRES function of certain transcripts, thus accounting for rapamycin-mediated increases in translation. We have detected similar rapamycin-induced increases in IRES function of cyclin D1 and c-myc in certain cell lines (40). Consistent with this hypothesis is the fact that several proapoptotic transcripts have been found to contain IRES structures (41, 42). However, the issue is not so clear-cut, as antiapoptotic transcripts, such as XIAP (43), can contain functioning IRES as well and HSPs are well translated in cells lacking eIF-4E (44). The depressive effect of rapamycin on HSP-27 expression may have been due to effects on transcription. However, the fact that XIAP translation was significantly depressed by rapamycin suggests that its IRES was not capable of maintaining translation in the face of mTOR inhibition in multiple myeloma cells. Collectively, these facts indicate that the modulation of IRES activity in proapoptotic and antiapoptotic transcripts during exposure of multiple myeloma cells to apoptotic stimuli plays a key role in cellular outcome. For example, although the XIAP IRES was incapable of maintaining translation in rapamycin-treated cells, its activity can be markedly enhanced by interleukin-6 (IL-6) with a resulting antiapoptotic effect (45). As IL-6 is a multiple myeloma growth factor that protects multiple myeloma tumor cell viability, the regulation of XIAP IRES function may have particular relevance.

Of additional interest is the ability of AKT activity to sensitize multiple myeloma cells to the enhanced apoptotic responses of treatment with rapamycin and dexamethasone. First, this may be clinically relevant, as myeloma is a disease with frequent heightened AKT activity in malignant plasma cells (46), further supporting the notion that a combination of mTOR inhibitors and dexamethasone may be efficacious in patients. Second, the ability of AKT activity to enhance apoptosis is reminiscent of studies done in the Eμ-myc murine lymphoma model (47). Murine lymphomas stably overexpressing BCL-2 or AKT grew more rapidly than nontransfected cells, resisted apoptosis, and displayed chemoresistance. When rapamycin, which by itself was ineffective, was added to chemotherapy, chemoresistance was reversed but only in tumors overexpressing activated AKT. Our recent work (40) on the ability of AKT to regulate IRES function could provide a possible mechanism by which heightened AKT and rapamycin presence may induce a markedly proapoptotic environment. In this theoretical scenario, AKT may enhance multiple myeloma cell apoptosis induced by combined mTOR inhibitor plus dexamethasone treatment through its ability to curtail IRES function of selected antiapoptotic transcripts.

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Huajun Yan, Patrick Frost, Yijiang Shi, et al.


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