Regulation of D-cyclin translation inhibition in myeloma cells treated with mammalian target of rapamycin inhibitors: rationale for combined treatment with extracellular signal–regulated kinase inhibitors and rapamycin

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Abstract
We have shown that heightened AKT activity sensitized multiple myeloma cells to the antitumor effects of the mammalian target of rapamycin inhibitor CCI-779. To test the mechanism of the AKT regulatory role, we stably transfected U266 multiple myeloma cell lines with an activated AKT allele or empty vector. The AKT-transfected cells were more sensitive to cytostasis induced in vitro by rapamycin or in vivo by its analogue, CCI-779, whereas cells with quiescent AKT were resistant. The ability of mammalian target of rapamycin inhibitors to down-regulate D-cyclin expression was significantly greater in AKT-transfected multiple myeloma cells due, in part, to the ability of AKT to curtail cap-independent translation and internal ribosome entry site (IRES) activity of D-cyclin transcripts. Similar AKT-dependent regulation of rapamycin responsiveness was shown in a second myeloma model: the PTEN-null OPM-2 cell line transfected with wild-type PTEN. Because extracellular signal–regulated kinase (ERK)/p38 activity facilitates IRES-mediated translation of some transcripts, we investigated ERK/p38 as regulators of AKT-dependent effects on rapamycin sensitivity. AKT-transfected U266 cells showed significantly decreased ERK and p38 activity. However, only an ERK inhibitor prevented D-cyclin IRES activity in resistant “low-AKT” myeloma cells. Furthermore, the ERK inhibitor successfully sensitized myeloma cells to rapamycin in terms of down-regulated D-cyclin protein expression and G1 arrest. However, ectopic overexpression of an activated MEK gene did not increase cap-independent translation of D-cyclin in “high-AKT” myeloma cells, indicating that mitogen-activated protein kinase/ERK kinase/ERK activity was required, but not sufficient, for activation of the IRES. These data support a scenario where heightened AKT activity down-regulates D-cyclin IRES function in multiple myeloma cells and ERK facilitates activity. [Mol Cancer Ther 2009;8(1):83–93]

Introduction
Mammalian target of rapamycin (mTOR) inhibitors have shown potential in preclinical studies as effective agents against multiple myeloma (1–3). In other tumor models, these drugs classically induce G1 arrest by alteration of cell cycle protein expression (4, 5). They also induce G1 arrest in myeloma cells when used alone (1), although they can synergize with other agents for heightened myeloma apoptosis (2, 3). In a xenograft model, we recently showed that the rapamycin analogue CCI-779 (temsirolimus) was an effective antmyeloma drug, inhibiting the in vivo tumor growth of OPM-2, 8226, and U266 cell lines (6). Interestingly, the level of AKT activity correlated with sensitivity to CCI-779 in these cell lines with the OPM-2 line, which expresses constitutively active AKT due to a PTEN mutation (7), being the most sensitive. Confirmation of a true regulatory effect of AKT on sensitivity was obtained when an activated AKT allele was stably transfected into U266 cells. This stably transfected myeloma line (U266AKT) was considerably more sensitive in vivo to the antitumor effects of CCI-779 than its empty vector–transfected (U266EV) control (6).

In the current study, the isogenic U266 transfected pair of cell lines was analyzed with the aim of investigating the mechanism by which AKT regulates responses in myeloma cells to mTOR inhibitors. By preventing cap-dependent translation, mTOR inhibitors abrogate D-cyclin expression and studies in other models implicated this inhibition in G1 arrest (8–10). Thus, we specifically focused on whether AKT regulates D-cyclin expression during mTOR inhibition. Our results show that AKT regulates D-cyclin expression during mTOR inhibition. The alternative mechanism of translation, so-called cap-independent translation mediated by internal ribosome entry site (IRES) structures, was prevented by heightened AKT activity. In addition, a mitogen-activated protein kinase/extracellular
signal–regulated kinase (ERK) kinase (MEK)/ERK inhibitor prevented D-cyclin IRES activity in the “low-AKT” myeloma cells. These latter results suggested the potential of combined therapy with mTOR and ERK inhibitors in resistant tumor targets. In support of that notion, exposure of the low-AKT rapamycin-resistant myeloma clone to rapamycin and ERK inhibitor resulted in sensitization to D-cyclin down-regulated expression and G1 arrest.

Materials and Methods

Myeloma Cell Lines and Transfections

Human U266 multiple myeloma isogenic cell lines were transfected as previously described (6). Briefly, the constitutively activated myristoylated AKT cDNA expression vector was purchased from Upstate. Stable transfection of U266 cells with activated AKT or empty vector (EV) control was accomplished by electroporation (230 V for 25 ms). Stable transfections were selected in neomycin (350 mg/mL) and successful transfection was determined by Western blotting with antibodies specific for total and phosphorylated AKT (serine 473). Transient transfections of cells with constitutively activated cDNA MEK1 expression vector (Upstate) or empty vector control was done using the AMAXA Nucleofection System (AMAXA, Inc.). The transfection efficiency was typically >80% as determined by transfection of cells with a green fluorescent protein plasmid vector. Successful transfection was determined by Western blotting with antibodies specific for phosphorylated ERK, a downstream substrate for MEK1.

Adenovirus expressing wild-type PTEN has been described previously (11). The genome of this vector has deletions of the El and E3 regions and protein IX gene and expresses its transgene under control of the human cytomegalovirus immediate-early promoter/enhancer. As corresponding control, we used the same virus containing enhanced green fluorescent protein (EGFP) as its transgene.

OPM-2 cells were transduced with adenovirus for 2 h with a multiplicity of infection of 100 (1). Virus-containing solutions were removed after 2 h and the cells were incubated in the presence or absence of rapamycin for an additional 48 h.

Reagents

All antibodies were purchased from Cell Signaling Technology, except the cyclin D1 antibody (BD Biosciences). The cyclin D1 antibody was specific for D1 and did not cross-react with D2 or D3. The ERK kinase assay kit was purchased from Cell Signaling Technology. Rapamycin was purchased from Calbiochem. CCI-779 was provided by Wyeth-Ayerst. The MEK inhibitor U0126 and the p38 inhibitor SB202190 were purchased from Promega. For testing cell survival, viability was determined by MTT assays as previously described (12).

Animals

Four- to six-week-old male nonobese diabetic severe combined immunodeficient (NOD/SCID) mice were obtained from The Jackson Laboratory. The mice were maintained at four per cage in pathogen-free conditions. All animal studies were conducted in accordance with protocols approved by the Animal Research Committee of the West Los Angeles Veterans Administration Medical Center.

Xenograft Model

We used the murine myeloma xenograft model of Leblanc et al. (13) with minor modifications as previously described (6). The cell lines were mixed with Matrigel and were then injected s.c. (200 μL/mouse containing 3 × 107 U266 cells). Mice were randomized into drug-treated or control groups (8–14 mice per group) when the tumor volume reached ~200 to 400 mm3. CCI-779 was injected i.p. daily ×5, followed by 2 d of no drug and then five additional injections, as previously described (6). Mice were routinely euthanized when tumors reached >2,000 mm3 in volume.

Immunohistochemistry

At day +13, mice were euthanized with CO2 and the tumor mass was excised. The tumor was bisected using a razor blade; one half of the tumor was immediately placed in 10% buffered formaldehyde overnight, and the other half was frozen for protein extraction. Formaldehyde-fixed tumors were embedded in paraffin and cut into 5-μm-thick serial sections using standard histologic procedures. Immunohistochemical staining with antihuman Ki-67 antibody was conducted using standardized automated methods (14). Sections were counterstained with H&E. The proliferation index was determined by assaying the area of Ki-67 staining using the MetaMorph software from 10 randomly selected fields at ×20 magnification, as previously described (6). Tumor nodules were primarily composed of tumor cells with ~2% of the area made up of vasculature (as determined by staining with anti-CD34 antibody) as reported in a previous study (15). In Ki-67–stained sections, stroma and vasculature (as determined by microscopy) were excluded from the analysis.

Western Blot Analysis

Protein was extracted in ice-cold lysis buffer and 25 μg of protein from each sample were boiled and separated by 10% SDS-PAGE. After blocking in 10% nonfat dried milk, the membranes were washed and then incubated with primary antibodies for 1 h, followed by incubation with 1 μg/mL horseradish peroxidase–labeled secondary antibodies.

Cell Cycle Analysis

Cell cycle analysis of hypotonic propidium iodide–stained cells was determined by fluorescence-activated cell sorting with a Becton-Dickinson FACS- Calibur. Histograms generated by fluorescence-activated cell sorting were analyzed by ModFit Cell Cycle Analysis Software (Verity) to determine the percentage of cells in each phase.

Polyosome Analysis

Extraction and display of polyosomes was done as previously described (9). Briefly, U266 isogenic cells (4 × 107) were lysed in ice-cold lysis buffer supplemented with 100 μg/mL cycloheximide at 4°C. Following removal of nuclei and mitochondria, supernatants were layered onto 15% to 50% sucrose gradients and spun at 38,000 rpm
for 2 h at 4°C in a SW-40 rotor (Beckman Instruments). The gradients were then fractionated into eleven 1-mL fractions using an ISCO density gradient fractionator at a flow rate of 3 mL/min. The polysome profile of the gradient was determined by UV absorbance at 260 nm. The RNA from individual fractions was extracted using phenol/chloroform and precipitated in ethanol. The RNA was then processed for Northern blots. Radioactive probes were generated using the Ambion MaxiScript kit (Ambion).

Dicistronic Reporter Assay

The parental dicistronic reporter construct used in these studies was pRF (a kind gift of A. Willis, University of Leicester, Leicester, United Kingdom; ref. 16). The pRCD1F construct, which contains the cyclin D1 5′-UTR (GenBank accession no. NM053056), and the pRp27F construct, which contains the 365-nucleotide IRES sequence (17) of p27Kip1, were subcloned into the intracistronic region of pRF as previously described (18). Cells were transfected with 25 μg plasmid DNA by electroporation. The cells were then incubated with or without drugs for 18 h, washed twice in PBS, and lysed in passive lysis buffer (Promega). The firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Transfection efficiency was measured by β-galactosidase activity using a β-galactosidase enzyme assay system (Promega).

Statistics

Student’s t test was used to determine significance of differences between groups.

Results

AKT Regulates the Antiproliferative Response to mTOR Inhibitors In vitro and In vivo

In previous studies, U266 multiple myeloma cells, stably expressing an activated AKT gene, were significantly more sensitive than empty vector control U266 cells to the in vitro antitumor effects of CCI-779 in a xenograft model (6, 15). In the current study, we used the same isogenic pair of myeloma cell lines to investigate the mechanisms of the AKT regulatory effects on rapamycin sensitivity. The expression of myristoylated AKT, constitutively phosphorylated on both threonine 308 and serine 473 residues, is shown in Fig. 1A. In contrast, empty vector cells (U266EV) express an AKT molecule that is minimally phosphorylated on threonine 308 and without phosphorylation of serine 473. Cells were treated with insulin-like growth factor I (400 ng/mL) as a positive control for AKT activation, and as shown, the empty vector control cells were capable of AKT phosphorylation on both residues when stimulated by insulin-like growth factor I. As shown in Fig. 1B, AKT clearly regulated the in vitro antiproliferative effect of rapamycin as measured by its effects on cell number (top) and the MTT assay (bottom). For the AKT-transfected cells in the MTT assay (U266AKT; bottom, open circles), the ED₅₀ was ~1 nmol/L, whereas the ED₅₀ was >100 nmol/L in the control empty vector–transfected cells (U266EV; closed circles). As has been reported in previous studies, flow cytometric analysis (data not shown) confirmed that rapamycin did not induce significant apoptosis in either isogenic cell line (1).

As an in vivo correlate of the above in vitro MTT proliferation data, we also analyzed the Ki-67 proliferation marker in U266 tumors harvested from CCI-779–treated mice. The cell lines were injected s.c. into NOD/SCID mice, and when the tumors reached 200 to 400 mm³ of volume, some mice were treated for 10 days with increasing doses of CCI-779 by i.p. injections (6). Tumors were then excised and immunostained for Ki-67 expression. As shown in Fig. 1C, CCI-779 had a much greater inhibitory effect on Ki-67 staining in AKT-transfected tumors (dark columns; ED₅₀ = 10 mg/kg) compared with empty vector controls (open columns; ED₅₀ > 20 mg/kg). At all concentrations of CCI-779, there was no significant effect on Ki-67 expression in the empty vector–transfected tumor cells. Thus, AKT regulates the cell cycle inhibitory effect of mTOR inhibitors in this myeloma model in vivo as well as in vitro.

We considered the possibility that an AKT-dependent regulation of sensitivity to mTOR inhibitors might be due to differences in the ability of rapamycin or CCI-779 to inhibit the mTOR pathway. To examine this possibility, we assayed the phosphorylation of the mTOR substrate, P70 S6 kinase (P70), after exposure to increasing concentrations of rapamycin. Figure 1D shows representative results from one of three independent experiments where phosphorylation of P70 was analyzed. Interestingly, although expressing little activated AKT (6), empty vector–transfected (U266EV) cells constitutively express significant amounts of basal phosphorylated P70 (Fig. 1D). This may be due to the interleukin-6 (IL-6) autocrine nature of these cells (19), which results in several activated signal cascades, some of which may induce downstream phosphorylating effects on P70 independently of AKT (20). The levels of P70 phosphorylation were only slightly more up-regulated in the AKT-transfected U266AKT cells (Fig. 1D). Nevertheless, in the presence of rapamycin, a comparable degree of inhibited P70 phosphorylation occurs between the two U266 cell lines. As shown in Fig. 1D, rapamycin induced ~50% inhibition at 0.1 nmol/L rapamycin and >95% inhibition at 1 nmol/L in both cell lines. Thus, these results rule out the possibility that differences in the ability of rapamycin to curtail mTOR activity can explain the AKT-dependent differential sensitivity of these multiple myeloma cell lines to growth inhibition.

AKT Activity Regulates Effects on Cyclin D1 Expression and Retinoblastoma Phosphorylation

In previous studies in other tumor models (8–10), down-regulation of D-cyclin expression induced by mTOR inhibitors played an important role in the observed cell cycle arrest. Thus, we evaluated the effects on D-cyclins in U266 cells. U266 cells predominantly express cyclin D1 [secondary to a t(11;14) translocation of cyclin D1] to drive G₁/S transition (21, 22). As shown in Fig. 2A (one of three separate comparable experiments), rapamycin was considerably more potent at inhibiting expression of cyclin D1 in U266AKT cells, decreasing expression by almost 50% at the...
lowest concentration tested (0.1 nmol/L) and by >75% at 1 nmol/L (bar graph below immunoblots). In contrast, rapamycin had little consistent effect on cyclin D1 expression in control U266EV cells (Fig. 2A, left). Basal levels of cyclin D1 (in the absence of rapamycin) were comparable between the two cell lines, indicating that the sensitization to mTOR inhibitors caused by heightened AKT activity is not due to a higher baseline level of an
mTOR-cyclin D1 pathway that is more important to growth of "high-AKT" myeloma cells.

To test if a differential effect on cyclin D1 expression was also present in the in vivo xenograft model, protein was extracted from tumors excised after the last CCI-779 treatment, followed by immunoblot assay for cyclin D1 and actin expression. As shown in Fig. 2B, a similar AKT-dependent effect on cyclin D1 expression was identified following in vivo treatment. CCI-779 effectively inhibited cyclin D1 expression in AKT-transfected tumors when used at 10 or 20 mg/kg per mouse, but similar doses had little effect on expression in empty vector–transfected tumors. Data shown are for pooled tumor lysate of four mice per group. The ratio of cyclin D1/actin expression measured by densitometry is shown below the representative Western blot.

Cyclins interact with cyclin-dependent kinases to phosphorylate retinoblastoma protein (Rb). Because D-cyclins are the major determinants of cyclin-dependent kinase 4/6 holoenzyme activity, decreased expression of cyclin D1 should prevent Rb phosphorylation in an AKT-dependent fashion. To test this notion, we also immunoblotted Rb, assaying its phosphorylation state in isogenic U266 cells treated with rapamycin in vitro (Fig. 2C). Although some U266 clones have loss of Rb expression (23), our U266 lines as well as others (24) do express Rb. As shown, phosphorylation of Rb residues (807/811 and 780) was inhibited in AKT-transfected U266AKT cells compared with empty vector controls (U266EV). This was best seen when testing phosphorylation at 807/811 residues, with 0.1 nmol/L rapamycin inhibiting phosphorylation by >50% and 10 nmol/L completely ablating phosphorylation in AKT-transfected cells. In contrast, rapamycin had little effect on Rb phosphorylation at the 807/811 residues in the U266EV cells. In experiments not shown, the ability of rapamycin to increase expression of the p27 cyclin-dependent kinase inhibitor was not AKT dependent, with approximately a doubling of p27 protein levels in both cell lines at 0.1 to 10 nmol/L concentrations. Thus, AKT activity determined the ability of mTOR inhibitors to down-regulate cyclin D1 expression and Rb phosphorylation while having no effect on up-regulation of p27.

**AKT Regulates Effects on Cyclin D Translation**

In other models, inhibition of mTOR causes an immediate downstream dephosphorylation of p70S6 kinase and 4E-BP1, which results in depressed translation of cell cycle proteins such as D-type cyclins (5). Thus, we tested for possible AKT-dependent effects on D-cyclin translation using the ribosomal loading assay described by Zong et al. (25). This assay is based on the fact that actively translated mRNA species are associated with multiple ribosomes (polysomes), whereas mRNAs sequestered with single ribosomes (monosomes) are inactive or poorly translated (25). Following separation of polysomes from monosomes, a Northern blot analysis done on their associated RNA can estimate translational state, defined as the ratio of polysome-associated RNA to monosome-associated RNA signal intensity. The two U266 isogenic cell lines were thus treated with or without 10 nmol/L rapamycin for 48 hours, and the cellular extracts prepared for polysome analysis as previously described (9). The polysomes and monosomes were separated by sucrose gradient centrifugation and fractionated into eleven 1-mL fractions. The UV absorbance (254 nm) of each fraction was measured to generate a polysome profile of the sucrose gradient that was used to differentiate between the polysome (fractions 1–3) and monosome (fractions 4–11) fractions.

**Figure 2.** Inhibition of cyclin D1 expression by mTOR inhibitors is AKT dependent. **A,** immunoblot assay for expression of cyclin D1 or actin in extracted protein from isogenic U266 cells cultured with increasing concentrations of rapamycin for 48 h. Densitometry was done on equally exposed autoradiographs and data are presented as cyclin D1/actin ratios compared with untreated cells (bottom). This experiment was repeated thrice with identical results. **B,** effects of CCI-779 on expression of cyclin D1. U266-challenged mice were treated with varying doses of CCI-779 (shown above blots as mg/kg) for 13 d, after which the tumor nodules were harvested and the extracted protein was immunoblotted for expression of cyclin D1 and actin. Data are from pooled extract (four mice per group) and are presented as in **A.** **C,** effects of rapamycin on Rb protein. Protein extracted from U266 cells cultured with increasing concentrations of rapamycin for 48 h was immunoblotted for total Rb and phosphorylated Rb at residues 780 and 807/811.
The Northern blot analysis of cyclin D1 or actin mRNA on total mRNA isolated from the corresponding fractions is shown in Fig. 3A. The percent of total mRNA present (measured by densitometry of equally exposed autoradiographs) in the polysomal fractions (fractions 4 – 11) is shown to the right of the autoradiographs. This experiment was done one additional time with identical results. B, summary of polysome analysis of isogenic U266 multiple myeloma cell lines from two separate experiments. Percentages of cyclin mRNA associated with the polysome (well translated) or monosome (poorly translated) fractions in U266 cells culture with or without 10 nmol/L rapamycin for 48 h. C, schematic diagrams of the dicistronic constructs used in this study. Constructs used are pRF, pRCD1F, which contains the 5'-UTR of human cyclin D1 in the intracistronic space; pRp27F, which contains the 5'-UTR of human p27 in the intracistronic space. 

AKT-Dependent Effects on Cyclin D1 IRES Activity

When mTOR inhibitors prevent cap-dependent translation, an alternative cap-independent mechanism of translation may be utilized, where the 40S ribosomal subunit binds a transcript via an IRES in the RNA 5'-UTR. Because AKT activity could regulate the ability of multiple myeloma cells to maintain cyclin D1 translation and expression in the face of mTOR inhibition, we theorized that the kinase achieved this by its effects on IRES activity. The cyclin D1 transcript has a previously described IRES sequence in its 5'-UTR (18). Thus, to test our hypothesis, we used the dicistronic reporter vector in which the cyclin D1 5'-UTR, containing its IRES, was subcloned into the intracistronic space to generate the pRCD1F vector. Prior work confirmed the presence of an IRES sequence in the p27 5'-UTR (17).

Following transfection of isogenic U266 multiple myeloma cell lines with the dicistronic vectors, the reporter constructs were tested for their ability to direct 5'-UTR-mediated cap-independent translation (firefly luciferase). Results were normalized for transfection efficiency by cotransfection with a β-galactosidase construct. Transfected cells were treated with or without rapamycin for 18 hours.
Figure 3D depicts reporter expression as a ratio of firefly/\textit{Renilla} luciferase activity, with the activity in the pRF vector normalized to a value of 1. As shown in the figure, the presence of the cyclin D1 5'-UTR IRES (pRCD1F) enhanced reporter expression >7-fold in empty vector–transfected cells (open columns) but only 1.8-fold in the AKT-transfected cell line (closed columns), showing that the overactive AKT had a significant inhibitory effect on cyclin D1 IRES activity ($P < 0.05$). The \textit{Renilla} luciferase activities were similar for both cell lines, indicating that cap-dependent translational mechanisms were independent of AKT activity and the marked change in firefly to \textit{Renilla} ratio was completely due to increased firefly luciferase expression. In contrast to the above, although the p27 5'-UTR of pRp27F vector also showed significant IRES activity (~6-fold over pRF), this was not AKT dependent because activity was comparable between the two isogenic multiple myeloma cell lines. This indicates the specificity of the AKT regulating effect on cyclin D1 IRES function. When cyclin D1 IRES activity was measured in the cells following exposure to rapamycin, \textit{Renilla} luciferase expression was decreased by 30% to 40% in both cell lines, attesting to the ability of rapamycin to inhibit mTOR and subsequent cap-dependent translation. The firefly luciferase expression was also slightly decreased in both cell lines such that the firefly-to-\textit{Renilla} ratios shown were minimally altered. Importantly, the AKT-dependent differences in cyclin IRES activity were maintained between the isogenic cell lines following exposure to rapamycin. The experiment shown in Fig. 3D was repeated thrice with similar results.

**Studies in a Second Myeloma Cell Model**

To rule out that the above results in isogenic U266 cell lines were a peculiarity of that particular multiple myeloma line, we also studied the OPM-2 myeloma cell line. In our previous study (6), PTEN-null OPM-2 expresses hyperactive AKT, and OPM-2 tumor grown in NOD/SCID mice is extremely sensitive to CCI-779, with a remarkable decrease in cyclin D1 expression observed even at the lowest CCI-779 dose (0.4 mg/kg) tested. To test whether AKT activation was responsible for this hypersensitivity to mTOR inhibition, we transiently transfected OPM-2 cells with wild-type \textit{PTEN} using an adenovirus vector. At a multiplicity of infection of 100:1, 90% of cells were successfully transduced. Figure 4A shows that adenovirus-mediated restoration of PTEN activity in OPM-2 cells results in a marked down-regulation of phosphorylated AKT (serine 473) after 24 hours compared with OPM-2 cells infected with control empty vector virus. We next tested if...
down-regulating AKT activity in OPM-2 cells affected cyclin D1 protein levels and IRES activity. As shown in Fig. 4B and C, whereas cyclin D1 expression in PTEN-null OPM-2 control cells (Fig. 4B, left) is extremely sensitive to treatment with rapamycin, with inhibition of protein expression evident after treatment with 1 nmol/L rapamycin, there is little effect on wild-type PTEN-expressing cells (right). In a similar manner, the dicistronic reporter expression (Fig. 4C) shows a significant increase in IRES activity in OPM-2 cells transfected with wild-type PTEN to down-regulate AKT activation ($P < 0.05$). These data collectively provide support that the AKT-dependent regulation of D-cyclin translation and rapamycin responsiveness in U266 cells is not restricted to that cell line.

An ERK Inhibitor Curtails D-Cyclin IRES Activity and Sensitizes Resistant Low-AKT Multiple Myeloma Cells to Rapamycin

Both ERK and p38 mitogen-activated protein kinase activity can facilitate IRES function of some transcripts, like c-myc (26, 27), and it is known that AKT can potentially down-regulate ERK (28) and p38 (29) activity. Thus, we tested the notion that the AKT regulatory role could be mediated via effects on these MAP kinases. In the U266 isogenic multiple myeloma clones, AKT also regulated these mitogen-activated protein kinase pathways. As shown in Fig. 5A, although the expression levels of total ERK and p38 were comparable between the two cell lines, the levels of phosphorylated ERK and phosphorylated p38 were significantly down-regulated in the AKT-transfected cells with complete ablation of ERK phosphorylation and an approximately 60% decrease in p38 phosphorylation. Thus, we next investigated whether AKT regulates cyclin D1 IRES activity via these inhibitory effects on the p38 or ERK pathways.

The dicistronic reporter assay was again used in multiple myeloma cells treated with either a p38 or a MEK/ERK inhibitor. After transfection, U266EV cells were cultured for 18 hours without or with U0126 (a MEK inhibitor) or SB202190 (a p38 inhibitor). Our results (Fig. 5B) show that treatment with the MEK/ERK inhibitor, but not p38 inhibitor, significantly diminished cyclin D1 IRES activity (measured by firefly luciferase activity) in these cells ($P < 0.05$). In contrast, there was no significant effect of the ERK inhibitor on Renilla luciferase expression. In comparison, U266AKT cells (Fig. 5B, right) treated with U0126 showed a complete abrogation of the relatively small cyclin D1 IRES activity in these cells (from 1.6- to 1-fold). The ability of the MEK inhibitor to abrogate concurrent ERK activity in the empty vector cells is shown in Fig. 5C. This inhibitor completely ablated ERK in vitro kinase activity in these cells. Although the p38 inhibitor at 25 μmol/L had no effect on IRES activity, it was capable of substantial inhibition of p38 phosphorylation (data not shown).

Effects of Combination Treatment

The above data indicated that MEK/ERK activity supports cyclin D1 IRES function and suggests that the ability of AKT to inhibit ERK played a role in its regulatory effects on IRES activity and subsequent sensitivity to mTOR inhibition. This suggested that ERK activity was an important determinant of the ability of rapamycin-resistant multiple myeloma cells to prevent D-cyclin down-regulated expression and G₁ arrest. To test this hypothesis, the isogenic cell lines were exposed to rapamycin or the U0126 MEK/ERK inhibitor and cell cycle analysis was done (Fig. 6A). In these experiments, we used the MEK/ERK inhibitor at 1 μmol/L, a concentration which inhibited ERK activity by >80% (Fig. 5C), because there was some modest cytotoxicity when U0126 was used alone at 10 μmol/L. As shown in Fig. 6A, although the empty vector–transfected myeloma cells were relatively resistant to the effects of either rapamycin or the ERK inhibitor used alone, exposure to the combination of drugs significantly induced G₁ arrest ($P < 0.01$). There was no detectable apoptosis resulting from combined therapy. The successful sensitization of the low-AKT multiple myeloma cells to G₁ arrest mirrored the successful down-regulation of cyclin D1 protein expression.
when combination therapy was used (Fig. 6B). In contrast to this sensitization, the ERK inhibitor did not cause an increase in G1 arrest when it was used in combination with rapamycin in the high-AKT multiple myeloma cell line (data not shown). This result was expected because these latter cells have barely detectable ERK activity (Fig. 5A). The results also argue against a nonspecific G1 toxic effect of the ERK inhibitor when used in combination.

The inhibitory effect of rapamycin on p70S6 kinase activity and the subsequent interference with a p70-induced negative feedback activity on IRS-1, with resulting PI3K/AKT activation, were previously characterized in multiple myeloma cell lines (30). It is also known that ERK activity can participate in p70S6K activation (20). Thus, one possible explanation for the sensitizing effect of the ERK inhibitor was its ability to activate AKT (mediated via p70 inhibition). Theoretically, it would convert the low-AKT resistant cells into high-AKT sensitive cells. Therefore, we tested the effects of the ERK inhibitor on AKT phosphorylation in empty vector–transfected U266 cells and compared them to the effects of rapamycin. As shown in Fig. 6C, the empty vector U266 line showed a significant up-regulation of AKT phosphorylation on both serine 473 and threonine 308 when it was exposed to either the U0126 MEK/ERK inhibitor or rapamycin. The effects were more marked for the serine 473 residue than for threonine 308. The ability of each inhibitor to increase AKT phosphorylation when used alone was comparable. There was no additive enhancement of phosphorylation when both were used together. We believe that this effect on AKT can be explained by the ability of both inhibitors to down-regulate p70 with the subsequent interruption of the negative feedback loop. Similar treatment of AKT-transfected U266 cells with the inhibitors did not increase AKT phosphorylation (not shown), probably because the AKT transgene is already constitutively activated in these cells.

Figure 6. Inhibition of ERK activity overcomes resistance to rapamycin in U266 cells. A, fluorescence-activated cell sorting analysis of hypotonic propidium iodide–stained U266EV cells treated with 1 nmol/L rapamycin, 1 μmol/L U0126 MEK/ERK inhibitor, or both drugs for 48 h. The percentages of cells in G1 (black columns) or S phase (open columns) were determined from the generated histograms using ModFit cell cycle analysis software. This experiment was repeated thrice with similar results. *, P < 0.05, compared with controls. B, immunoblot assay for expression of cyclin D1 or actin in extracted protein from U266EV cells treated with indicated concentrations of rapamycin (1 nmol/L) or U0126 (1 μmol/L) as described above. C, Western blot analysis of total and phosphorylated AKT expression in U266EV cells treated with increasing concentrations of rapamycin (1 nmol/L) or U0126 (1 μmol/L) as described above. D, U266 EV cell lines were cotransfected with either constitutively active MEK1 plasmid or empty vector control and either pRF or the pRCDF construct containing the cyclin D1 IRES (CCND1). The successful activation of ERK by the MEK1 construct was determined by immunoblot for total and phospho-ERK in lysate from U266AKT cells transfected with either green fluorescent protein (GFP) transfection control plasmid, the empty vector control plasmid (pUSE-EV), or the constitutively activated MEK1 expression plasmid (pUSE-actMEK). The cells were lysed after 18 h and the firefly and Renilla luciferase activities were measured as described above. Data are shown as relative Renilla luciferase (white columns) or firefly luciferase (black columns) activity. *, P < 0.05.
Next, we asked if reactivation of the MEK/ERK pathway conferred resistance to rapamycin in high-AKT cell lines by enhancing cyclin D1 IRES activity. To test this, we transiently transfected U266 isogenic cell lines with a constitutively activated MEK1 plasmid or empty vector control and either pRF or pRCD1F dual luciferase reporter vector. Successful transfection of constitutively activated MEK1 was confirmed by Western blot for up-regulated ERK phosphorylation in U266AKT cells (Western blot shown in inset). As shown in Fig. 6D, up-regulation of ERK activity in U266EV cells increased cyclin D1 IRES activity by ~1.5-fold. However, constitutively activated MEK1 had little effect on IRES activity in the AKT-transfected cells. Thus, although ERK activity is required for IRES function (Fig. 5B), it is not sufficient. As described in Discussion, there is another potential reason for why MEK/ERK activation was not capable of enhancing IRES activity in the AKT-transfected cells.

Discussion
The results of this study indicate that the level of AKT activity determines the sensitivity of multiple myeloma cells to the cytostatic effects of mTOR inhibitors, and this is associated with AKT-dependent differential effects on D-cyclin expression and translational efficiency. Furthermore, the data indicate that AKT regulates sensitivity through its inhibitory effect on the D-cyclin IRES, preventing the fail-safe mechanism of protein translation when mTOR activity is inhibited. An AKT-dependent regulation of translation was found in two independent multiple myeloma cell lines where AKT could be differentially activated or inhibited by gene transfer. Effects on Rb phosphorylation mirrored the AKT-dependent effect on cyclin D1. In contrast, rapamycin resulted in up-regulation of p27 levels as has been seen before (2), but these effects were not regulated by AKT.

The results of this study have clinical significance, further supporting potential efficacy of mTOR inhibitors against myeloma clones that express excessive levels of activated AKT. Furthermore, the ability of ERK activity to participate in resistance to rapamycin and successful sensitization of rapamycin-resistant cells to G1 arrest with the addition of an ERK inhibitor suggests that such combination therapy might be efficacious for multiple myeloma clones that express low levels of activated AKT. Because ERK activity is stimulated by the myeloma growth factor IL-6 (31), up-regulation of cyclin D1 translation may occur frequently in primary myeloma cells in vivo if AKT is not coactivated.

Cyclin D1 RNA is frequently overexpressed in myeloma due to an IgH/cyclin D1 translocation (21). The regulatory effects of AKT and ERK on responsiveness to mTOR inhibitors rely on the function of the IRES, located in the 5′UTR of the D-cyclin transcripts. Thus, the AKT and ERK regulatory effects on translation of the cyclin-D1 transcript should be present even when the RNA is overexpressed from an IgH/D-cyclin D1 translocation, as long as the 5′-UTR sequence (containing the IRES) is not disrupted. Our U266 cell lines used in this study are good examples of this because they also contain the IgH/cyclin translocation and up-regulated RNA expression (21).

Our mechanistic studies delineating a role for the MEK/ERK cascade in D-cyclin IRES activity supported a rationale for combined therapy with ERK and mTOR inhibitors. MEK/ERK inhibitors have been used previously in preclinical myeloma studies with varying results. MEK/ERK inhibition will clearly prevent an IL-6–induced proliferative response (31) and will prevent multiple myeloma cell growth of strictly IL-6–dependent lines such as ANBL-6 cells. However, they have little effect on IL-6–independent myeloma cell lines (32). The inhibitors also have little apoptotic effect when used alone but significantly synergize with a G2-M checkpoint abrogator for enhanced myeloma cell apoptosis (33). The U266 clone that we have used secretes IL-6 and expresses constitutive MEK/ERK activity, which is abrogated in the presence of anti-IL-6/IL-6 receptor antibodies (data not shown). However, these blocking antibodies have no effect on cell growth of the parental or transfected U266 cell lines. Thus, it is clear that MEK/ERK activity is not crucial for growth of these U266 cell lines, nor is it crucial for cyclin D1 translation by cap-dependent pathways. However, MEK/ERK activity becomes crucial for D-cyclin translation during mTOR inhibition.

The mechanism by which the ERK inhibitor down-regulates IRES activity is unknown. Because AKT clearly regulates IRES activity, it is possible that the effect of the ERK inhibitor is simply mediated via its ability to increase AKT activity, as shown in Fig. 6C. Previous work (30) has shown that inhibition of p70S6K activity by mTOR inhibitors interrupts a negative feedback loop, which results in AKT activation. It is likely that the ability of ERK inhibitor to activate AKT is also mediated via its well-known inhibitory effects on p70S6K (20). A second possibility is that ERK has independent effects on IRES trans-acting factors (ITAF), which are critical for IRES function, and the ERK inhibitor prevents this effect. ITAFs bind to the IRES and induce conformational changes that facilitate recruitment of the ribosome to the IRES (34). The ERK cascade may enhance ITAF function via enhanced ITAF expression or posttranslational modification that enhances ITAF-IRES binding.

Although a MEK/ERK inhibitor prevented D-cyclin IRES translation in the low-AKT multiple myeloma cells, thus sensitizing them to rapamycin, a constitutively active MEK allele could not enhance IRES activity in high-AKT cells, nor could it induce resistance in these rapamycin-sensitive cells. Thus, MEK/ERK activity is required, but not sufficient, for AKT-mediated regulation of IRES activity in multiple myeloma cells. The fact that the constitutive MEK allele could induce a 1.5-fold increase in low-AKT U266EV cell IRES activity (Fig. 6D) but not in the high-AKT isogenic clone indicates an additional inhibitory effect of AKT on the D-cyclin IRES. A recent study (35) showed a possible explanation: AKT-induced phosphorylation of hnRNP A1, a newly discovered ITAF for D-cyclin IRES function, prevented its ability to promote cap-independent
translation. Thus, AKT has two separate effects that could regulate IRES function: (a) It down-regulates ERK activity, and (b) it paralyzes hnRNP A1 activity.

In summary, our study shows that AKT regulates the sensitivity of multiple myeloma cells to mTOR inhibitor–induced cytostasis. This is associated with an AKT-dependent effect on cyclin D translation and IRES activity that is mediated, in part, by regulation of ERK activity. AKT hyperactivity is a frequent occurrence in myeloma (36), and heightened AKT activity confers resistance to a variety of other anticancer therapies. Thus, the ability of mTOR inhibitors to exploit overactive AKT for heightened cyto- reductive effects could have considerable therapeutic potential in multiple myeloma. Furthermore, our results suggest that assessment of pretreatment AKT activity and monitoring the effect on D-cyclin expression may be predictive in clinical trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Regulation of D-cyclin translation inhibition in myeloma cells treated with mammalian target of rapamycin inhibitors: rationale for combined treatment with extracellular signal–regulated kinase inhibitors and rapamycin

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