Enhanced Sensitivity of Multiple Myeloma Cells Containing *PTEN* Mutations to CCI-779[1]

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ABSTRACT

Recent work identifies the AKT kinase as a potential mediator of tumor expansion in multiple myeloma. The finding of PTEN mutations in several myeloma cell lines suggests that loss of PTEN function may be one mechanism by which AKT activity is increased in this disease. Because PTEN-deficient myeloma cells may have up-regulated activity of the mammalian target of rapamycin (mTOR), downstream of AKT, they may be particularly sensitive to mTOR inhibition. To test this hypothesis, we challenged myeloma cell lines with CCI-779, a newly developed analogue of rapamycin and an efficient inhibitor of mTOR. Three of four PTEN-deficient cell lines with constitutively active AKT were remarkably sensitive to cytoreduction and G_1 arrest induced by CCI-779 with ID_{50} concentrations of <1 nM. In contrast, myeloma cells expressing wild-type PTEN were >1000-fold more resistant. Acute expression of a constitutively active *AKT* gene in CCI-779-resistant myeloma cells containing wild-type PTEN and quiescent AKT did not convert them to the CCI-779-sensitive phenotype. Conversely, expression of wild-type PTEN in CCI-779-sensitive, PTEN-deficient myeloma cells did not induce resistance. Differential sensitivity did not appear to be due to differences in the ability of CCI-779 to inhibit mTOR and induce dephosphorylation of p70S6kinase or 4E-BP1. However, CCI-779 inhibited expression of c-myc in CCI-sensitive PTEN-null myeloma cells but had no effect on expression in CCI-resistant cells. In contrast, cyclin D1 expression was not altered in either sensitive or resistant cells. These results indicate that PTEN-deficient myeloma cells are remarkably sensitive to mTOR inhibition. Although the results of transfection studies suggest that the level of PTEN and AKT function *per se* does not regulate sensitivity, PTEN/AKT status may be a good predictive marker of sensitivity.

INTRODUCTION

Recent work identifies the PI3-K[3]/AKT kinase pathway as a potential mediator of tumor expansion in MM. AKT is phosphorylated (activated) *in situ* in myeloma cells of patient bone marrow (1), and selective inhibition of the pathway in myeloma cell lines results in suppression of myeloma cell proliferation (2) or sensitization to apoptosis (3).

The activation of AKT in marrow-based myeloma cells may be due to *in situ* cytokine stimulation. We (2) and others (4) have shown that the myeloma growth factors IL-6 and IGF-I activate PI3-K/AKT in myeloma cell lines, supporting this notion. However, a second possible mechanism of AKT activation is PTEN mutation. *PTEN* is a tumor suppressor gene that encodes a phosphatase that regulates

signaling through the PI3-K/AKT pathway. Thus, loss of PTEN function results in up-regulated AKT activation and downstream signaling. Recently, Hyun *et al.* (5) identified loss of function PTEN mutations in human myeloma cells.

Although the antiapoptotic effect of the PI3-K/AKT pathway is mediated through several possible AKT targets, most evidence indicates that the proliferative effect is uniquely mediated via downstream signaling through the mTOR [also known as FKBP12 rapamycin-associated protein or FRAP (6)]. It is not clear whether mTOR is immediately downstream of PI3-K/AKT in a linear pathway or is regulated by PI3-K/AKT in a more complex fashion (6). In any case, PI3-K/AKT-dependent mTOR activity, in turn, results in phosphorylation of p70 and 4E-BP1 translational repressor, and these phosphorylation events are critical for the up-regulated translation required for cell cycle transit (7). Phosphorylation of p70 is critical for ribosome biogenesis, and phosphorylation of 4E-BP1 disrupts its interaction with the eIF-4E initiation factor, allowing eIF-4E to participate in assembly of a translation initiation complex. Our preliminary work supports a role for these downstream targets of PI3-K/AKT because p70 and 4E-BP1 are frequently phosphorylated in MM cells (8, 9).

Because mTOR appears to be a critical signal protein in myeloma cells, the use of mTOR inhibitors has appeal. Rapamycin and its newly developed analogue, CCI-779, are specific inhibitors of mTOR. They form a complex with FKBP12 that binds specifically to mTOR and prevents its ability to induce phosphorylation of p70 and 4E-BP1. These drugs have shown antitumor efficacy in preclinical models (10, 11) and are currently in Phase I trials (12).

Because PTEN-deficient tumor cells may have a selective up-regulation of the AKT/mTOR/p70/4E-BP1 pathway, they may be particularly sensitive to mTOR inhibition. Recent work in prostate cancer cells (11, 13) supports this hypothesis. Furthermore, transfection of a constitutively active *AKT* gene into wild-type PTEN-containing cells resulted in enhanced sensitivity (11), supporting the hypothesis that the up-regulated signaling downstream of mutated PTEN determines sensitivity to mTOR inhibition. Because rapamycin or CCI-779 may be particularly effective in tumors with up-regulated AKT activation and our prior work established frequent AKT activation in myeloma cells (1), we initiated the current study, investigating CCI-779 as a potential therapeutic agent in PTEN-deficient myeloma cells. Our results are consistent with the previous work (11) documenting enhanced sensitivity to mTOR inhibition. However, there was no alteration of sensitivity when a constitutively activated *AKT* gene was introduced into wild-type PTEN-expressing tumor cells or when a wild-type *PTEN* gene was introduced into PTEN-deficient tumor cells.

MATERIALS AND METHODS

Myeloma Cells. The MM cell lines 8226, OCI-My5, and AF-10 were kind gifts from Drs. J. Epstein (University of Arkansas), H. Messner (University of Toronto), and James Berenson (University of California Los Angeles). The lines were maintained *in vitro* as described previously (2). HS-Sultan, ARH-77, OPM 2, and Δ47 cell lines were purchased from American Type Culture Collection. The culture conditions for OPM 2 and Δ47 have been described previously (5). UCLA #1 and UCLA #2 cell lines

were recently initiated from isolated CD38-expressing plasma cells of two patients as described previously (1).

Reagents. Recombinant IL-6 and IGF-I were purchased from R&D Systems (Minneapolis, MN). Phospho-specific antibodies against phosphorylated AKT, p70, and 4E-BP1 (on serine 65) were purchased from New England Biolabs or Upstate Biotechnology, Inc.). Antibody detecting total 4E-BP1 was a kind gift of N. Sonenberg and C. Lister (Montreal, Canada). CCI-779 was provided by Wyeth-Ayerst (Pearl River, NY). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise described.

Cell Treatment and MTT Assays. For testing effects on cell signaling, MM lines were incubated with CCI-779 at varying concentrations for 6 h. For testing effects on cell survival, MM cells were continuously cultured with varying concentrations of CCI-779 for 48 h. Cells were then harvested, and viable recovery was determined by MTT assays as described previously (14). The percentage of cell survival was determined as follows: $A_{exp\ group}$/$A_{control} \times 100$. An ID_{50} (concentration of drug inducing 50% decrease in survival) was determined by extrapolation of results of MTT assays whereby percentage survival was plotted against $\log_{10}$ concentration.

Western Blot Analysis. Protein was extracted and separated by 12.5% SDS-PAGE as described previously (2). Proteins were transferred to polyvinylidene difluoride membranes and phosphorylated, and total proteins were detected as previously described (2). Relative expression of proteins was determined by densitometry.

Northern Blot Analysis. Total RNA isolation and Northern blot analysis were performed as described previously (15). Full-length PTEN cDNA was used as the probe.

Cell Cycle Analysis. MM cells were stained with hypotonic propidium iodide (50 μg/ml in 0.1% sodium citrate) and 0.1% Triton X-100 for 1 h at 4°C. Cells were kept in the dark before analysis. Cell cycle distribution was determined by analyzing 1,000–15,000 events on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The DNA data were fitted to a cell cycle distribution analysis by use of the MODFIT program for MAC V2.0.

Apoptosis Assay. Cells were stained with 7-AAD. The percentage of apoptosis was identified by flow cytometry as described previously (16). To induce apoptosis, cells were treated for 18 h with anti-fas antibody (clone CH11; Upstate Biotechnology, Inc.).

p70 *in Vitro* Kinase Assay. As described previously (17), p70 was immunoprecipitated from MM cells with C18 antibody (Santa Cruz Biotechnology). p70 was then mixed with S6 substrate peptide (Upstate Biotechnology, Inc.) in kinase buffer in the presence of 200 μCi/ml γ-^{32}P. The reaction was stopped by the addition of 20 μl of 250 mM EDTA, followed by boiling for 5 min. The reaction mixture was then transferred to phosphocellulose columns (Pierce) that were centrifuged to separate free ^{32}P from S6-labeled ^{32}P. The amount of labeled S6 peptide that adhered to the columns was then assayed by liquid scintillation. For each cell line, equivalent amounts of cell lysate were immunoprecipitated with an anti-Grb2 antibody (similar isotype) as a negative control, and these precipitates were also tested for phosphorylating activity against the S6 substrate. The activity of this control immunoprecipitate (in cpm) was considered background and subtracted from the activity in p70-anti-p70 immunoprecipitates to give specific p70 activity, which is what is presented in Figs. 3 and 4.

Expression Constructs and Transductions. The hemagglutinin-tagged E40K construct, which contains a point mutation in its PH domain and possesses enhanced constitutive AKT activity, was described previously (18). A recombinant adenovirus that encodes E40K was generated by homologous recombination in bacteria as described previously (19). The construct was first cloned into pAdTrack-CMV, which also contains the green fluorescent protein gene. The resultant construct was linearized and transformed with the super-coiled adenoviral vector, pAdEasy-1, into *Escherichia coli,* and recombinants were selected in kanamycin and screened by restriction endonuclease digestion. The recombinant adenovirus was then transfected into 293 cells. Control pAdTrack-CMV recombinant virus was generated in an identical fashion, but without transgene insertion. Adenovirus expressing wild-type PTEN has been described previously (20). As a corresponding control, we used the same virus with EGFP instead of PTEN as the transgene. Myeloma cells were transduced with adenovirus at varying MOIs for 2 h. Adenovirus was then washed away, and cells were resuspended in media with low serum concentration (1% FCS) to minimize proliferation. At 24 and 48 h, EGFP fluorescence demonstrated that >85% of cells were successfully transfected when using MOIs between 10

and 100. Expression of PTEN and E40K was also confirmed by immunoblot assay.

Sequencing Analysis. Reverse transcription-PCR reactions were performed as described previously (5) using primers flanking the translation start codon and stop codon allowing amplification of 1.2-kb cDNAs. The PCR products were subcloned into the PCR4-Topo cloning vector (Invitrogen) for sequence analysis.

Binding of 4E-BP1 to m^{7}GTP-Sepharose. 4E-BP1 binding to m^{7}GTP-Sepharose was performed as described previously (21). 4E-BP1 bound to beads was eluted by boiling in SDS-PAGE sample buffer. The samples were subjected to electrophoresis, and 4E-BP1 was detected by immunoblotting.

Statistics. The *t* test was used to determine significance of difference.

RESULTS

PTEN Mutations in Myeloma Cell Lines Correlate with Heightened AKT Phosphorylation. Loss of function PTEN mutations have been detected recently in two human myeloma cell lines, OPM 2 and Δ47 (5). In a further screening, we sequenced PTEN reverse transcription-PCR products in additional myeloma cell lines. AF-10, 8226, OCI-My5, UCLA #1, and ARH-77 MM cells only contained wild-type PTEN sequence. However, PTEN mutations were detected in two additional myeloma cell lines. In HS-Sultan cells, PTEN was mutated with a C to T transition at the first base of codon 17, resulting in a substitution of glutamine with a premature termination signal. This point mutation in HS-Sultan cells has actually been described previously (22). However, we additionally detected a deletion in exon 6 in the HS-Sultan PTEN sequence. As described previously (22), HS-Sultan cells also showed loss of the wild-type allele. In UCLA #2, a MM cell line recently initiated from a patient with plasma cell leukemia, sequencing demonstrated two separate PTEN mutations: (*a*) a C to T transition in exon 3, resulting in a leucine to proline substitution; and (*b*) an A to G transition in exon 8, resulting in an aspartic acid to serine substitution. As in HS-Sultan cells, there was loss of the wild-type allele. Northern blot analysis demonstrated that the former alterations in HS-Sultan cells (Fig. 1*A, HS*) resulted in loss of expression of the PTEN 5.5-kb transcript (Fig. 1*A*). In contrast, normal-sized PTEN RNA was expressed in UCLA #2 cells (Fig. 1*A, UC2*) as well as in AF-10, 8226, and UCLA #1 cells (Fig. 1*A*) and in OCI-My5 and ARH-77 cells (data not shown). In Western blot analysis, which used AF-10 cells stably transfected with the wild-type *PTEN* gene as a positive control (Fig. 1*B*), we also demonstrated the absence of PTEN protein expression in HS-Sultan cells. Varying levels of normal-sized PTEN protein expression were detected in AF-10, 8226, UCLA #1, and UCLA #2 cells (Fig. 1*B*) as well as in OCI-My5 and ARH-77 cells (data not shown). Although the detected mutations in UCLA #2 cells did not prevent PTEN RNA and protein expression (Fig. 1, *A* and *B*), these mutations are in critical areas of the gene. The former substitution (exon 3) is in an area that regulates phosphatase activity, and the latter (exon 8) is in the carboxy terminus, which mediates protein-protein interactions and PTEN targeting to the cell membrane. Either mutation or both mutations could theoretically inhibit normal PTEN function.

To test whether the PTEN alterations described by Hyun *et al.* (5) in OPM 2 and Δ47 cell lines and the above-described alterations in HS-Sultan and UCLA #2 cells correlated with heightened AKT activation, we performed immunoblot assays using antibodies that specifically detect phosphorylated/activated AKT and total AKT protein. As shown in Fig. 1*C,* comparable amounts of total AKT were expressed in all of the screened myeloma lines. However, phosphorylated AKT was only detected in the four human MM cell lines with PTEN mutations (UCLA #2, Δ47, OPM 2, and HS-Sultan). Furthermore, we were able to infect PTEN-mutated HS-Sultan and UCLA #2 cells with an adenovirus expressing wild-type PTEN. As shown in
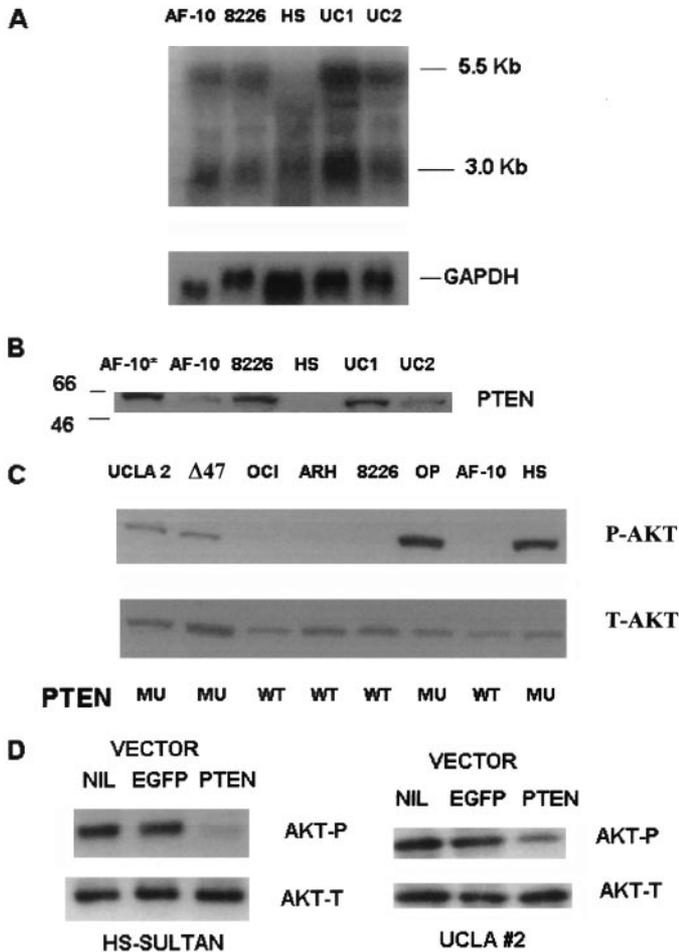
A

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C

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Fig. 1. Analysis of PTEN/AKT status in myeloma cells lines. *A,* Northern analysis of PTEN RNA expression in AF-10, 8226, HS-Sultan (*HS*), UCLA#1 (*UC1*), and UCLA#2 (*UC2*) cells. *B,* immunoblot assay for PTEN protein expression in AF-10, 8226, HS-Sultan (*HS*), UCLA#1 (*UC1*), and UCLA#2 (*UC2*) cell lines. *AF-10** , AF-10 cells stably transfected with wild-type *PTEN* gene. *C,* immunoblot assay for phosphorylated AKT (*P-AKT*) and total AKT (*T-AKT*) in myeloma cell lines. *OCI,* OCI-My5; *ARH,* ARH-77; *OP,* OPM 2; *HS,* HS-Sultan. PTEN status is shown *below* the blot as mutated (*MU*) or wild type (*WT*). *D,* effect of PTEN transfection in HS-Sultan or UCLA #2 cell lines. Wild-type *PTEN* gene or control empty vector (EGFP) was transfected by adenoviral vector. Twenty-four h later, an immunoblot assay was performed for phosphorylated AKT (*AKT-P*) or total AKT (*AKT-T*).

Fig. 1*D,* ectopic PTEN expression resulted in loss of AKT phosphorylation in HS-Sultan cells (*left panel*) and a significant decrease of AKT phosphorylation in UCLA #2 cells (*right panel*), confirming that the respective PTEN mutations were responsible for the uncontrolled AKT activation.

Sensitivity of PTEN-null MM Cells to Cytoreduction Induced by CCI-779. Previous work (11) suggests that the PTEN-deficient state may result not only in heightened AKT activation but also in enhanced downstream signaling through the mTOR. mTOR activity, in turn, results in phosphorylation of the p70 and the 4E-BP1 translational repressor, events that are critical for the up-regulated translation of transcripts required for cell cycle transit. To examine the effects of mTOR inhibition on the growth of PTEN-deficient MM cells, we exposed cells to varying concentrations of CCI-779 *in vitro.* CCI-779 is a newly developed analogue of rapamycin, the classical inhibitor of mTOR. The effect of CCI-779 on mTOR is comparable with that of rapamycin in potency and specificity, and CCI-779 has recently entered Phase I trials (12). Furthermore, recent work (11) demonstrated a heightened sensitivity of PTEN-deficient tumors to the cytoreductive effects of CCI-779. To test whether our PTEN-

mutated myeloma lines were also sensitive to CCI-779, we cultured MM cells with increasing concentrations of CCI-779 for 48 h in MTT assays. As shown in Fig. 2, there was a clear distinction in sensitivity with three of the four PTEN-mutated MM cell lines (UCLA #2, HS-Sultan, and OPM 2) demonstrating remarkable sensitivity to CCI-779 with ID_{50}s of 0.1–1.0 nM. The exception was the PTEN-mutated $\Delta 47$ cell line, which was resistant to CCI-779. In contrast, MM cells containing wild-type PTEN (ARH-77, AF-10, and OCI-My5) were all resistant to CCI-779, and 50% inhibition of growth was never achieved, even with concentrations of 1000 nM CCI-779. Thus, there is a general correlation between the PTEN-null state of myeloma cells and sensitivity to CCI-779, the exception being the $\Delta 47$ cell line. These results are in keeping with that of Neshat *et al.* (11).

To examine whether the cytoreductive effect of CCI-779 on PTEN-mutated MM cells was due to induction of G_1 block or apoptosis, we analyzed cells stained with propidium iodide for cell cycle analysis and cells stained with 7-AAD to assess induction of apoptosis. Anti-fas treatment was used as a positive control for induction of and detection of apoptosis. Data in Table 1 clearly indicate that the mTOR inhibitors induced a G_1 cell cycle block without induction of apoptosis in the PTEN-deficient OPM 2, HS-Sultan, and UCLA #2 cell lines. This block in the proliferative cycle of the PTEN-deficient lines was much more impressive than that seen in the wild-type PTEN-containing ARH-77 cell line (Table 1).

p70 Activity and Phosphorylation in PTEN-mutated MM Cells. Because PTEN-deficient MM cells have elevated AKT activation, we next asked whether these cells also have enhanced mTOR activity. This might be an explanation for the enhanced sensitivity of PTEN-
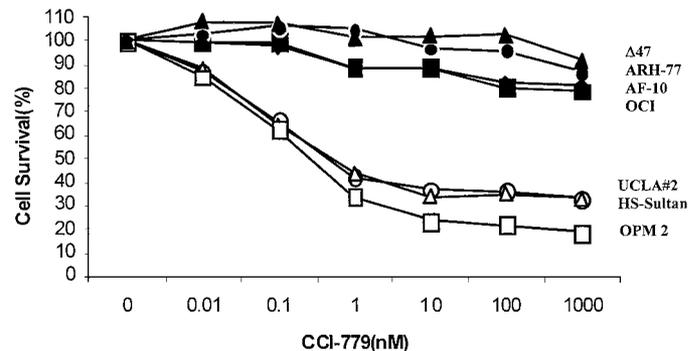

Fig. 2. Differential sensitivity to CCI-779. MTT assay was performed after a 48-h culture of cell lines with increasing concentrations of CCI-779.

Table 1 *CCI-779 induces G_1 arrest in PTEN-deficient myeloma cells[a]*

Cell line	Conditions[b]	% G_1[c]	% S[c]	% Apoptosis[c]
OPM 2		40	42	5
	CCI-779, 0.05	60[d]	27[d]	4
	CCI-779, 0.1	68[d]	22.1[d]	3.1
	CCI-779, 10	78[d]	17[d]	4.5
	Anti-fas			21[d]
HS-Sultan		33	61	4.4
	CCI-779, 0.05	74[d]	20[d]	3.9
	Anti-fas			15[d]
UCLA #2		45	39	3
	CCI-779, 0.05	69[d]	17[d]	4
ARH-77		45	38	6
	CCI-779, 0.05	52	32	5
	CCI-779, 0.1	54[d]	31[d]	5

[a] Cell lines were cultured with or without drugs for 20 h, and cell cycle distribution was determined by fluorescence-activated cell-sorting analysis of hypotonic propidium iodide-stained cells, and apoptosis was determined by 7-AAD-stained cells.

[b] CCI-779 concentration shown in nM. Anti-fas antibody was used at 0.5 μg/ml.

[c] Results are percentage of G_1 or S phase or apoptosis, means of four experiments. The SDs were all <5% of the respective means.

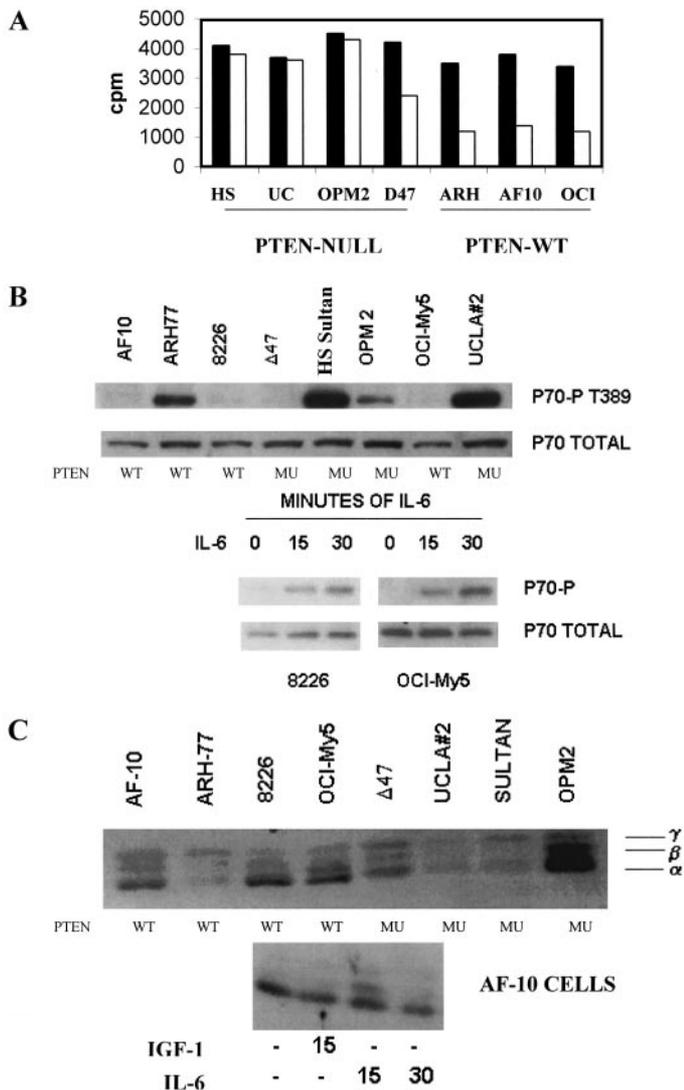[d] Significantly different ($P < 0.05$) from control (culture without drugs).

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Fig. 3. Analysis of p70 and 4E-BP1 in myeloma lines. *A, in vitro* kinase assay for p70 activity in cell lines cultured in serum (■) or after serum depletion (overnight, □). *HS,* HS-Sultan; *UC,* UCLA #2; *D47,* Δ47; *ARH,* ARH-77; *OCI,* OCI-My5; *PTEN-WT,* expressing wild-type PTEN. Results are specific kinase activity (calculated as described in "Materials and Methods") and are the means of three separate experiments. The SDs were <5% of the means in all groups. *B, top two panels,* immunoblot assay for phosphorylated p70 on threonine 389 (*P70-P T389*) or total p70; *bottom panels,* similar immunoblot assay on 8226 or OCI-My5 cells after 0, 15, or 30 min of incubation with IL-6 (100 units/ml). *C,* immunoblot for 4E-BP1 expression in myeloma cells is shown in the *top panel.* γ, β, and α designate differentially phosphorylated forms of 4E-BP1. PTEN status is given *below* the immunoblot as wild type (*WT*) or mutated (*MU*). In the *bottom panel,* AF-10 cells were stimulated with IGF-I (400 ng/ml) or IL-6 (100 units/ml) for 15 or 30 min (as shown), and an immunoblot assay for 4E-BP1 expression was performed.

null MM cells to CCI-779. We tested this notion by examining p70 and the 4E-BP1 translational repressor, which are downstream targets of mTOR. p70 activity in the four PTEN-null cell lines (HS-Sultan, Δ47, UCLA #2, and OPM-2) was not significantly greater than that in the ARH-77, AF-10, or OCI-My5 MM cell lines that express wild-type PTEN (Fig. 3A). However, activity in the latter cell lines was significantly lost by serum depletion (Fig. 3A, □), whereas p70 activity in PTEN-mutated HS-Sultan, UCLA #2, and OPM-2 cells was not affected by serum depletion. The activity in PTEN-mutated Δ47 cells was modestly affected.

p70 activity correlates well with phosphorylation of p70 on threonine 389 (23). To test phosphorylation, we used a phospho-specific antibody that only recognizes p70 when it is phosphorylated on threonine 389. As shown in Fig. 3B, constitutive phosphorylation of p70 on threonine 389 was demonstrated in three of the four PTEN-mutated cell lines, HS-Sultan, OPM 2, and UCLA #2. In contrast, the PTEN-mutated Δ47 cell line did not express phosphorylated p70, although the total amount of p70 expressed was comparable with that of the other three MM lines with PTEN mutations. Of the other four MM lines we tested that contained wild-type PTEN, only the ARH-77 cell line constitutively expressed a considerable amount of phosphorylated p70. Although the other MM cells with wild-type PTEN contained little phosphorylated p70 in their basal state, the myeloma growth factor, IL-6, rapidly induced p70 phosphorylation in these cell lines (Ref. 8 for AF-10 cells; data shown for 8226 and OCI-My5 in Fig. 3B, *bottom panel*).

4E-BP1 Phosphorylation in PTEN-mutated Myeloma Cells. In addition to p70 activity, mTOR also regulates phosphorylation of the 4E-BP1 translational repressor (6). To investigate this additional potential molecular target, we used immunoblot assays to detect 4E-BP1 expression. 4E-BP1 was resolved into between one and three separate bands, as shown in Fig. 3C. These separate 4E-BP1 forms arise from differences in the phosphorylation state, with an increased phosphorylation causing a decrease in mobility. Thus, as shown in Fig. 3C, the bands designated γ and β represent the more phosphorylated species of 4E-BP1. Wild-type PTEN-containing MM cell lines AF-10, 8226, and OCI-My5 demonstrate that most of their 4E-BP1 is present in the α hypophosphorylated form. This is expected because there should be minimal constitutive upstream activation of AKT and mTOR due to the presence and restraining activity of wild-type PTEN. However, when AF-10 MM cells are exposed to IL-6, 4E-BP1 becomes phosphorylated, as shown by the development of two slower-migrating bands at the 15 min time point (Fig. 3C, *bottom panel*). The induction of 4E-BP1 phosphorylation by IL-6 in AF-10 cells is even more impressive than that induced by IGF-I. In contrast, considerable proportions of 4E-BP1 are constitutively expressed as the hyperphosphorylated γ and β forms in the PTEN-mutated cell lines OPM 2, HS-Sultan, UCLA #2, and Δ47. The ARH-77 cell line, with wild-type PTEN expression, also expresses increased hyperphosphorylated 4E-BP1. Although this is difficult to explain in light of its wild-type PTEN status and lack of AKT activation, this finding correlates with ARH-77's constitutive expression of phosphorylated p70 (Fig. 3B), the other mTOR target.

Sensitivity of p70 Activation/Phosphorylation and 4E-BP1 Phosphorylation to CCI-779. We next tested the concentration dependence and specificity of the ability of CCI-779 to inhibit the p70. Relatively low concentrations of drug inhibited p70 activity in all four PTEN-mutated cell lines (Fig. 4A). This inhibition correlated nicely with the ability of CCI-779 to also inhibit p70 phosphorylation on threonine 389 (Fig. 4B). Inhibition of p70 activity/phosphorylation was relatively specific, with similar concentrations of CCI-779 having no effect on the heightened AKT phosphorylation in these cell lines (data shown for HS-Sultan cells in Fig. 4C).

We tested similar concentrations of CCI-79 for their effects on 4E-BP1 phosphorylation. Because 4E-BP1 phosphorylation inhibits its binding to eIF-4E, we also tested the effect of CCI-779 on the ability of 4E-BP1 to bind eIF-4E. After exposure to CCI-779, cell extracts were first immunoblotted with antibodies to detect total 4E-BP1, phosphorylated 4E-BP1 (*P-4E-BP1,* 4E-BP1 phosphorylated on serine 65), or actin (Fig. 5). To test binding of 4E-BP1 to eIF-4E, eIF-4E was first precipitated using m⁷GTP-Sepharose, followed by immunoblot detection of 4E-BP1 in the precipitates. Results are shown in Fig. 5A for two PTEN-deficient MM cell lines, HS-Sultan and OPM 2. Although there is little expression of total 4E-BP1 in HS-Sultan cells, the faint hyperphosphorylated γ 4E-BP1 band becomes lost after exposure to high concentrations of CCI-779 (10 and 100 nM; Fig. 5A, *top panel*). This occurs in conjunction with decreased
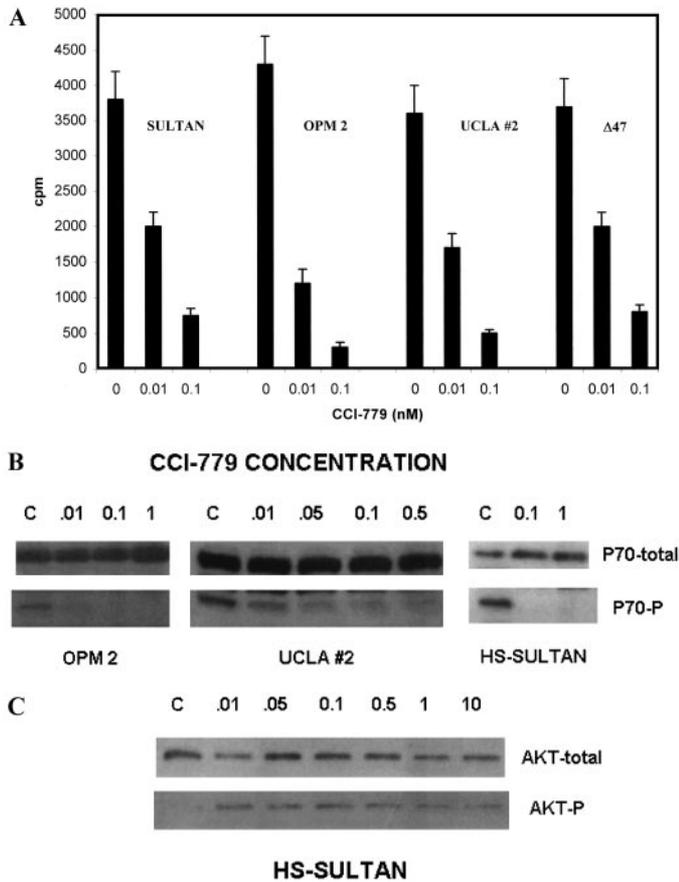
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Fig. 4. Effect of CCI-779 on p70. *A,* PTEN-deficient cell lines were cultured for 6 h in increasing concentrations of CCI-779, and an *in vitro* kinase assay was performed for specific p70 activity. Results are the means ± SD of three separate experiments. All levels of activity after CCI-779 exposure are significantly lower ($P < 0.05$) than those of the corresponding control (no CCI-779). *B,* OPM 2, UCLA #2, or HS-Sultan cells were incubated with CCI-779 for 6 h at the designated concentration (in nM), and then an immunoblot assay was performed for expression of phosphorylated p70 (*P70-P*) or total P70 (*P70-total*). *C,* HS-Sultan cells were exposed to CCI-779 (concentration in shown above the panels in nM) for 6 h, and then an immunoblot assay was performed for expression of phosphorylated AKT (*AKT-P*) or total AKT (*AKT-total*).

specific immunodetection of phosphorylated 4E-BP1 in HS-Sultan cells (Fig. 5*A, second panel, P-4E-BP1*), which is slightly decreased at 1 nM CCI-779 and barely detected at 10 and 100 nM CCI-779. For OPM 2 cells, there is little change in 4E-BP1 phosphorylation (Fig. 5*A, top panel*). With increasing concentrations of CCI-779 (1–100 nM), however, more rapidly migrating forms of phosphorylated 4E-BP1 (Fig. 5*A, second panel*) become expressed. This suggests that, in OPM 2 cells, although CCI-779 has little effect on phosphorylation of 4E-BP1 at serine 65, it is capable of dephosphorylating other residues, which results in faster electrophoretic migration. Analysis of 4E-BP1 binding to eIF-4E demonstrates that, in HS-Sultan cells, CCI-779-induced dephosphorylation of 4E-BP1 is associated with increased binding of 4E-BP1 to eIF-4E. Although this is difficult to see in the *fourth panel* of Fig. 5*A* (labeled *PPT-4E-BP1*), upon longer exposure of the film (Fig. 5*A,* **), this conclusion is clear. Slightly increased 4E-BP1 is detected in the m^7GTP-eIF-4E precipitate after exposure to 0.1 and 1 nM CCI-779, and a greater amount of bound 4E-BP1 is detected after exposure to 10 and 100 nM CCI-779. In contrast, there is little effect of CCI-779 on 4E-BP1 binding to eIF-4E in OPM 2 cells (Fig. 5*A, fourth panel*). Reprobing with an antibody to eIF-4E (Fig. 5*A, bottom panel*, labeled *PPT-eIF-4E*) demonstrates that equal amounts of eIF-4E are present in each precipitate. In summary, exposure of OPM 2 cells to CCI-779 had little effect on 4E-BP1

phosphorylation at serine 65 and little effect on binding of 4E-BP1 to eIF-4E. In contrast, CCI-779 induced dephosphorylation of 4E-BP1 and increased the binding of 4E-BP1 to eIF-4E in HS-Sultan cells.

Relative Sensitivity of CCI-779-resistant *versus* CCI-779-sensitive MM Cells to Drug-induced Dephosphorylation of p70 or 4E-BP1. Another potential explanation for the differential sensitivity of PTEN-deficient MM cells to CCI-779 is a differential sensitivity to the ability of the drug to induce dephosphorylation of p70 or 4E-BP1. We tested that hypothesis by directly comparing the CCI-779-sensitive, PTEN-mutated HS-Sultan and OPM 2 MM cell lines with the CCI-779-resistant, wild-type PTEN-expressing ARH-77 cell line. The CCI-779-resistant ARH-77 cell line was an appropriate comparison because it expressed phosphorylated p70 and phosphorylated 4E-BP1 in its basal state (Fig. 3), which was comparable to that present on the PTEN-deficient cells. As shown in Fig. 5*A,* ARH-77 cells were exquisitely sensitive to dephosphorylation of 4E-BP1. After exposure to only 0.1 nM CCI-779, the majority of 4E-BP1 became expressed as the lower, faster-migrating hypophosphorylated α band (Fig. 5*A, top panel*), and specific immunodetection of phosphorylated 4E-BP1 (Fig. 5*A, second panel*, labeled *P-4E-BP1*) was lost. There was a corresponding increase in the amount of 4E-BP1 bound to eIF-4E first present after exposure to 0.1 nM CCI-779, which increased with increasing concentrations of drug (*fourth panel,* labeled *PPT-4E-BP1*). Thus, the ability of CCI-779 to induce dephosphorylation of 4E-BP1 and increase binding of 4E-BP1 to eIF-4E in CCI-779-resistant ARH-77 cells was at least comparable with that in the CCI-779-sensitive HS-Sultan cell line and much more impressive than that in the CCI-779-sensitive OPM 2 cell line.

Similar results were seen when p70 phosphorylation was examined. As shown in Fig. 5*B,* CCI-779 was extremely effective in dephosphorylating p70 in ARH-77 cells. This was comparable with that seen in CCI-779-sensitive HS-Sultan and OPM 2 targets when the three cell lines were compared head to head and occurred at comparable low concentrations of CCI-779 (0.01 nM).

Effects on Expression of c-myc and Cyclin D1. To test whether CCI-779 sensitivity correlates with the drug's ability to reduce expression of proteins that are important for cell cycle transit, we performed immunoblot analysis of cyclin D1 and c-myc expression, comparing the effects in CCI-779-sensitive HS-Sultan *versus* CCI-779-resistant ARH-77 cells. As shown in Fig. 6*A,* exposure to CCI-779 for 48 h had no inhibitory effect on cyclin D1 expression in either cell line. In fact, there was even a modest increase in expression in the CCI-779-sensitive HS-Sultan cells. Because these data were somewhat surprising, we repeated the experiment with CCI-779-sensitive HS-Sultan cells, studying expression at several time points. As shown in Fig. 6*B,* little effect on cyclin D1 expression was present at 6 or 24 h, and at 48 h, a modest increase in expression was again noted. In contrast, CCI-779 did have a differential effect on c-myc expression. As shown in Fig. 6*C,* a significant decrease in expression was induced in CCI-779-sensitive HS-Sultan cells, whereas expression was unaffected in the CCI-779-resistant ARH-77 cells.

Enhancing or Inhibiting AKT Function Does Not Affect CCI-779 Sensitivity. When wild-type PTEN-containing prostate cancer cells are stably transfected with a constitutively active *AKT* gene, they become considerably more sensitive to the antitumor effect of CCI-779 *in vivo* (11). To attempt similar studies in MM cells lines, we expressed a constitutively active AKT mutant, E40K, in wild-type PTEN-containing MM cells using an adenoviral vector. Our adenoviral vector expresses EGFP, which allowed us to test transduction efficiency. Fluorescence microscopy demonstrated very effective transduction at low MOI (>90% at MOI 10) in AF-10 and OCI-My5 MM cells. Twenty-four h after infection of these cell lines, an immunoblot assay demonstrated that the transfected AKT was constitu-
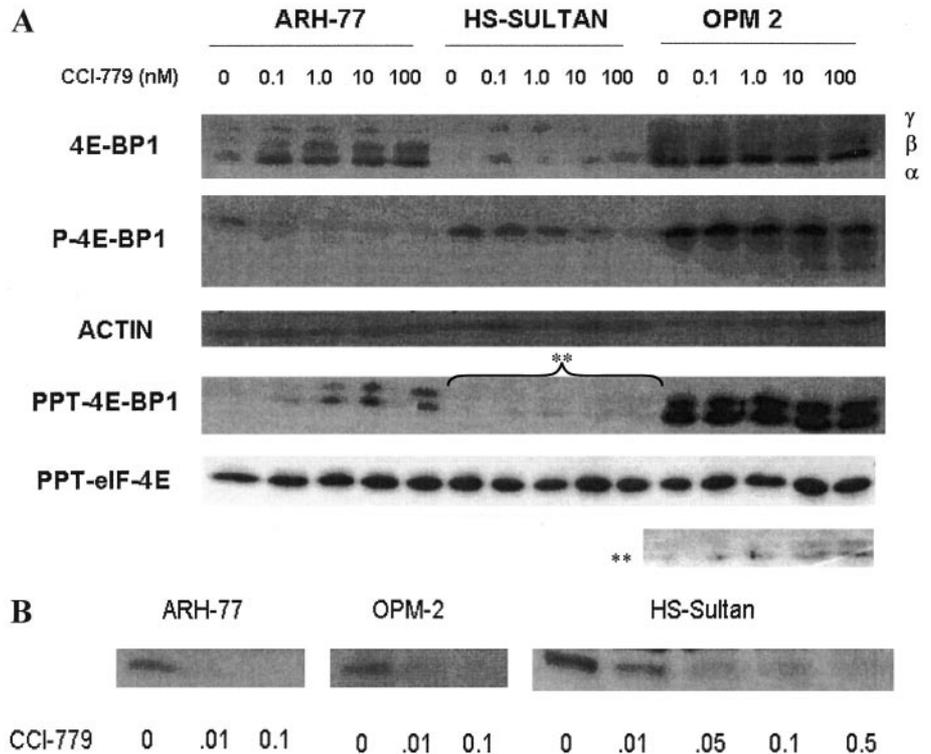
5031

Fig. 5. Comparison of the CCI-779-resistant ARH-77 cell line with the CCI-779-sensitive HS-Sultan and OPM 2 cell lines. *A,* the three cell lines were exposed to increasing concentrations of CCI-779 for 6 h, and then an immunoblot assay was performed for expression of 4E-BP1 *(top panel),* phosphorylated 4E-BP1 (on serine 65, *P-4E-BP1, second panel),* or actin *(third panel).* In addition, cell extracts were incubated with m^{7}GTP-Sepharose to precipitate eIF-4E, and an immunoblot assay was performed on the precipitate for presence of 4E-BP1 (*PPT-4E-BP1, fourth panel*). Results on HS-Sultan cells are shown below (∗∗) after a longer exposure. Membrane was reprobed for the presence of eIF-4E (*PPT-eIF-4E, bottom panel*). *B,* the three cell lines were exposed to increasing concentrations of CCI-779 (in nM) for 6 h, and an immunoblot assay was performed for phosphorylated p70 (total p70 expression in all lines was equal; data not shown).

tively phosphorylated (Fig. 7) and that infection also resulted in downstream phosphorylation of p70 on serine 411. Expression of 4E-BP1 in these cells (Fig. 7, *T-4E-BP1*) was mostly resolved into two bands. Transduction of cells with E40K resulted in an increase in the portion of 4E-BP1 expressed as a hyperphosphorylated, slower-migrating species in both AF-10 and OCI-My5 cells. Despite enhanced expression of phosphorylated AKT, p70, and 4E-BP1, E40K-infected cells were not altered in their sensitivity to CCI-779 when compared with control-infected cells (Fig. 8).

We next used the adenoviral vector to express wild-type PTEN in the PTEN-null OPM 2 MM cell line. Expression of EGFP in the control adenoviral vector allowed us to determine that transduction efficiencies of >80% were achieved with a MOI of 40. After infection with wild-type PTEN, OPM 2 cell growth was modestly decreased by 20% compared with control adenovirus infection. Twenty-four h after
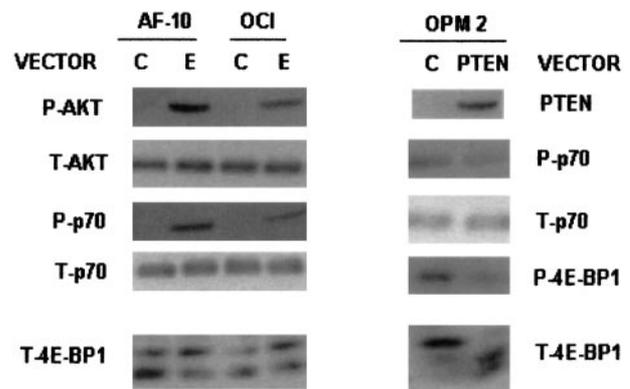

Fig. 7. Effects of transduction. *Left panels,* AF-10 or OCI-My5 (*OCI*) cells were transduced with control (empty vector, *C*) or E40K (*E*) gene, and 24 h later, an immunoblot assay was performed for phosphorylated AKT (*P-AKT*), total AKT (*T-AKT*), phosphorylated p70 (*P-p70*), total p70 (*T-p70*), or 4E-BP1. *Right panels,* OPM 2 cells were transduced with control (empty vector, *C*) or wild-type *PTEN* gene, and, 24 h later, an immunoblot assay was performed for PTEN, phosphorylated p70 (*P-p70*), total p70 (*T-p70*), phosphorylated 4E-BP1 on serine 65 (*P-4E-BP1*), or total 4E-BP1 (*T-4E-BP1*).

infection, immunoblot assays confirmed ectopic PTEN expression (Fig. 7, *top right panel*) and dephosphorylation of AKT (data not shown), dephosphorylation of p70 (Fig. 7, *P-p70*), and decreased expression of 4E-BP1 phosphorylated on serine 65 (Fig. 7, *P-4E-BP1*). Immunoblot assay of total 4E-BP1 (Fig. 7, *T-4E-BP1*) demonstrated that the γ hyperphosphorylated 4E-BP1 band was markedly decreased after PTEN transduction and that the α and β hypophosphorylated bands were increased. However, this significant inhibition of AKT, p70, and 4E-BP1 phosphorylation had no effect on sensitivity to CCI-779 (Fig. 8).

DISCUSSION

This study demonstrates a correlation between the PTEN-null state, heightened AKT phosphorylation, and sensitivity to the cytoreductive
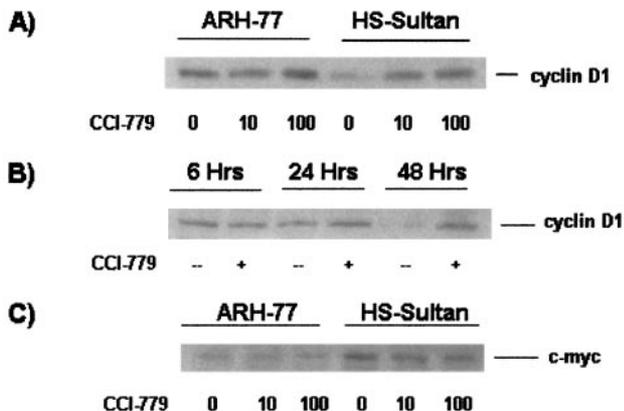

Fig. 6. Immunoblot assay for expression of cyclin D1 and c-myc. *A,* ARH-77 or HS-Sultan cells were treated with 0, 10, or 100 nM CCI-779 for 48 h, and extracts were assayed for cyclin D1 expression. *B,* HS-Sultan cells were treated with 0 or 100 nM CCI-779 for 6, 24, and 48 h and assayed for cyclin D1 expression. *C,* ARH-77 or HS-Sultan cells were treated with 0, 10, or 100 nM CCI-779 for 48 h and then assayed for c-myc expression.
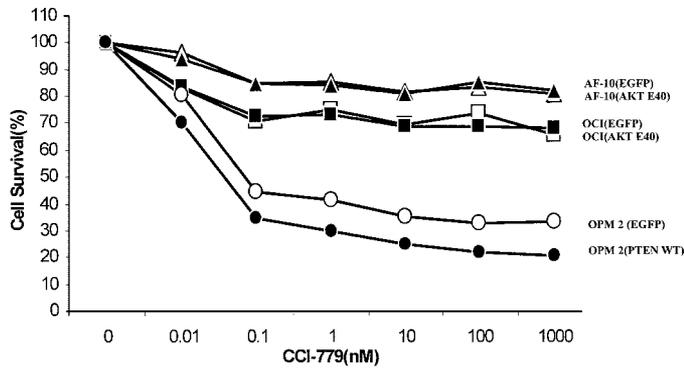
Fig. 8. MTT assay of transduced cells. AF-10 and OCI-My5 (*OCI*) cells were transduced with control *EGFP* (*EGFP*) or *E40K* (*AKTE40*) genes and treated with increasing concentrations of CCI-779 for 48 h, and the MTT assay was performed. The same assay was performed for OPM 2 cells transfected with control *EGFP* gene (*EGFP*) or wild-type *PTEN* gene (*PTEN WT*). Results are the means of three separate transduction experiments.

effects of CCI-779, a newly developed rapamycin analogue that inhibits mTOR. This finding is consistent with previous work using other cell model systems (11, 13). However, because acute expression of a constitutively active *AKT* gene or wild-type *PTEN* gene had no effect on CCI-779 sensitivity, our data suggest that the PTEN-null state and AKT activation are markers for CCI-779 sensitivity rather than the cause of heightened sensitivity.

To date, PTEN mutations have been detected in four human MM cell lines: Δ47, OPM 2, UCLA #2, and HS-Sultan. These mutations resulted in loss of PTEN expression in Δ47 (5), OPM 2 (5), and HS-Sultan (Fig. 1). Although UCLA #2 MM cells expressed normal-sized transcripts and protein, the detected PTEN mutations were in areas of the gene critical for normal PTEN phosphatase activity. Because ectopic expression of wild-type PTEN in UCLA #2 cells resulted in significant inhibition of the up-regulated phosphorylation of AKT [and p70 phosphorylation (data not shown)], the data indicated that a PTEN-deficient state is the cause of heightened activation of this pathway downstream of PI3-K. The prevalence of PTEN mutation in patient myeloma specimens is unknown. However, the fact that the UCLA #2 cell line was recently initiated from a clinical specimen suggests that PTEN mutations may be present in myeloma cells of some patients.

The enhanced sensitivity of PTEN-deficient myeloma cells was remarkable, with at least a 1000-fold lower ID_{50} than wild-type PTEN-containing MM cells. The cytoreductive effect in PTEN-deficient cells was primarily one of G_1 arrest rather than induction of apoptosis. This block in cell cycle transit was also more impressive in PTEN-deficient MM cells when compared with ARH-77 cells containing wild-type PTEN. It is possible, however, that inhibition of mTOR may result in enhanced apoptosis under some conditions. Because mTOR results in p70 activation, and p70 may inhibit apoptosis via inactivation of BAD (24), mTOR inhibition and the resulting p70 inhibition could potentially induce or sensitize a target cell for enhanced apoptosis. We are currently evaluating the effects of CCI-779 and rapamycin on BAD in myeloma cells. If they prevent inactivation of BAD, these drugs may sensitize targets to other apoptosis inducers in combination therapy. Of note, use of CCI-779 *in vivo* resulted in decreased proliferation as well as enhanced apoptosis in PTEN-deficient tumors (11).

To investigate the determinants of enhanced sensitivity to CCI-779 in PTEN-deficient MM cells, we first asked whether downstream signaling via AKT/mTOR/p70 and 4E-BP1 was enhanced. Immunoblot assays evaluating p70 and 4E-BP1 phosphorylation are somewhat supportive of this notion in that p70 and 4E-BP1 phosphorylation

roughly correlate with PTEN mutation, AKT activation, and CCI-779 sensitivity. However, the correlation is not perfect. For example, CCI-779-resistant ARH-77 MM cells, which have wild-type PTEN and quiescent AKT, demonstrate heightened phosphorylation of p70 and 4E-BP1 that is sensitive to mTOR inhibition. It is possible that mTOR activation in ARH-77 cells is achieved by an upstream pathway different from PI3-K/AKT that is unregulated by PTEN. Nevertheless, in the face of phosphorylated p70 and 4E-BP1, ARH-77 cells are resistant to CCI-779. Furthermore, although there are some differences in p70 phosphorylation, p70 activity was comparable between CCI-779-sensitive and CCI-779-resistant MM target cell lines (Fig. 3). The finding that the p70 activity in the CCI-779-sensitive lines was more resistant to serum depletion is probably not relevant because our MTT and cell cycle distribution assays are run in the presence of serum. An additional inconsistency is the fact that the Δ47 MM cell line, which contains a PTEN mutation and constitutively activated AKT, was completely resistant to CCI-779. The major difference between Δ47 and other CCI-779-sensitive, PTEN-deficient MM lines was an absence of p70 phosphorylation on threonine 389. Despite this, p70 activity in Δ47 was equal to that of other PTEN-deficient MM lines and comparably sensitive to CCI-779-induced inhibition (Fig. 4). Further evidence that it is not simply the level of downstream signaling which determines sensitivity to CCI-779 was obtained by gene transfer studies. Expression of an activated *AKT* gene, which resulted in downstream phosphorylation of p70 and 4E-BP1, could not induce sensitivity to CCI-779 in AF-10 or OCI-My5 cells, and expression of wild-type PTEN, which resulted in dephosphorylation of AKT, p70, and 4E-BP1, could not induce resistance.

Although our results do not support the hypothesis that differences in basal mTOR-dependent p70 activity and 4E-BP1 phosphorylation account for differences in the response to CCI-779, it was possible that a differential sensitivity to the mTOR-inhibiting effects of CCI-779 was key. We tested this by directly comparing CCI-779-resistant ARH-77 cells with CCI-779-sensitive OPM 2 and HS-Sultan cells. However, the results shown in Fig. 5 rule out this hypothesis because when CCI-779-resistant ARH-77 cells are compared with CCI-779-sensitive cells, there is no difference in the ability of CCI-779 to induce dephosphorylation of p70, dephosphorylation of 4E-BP1, and binding of 4E-BP1 to eIF-4E. In fact, CCI-779 effectively inhibits growth of OPM 2 cells without having a significant effect on 4E-BP1 phosphorylation at serine 65 or on 4E-BP1 binding to eIF-4E. These latter results are similar to those of a previous study by Neshat *et al.* (11), in which treatment of PTEN-null fibroblasts with CCI-779 results in inhibited proliferation and decreased 4E-BP1 phosphorylation, but no alteration on 4E-BP1 binding to eIF-4E. Thus, our data as well as the study of Neshat *et al.* (11) suggest that effects on 4E-BP1 binding to eIF-4E do not regulate sensitivity to CCI-779-induced cytoreduction.

Because CCI-779 was very efficient in inducing dephosphorylation of p70 and 4E-BP1 in HS-Sultan and ARH-77 cells as well as in reducing the amount of free, unbound eIF-4E, we were surprised to see no inhibitory effects on expression of cyclin D1 in either of these cell lines. Because we presume that the inhibitory effects of CCI-779 should significantly curtail cap-dependent translation, these data suggest an alternative cap-independent mechanism for cyclin D1 translation. One possibility is that cyclin D1 RNA can, under certain conditions, use an IRES to allow for cap-independent translation. Cyclin D1 RNA has a 5′-untranslated region that is relatively long and highly structured (25) and also contains a second additional upstream translation initiation codon. These are molecular characteristics that have been noted in RNAs previously shown to contain an IRES (26). Whatever the mechanism for cap-independent expression, it is clear

that, in our myeloma cells, down-regulation of cyclin D1 expression is not needed for CCI-779-mediated induction of G_1 arrest.

In contrast to cyclin D1, CCI-779 differentially affected c-myc expression, inhibiting it in CCI-779-sensitive cells but having little effect in CCI-779-resistant cells. c-myc RNA is known to contain an IRES (27), which could allow cap-independent translation in resistant cells. Thus, one potential explanation for the CCI-779-sensitive *versus* CCI-779-resistant phenotype is that CCI-779-sensitive cells cannot use the c-myc IRES for effective cap-independent translation, but CCI-779-resistant cells can.

As mentioned above, the results of our gene transfer studies are contrary to those of Neshat *et al.* (11), who demonstrated that ectopic expression of a constitutively activated *AKT* gene significantly sensitized cells to CCI-779. One major difference in our experimental design that could explain this inconsistency is that we acutely expressed our *AKT* gene by adenoviral infection, whereas Neshat *et al.* (11) used retroviral transfection and *in vitro* selection to ectopically express activated AKT in stably transfected lines. It is thus possible that in Neshat's stable transfectants, adaptation to a high level of AKT/mTOR activation during selection significantly altered sensitivity to CCI-779. Thus, one key determinant of sensitivity could be the degree for which a cell depends on mTOR for its proliferative potential. If cells with PTEN mutation and constitutive AKT activation are completely dependent on the AKT/mTOR/p70/4E-BP1 cascade for translation of proteins required for cell cycle transit such as c-myc, inhibition of that cascade by CCI-779 would induce G_1 arrest. In contrast, other cell populations without PTEN mutation/AKT activation might have additional pathways for enhancing expression of these cell cycle proteins (*i.e.*, cap-independent mechanisms as described above), and, thus, CCI-779, although effectively ablating any mTOR/p70/4E-BP1 signaling, would have little effect on cell growth. A similar difference in the mTOR dependence of c-myc expression explained the development of resistance to rapamycin in a previous study (28), which is consistent with our data regarding expression of c-myc. This theory would explain the lack of effect of acute expression of a constitutively active *AKT* gene or wild-type PTEN on sensitivity. In contrast, during selection of cells transfected with active *AKT* genes in a retroviral vector, the non-mTOR pathways (*i.e.*, cap-independent translation) could become down-regulated, and ultimate transfectants could be solely dependent on AKT/mTOR, thus explaining their increase in CCI-779 sensitivity.

Although the results of our gene transfer experiments suggest that the level of AKT activation *per se* does not regulate sensitivity to CCI-779, AKT status may be a good predictive marker of sensitivity. In keeping with this idea, myeloma cells that express activating mutations of ras and up-regulated downstream signaling through PI3-K and AKT also demonstrate enhanced sensitivity to mTOR inhibition (29). Because immunochemistry can identify AKT phosphorylation in myeloma cells within marrow biopsies (1), this may be an effective tool for predicting efficacy in future trials.

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Enhanced Sensitivity of Multiple Myeloma Cells Containing *PTEN* Mutations to CCI-779

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