Genetic and Pharmacologic Evidence That mTOR Targeting Outweighs mTORC1 Inhibition as an Antimyeloma Strategy

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Abstract

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates cell growth, proliferation, metabolism, and cell survival, and plays those roles by forming two functionally distinct multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Deregulation of the mTOR pathway has been found in different cancers, including multiple myeloma. Agents acting on mTORC1, such as rapamycin and derivatives, are being explored as antitumoral strategies. However, whether targeting mTOR would be a more effective antimyeloma strategy than exclusively acting on the mTORC1 branch remains to be established. In this report, we explored the activation status of mTOR routes in malignant plasma cells, and analyzed the contribution of mTOR and its two signaling branches to the proliferation of myeloma cells. Gene expression profiling demonstrated deregulation of mTOR pathway-related genes in myeloma plasma cells from patients. Activation of the mTOR pathway in myelomatous plasma cells was corroborated by flow cytometric analyses. RNA interference (RNAi) experiments indicated that mTORC1 predominated over mTORC2 in the control of myeloma cell proliferation. However, mTOR knockdown had a superior antiproliferative effect than acting only on mTORC1 or mTORC2. Pharmacologic studies corroborated that the neutralization of mTOR has a stronger antimyeloma effect than the individual inhibition of mTORC1 or mTORC2. Together, our data support the clinical development of agents that widely target mTOR, instead of agents, such as rapamycin or its derivatives, that solely act on mTORC1. Mol Cancer Ther; 13(2); 504–16. ©2014 AACR.

Introduction

Multiple myeloma is a malignant plasma cell disorder, characterized by an abnormal accumulation of plasma cells in the bone marrow. As a consequence, bone lesions are generated and, in many cases, overproduction of paraprotein/antibodies may cause kidney dysfunction (1). The introduction of new agents such as the proteasome inhibitor bortezomib or the thalidomide analog lenalidomide in the treatment of patients with myeloma has resulted in improved response rates and longer overall survival (2–5). However, most patients eventually relapse, making this malignancy incurable. For these reasons, the development of novel therapies is absolutely required to provide more treatment options.

Mammalian target of rapamycin (mTOR) is a kinase that regulates cell growth, survival, proliferation, and metabolism (6). mTOR functions by forming two protein complexes, mTORC1 and mTORC2, that share common proteins like mTOR, mammalian lethal with SEC13 protein 8 (mLST8), and DEP domain-containing mTOR-interacting protein (DEPTOR), but also contain complex-specific components, as is the case of regulatory-associated protein of mTOR (raptor) and proline-rich AKT1 substrate 1 (PRAS40) for mTORC1, and rapamycin-insensitive companion of mTOR (rictor), mSin1, and protor1/2 for mTORC2, respectively (7). As for the downstream effectors of these complexes, S6 kinases (S6K) and eIF4E-binding proteins (4E-BP) are well-identified mTORC1 downstream targets (7, 8). The kinases S6K1 and S6K2 are members of the S6Ks family, which enhance the mRNA translation by phosphorylation and activation of ribosomal protein S6 (S6) and eIF4B. The 4E-BP family includes 4E-BP1, 4E-BP2, and 4E-BP3, which function as translational repressors through binding to eIF4E, a protein required for the assembly of the translation machinery. mTORC1 may phosphorylate and prevent 4E-BPs from binding to eIF4E and, thereby, enhance the cellular mRNA translation (9). On the other hand, several members of the AGC subfamily of kinases including Akt and the serum- and glucocorticoid-regulated kinase 1 (SGK1) have been identified as mTORC2-specific substrates that regulate cell survival, growth, and metabolism (7, 8). mTORC2 directly phosphorylates Akt at Ser473 and such phosphorylation is required for its maximal activation (10).
Several lines of evidence indicate that targeting the mTOR pathway may represent an efficient strategy against multiple myeloma. Activation of Akt has been reported in a substantial proportion of plasma cells from patients with multiple myeloma, and may be linked to the progression of the disease (11). In addition, overexpression of DEPTOR in multiple myeloma is a frequent event and its downregulation by gene knockdown results in an impaired growth of the multiple myeloma cells (12).

Several types of mTOR inhibitors have been used in the clinic (13). Rapamycin and its analogs were the first generation of mTOR inhibitors. These allosteric inhibitors were initially used in transplantation as immunosuppresant drugs and have been approved in several oncology settings such as advanced renal cell carcinoma, pancreatic neuroendocrine tumors, advanced breast cancer, or acute myeloid leukemia (14, 15). These rapalogs influence the mTOR pathway by binding to FK506-binding protein of 12 kDa (FKBP12). Subsequently, the resulting rapamycin-FKBP12 complex interacts with mTOR FKBP12-rapamycin–binding (FRB) domain and interferes with the binding of raptor to mTOR (16). However, recent studies indicated rapalogs’ lack of efficacy in several contexts, which may be related to the rapamycin-insensitive mTOR actions (15). Thus, although in some cell lines, prolonged treatment with rapamycin may block mTORC1 and mTORC2 complexes (17), in other cell types, rapamycin does not affect mTORC2 signaling. Moreover, rapamycin may provoke Akt activation in some cells, probably caused by a negative feedback exerted by mTORC1 over mTORC2 (18–20). In addition, in some cell types, rapamycin fails to affect the phosphorylation of 4E-BP1 (18, 21), raising doubts about the capacity of the drug to act as an efficient mTORC1 inhibitor. To overcome the inefficacy of rapamycin, a new type of active-site mTOR inhibitors, also known as second-generation mTOR inhibitors, is being developed (13, 22–24). These inhibitors directly target the ATP-binding site of mTOR, blocking its catalytic activity and were found to be able to completely inhibit the mTOR pathway. Nevertheless, their use and advantage over the first-generation mTOR inhibitors in the myeloma context needs to be studied.

Through analysis of gene expression as well as cytometric and biochemical data, we show here, that deregulation of the mTOR pathway is a frequent event in multiple myeloma. Moreover, by using a genetic approach, we have evaluated the relevance of targeting mTORC1, mTORC2 or acting on both mTOR-derived routes as an antmyeloma strategy. These experiments revealed the predominance of mTORC1 over mTORC2 in controlling malignant plasma cell proliferation. However, a better antiproliferative action was obtained when total mTOR activity was downregulated, as compared with exclusively acting on mTORC1 or mTORC2. Finally, we validated the possibility of targeting the mTOR pathway using different mTOR inhibitors available in the clinic on preclinical multiple myeloma models.

Materials and Methods

Reagents and antibodies

CC214-1 was provided by Celgene and rapamycin was purchased from LC Laboratories. Cell culture media, FBS, and the CellTraceTM CFSE Cell proliferation kit were from Life Technologies and the JetPEI reagent from Polyplus-transfection. Generic chemicals were from Sigma-Aldrich, GE Healthcare Life Sciences, BD Biosciences, Roche Biochemicals, or Merck.

The anti-protor-1, anti-GAPDH, anti-Cyclin E, anti-CDK2, anti-PARP, anti-AIF, and anti-p27 antibodies were purchased from Santa Cruz Biotechnology. The anti-mTOR, anti-raptor, anti-COX IV, anti-S6, anti-pS6 (S240/244), anti-p4E-BP1 (T37/46), anti-Akt, and anti-pS6 (S235/236)–Alexa Fluor 488 antibodies were from Cell Signaling Technology. The anti-active-caspase-3, anti-caspase-3, anti-Rb, anti-Smac, anti-cytochrome c, anti-cycillin A, anti-pAkt (S473)-PE, anti-CD19-PerCP-Cy5.5, and anti-CD38-APC were obtained from BD Biosciences. The anti-DEPTOR, anti-mLST8, and anti-Sin1 antibodies were from Millipore Corporation. The anti-rictor was from Bethyl Laboratories. The anti-pAkt (S473) antibody was generated in the laboratory and has already been described (25).

Patient samples

The detection of mTOR pathway activation on patient samples was carried out by flow cytometry. That study included patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma diagnosed according to the International Myeloma Working Group criteria (26). Samples were collected after informed consent in accordance with the Declaration of Helsinki, and with the approval from the ethical committee of the local university hospital.

Cell lines and culture, transfections, generation of lentiviruses, and infection

The HEK-293T and the human stromal cell line HS-5 were obtained from the American Type Culture Collection and maintained at low passages. All the human multiple myeloma cell lines used were obtained in 2003 as described in refs. (27, 28) and maintained at low passages. No authentication of the myeloma cell lines was done by the authors. HEK-293T cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) and all the other cells in RPMI 1640 medium, both supplemented with 10% FBS and antibiotics. All cells were cultured at 37°C in a humidified atmosphere in the presence of 5% CO2–95% air.

The lentiviral vectors containing short hairpin RNA (shRNA) for raptor, rictor, and mTOR have already been described (10, 18) and obtained from Addgene. For the production of lentiviruses, vectors were cotransfected in HEK-293T cells with the pMDLG/RRE, pRSV-Rev, and pMD2.G plasmids by JetPEI transfection using a previously described protocol (27). Forty-eight hours after the transfection, the lentivirus-containing supernatants were filtered and either used to infect target cells or stored at −80°C in aliquots. For the infection, target myeloma cells
were incubated with the lentiviruses in the presence of 8 μg/mL polybrene. Two days after the infection, cells were selected with puromycin.

Detection of intracellular phospho-antigens by multicolor flow cytometry

The detection of phospho-antigens by flow cytometry was based on previous works (29) with some modifications. Briefly, cells were collected, fixed in 2% paraformaldehyde (PFA), permeabilized in 50% ice-cold methanol/PBS and stained with the adequate phospho-specific antibodies and, if necessary, anti-surface lineage antibodies. For patient samples’ staining, whole bone marrow samples were fixed by adding 10% PFA. After washing out the PFA, samples were incubated in 0.1% Triton X-100 to remove the red blood cells. The remaining cells were then incubated in 50% methanol/PBS and stained with the combination of antibodies pS6–Alexa Fluor 488, pAkt-PE, CD19-PerCP-Cy5.5, and CD38-APC. As a negative control, phospho-specific antibodies were competed with their corresponding phospho-peptides before the staining. Data were acquired using a FACSCalibur flow cytometer (BD Biosciences). The same number of cells (100,000) was acquired for each sample.

Immunoprecipitation, Western blotting, and cell fractionation

To prepare cells for protein analyses, they were collected by centrifugation, washed in PBS, and lysed in ice-cold lysis buffer. Immunoprecipitation experiments, SDS-PAGE, and Western blot analysis were performed as described in ref. (18). Cell fractionation experiments were carried out following previously published indications (30).

Cell proliferation, cell cycle, apoptosis assays, and mitochondrial membrane potential measurement

To assess cell proliferation, cells were plated in 6-well plates and counted at different time points with a Z1 Coulter Particle Counter (Beckman Coulter, Inc). To evaluate the cytotoxicity of the drugs, conventional MTT assays were used (27). For cell-cycle profiling, cells fixed and permeabilized in 70% ice-cold ethanol were resuspended in PBS containing 50 μg of propidium iodide (PI) and 200 μg of DNase-free RNase A and incubated for 1 hour at room temperature. For the bromodeoxyuridine (BrdUrd) incorporation assay, 10 μmol/L BrdUrd was added during the last 2 hours of incubation. Cells were then fixed, permeabilized, treated with DNase, and stained using the FITC (fluorescein isothiocyanate) BrdU Flow Kit according to the provider instructions (BD Biosciences). To determine apoptosis, cells were resuspended in the Binding Buffer (10 mmol/L HEPES/NaOH, 140 mmol/L NaCl, 2.5 mmol/L CaCl2, pH 7.4) containing 5 μL of Annexin V-FITC (BD Biosciences) and 5 μL of 50 μg/mL PI and stained for 15 minutes (18). To detect the integrity of mitochondrial membrane potential, cells were treated as indicated, washed, and resuspended in PBS containing 50 nmol/L of tetramethyl rodamine ethyl ester (TMRE; ref. 31). All the stained cells were analyzed in a FACSCalibur flow cytometer.

Coculture model

Human stromal HS-5 cells were labeled with the cell tracker carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the provider instructions and plated in 12-well plates. Myeloma cells were then added to the HS-5 layer and incubated with the drugs. Supernatants containing floating cells were collected together with the attached cells that had been previously trypsinized. After washing, cells were stained with Annexin V allophycocyanin (APC) and 7-aminoactinomycin D (7-AAD). Cell death for HS-5 (CFSE(+) ) and myeloma cells (CFSE(−) ) was evaluated.

Ex vivo evaluation of drugs on patient cells

The effect of drugs on primary patient samples was performed in an ex vivo model as described in ref. (32). Briefly, once red blood cells were removed, myeloma patient bone marrow cells were plated and incubated in the presence of the drugs for 2 days. The cell mixture was collected and stained with the combination of anti-Annexin V FITC, 7-AAD, and anti-CD38-APC to evaluate the effect of the treatment on plasma cells (CD38+) by flow cytometry. On another set of experiments, the FITC BrdU Flow Kit was used to detect BrdUrd incorporation specifically in plasma cells according to the provider’s instructions.

Gene expression analyses

For gene expression analysis, a dataset from the Mayo Clinic, obtained through the Multiple Myeloma Genomics Portal, was used (33). The robust multiarray average (RMA)-normalized, log2-transformed expression values were directly loaded into the R environment for further analysis (http://www.R-project.org; ref. 34). We mapped the genes of interest with the probe set IDs through NetAffx (Affymetrix). Finally, the extracted expression matrix corresponding to the mTOR pathway–related genes in patients was obtained for further analysis.

Statistical analyses

A two-sample t test was used to compare the differences of mean between the two groups. The Fisher exact test was applied on a 2 × 2 contingency table to compare the differences of probability. Significant difference was considered when P < 0.05. All the statistical analyses were performed in the statistical software SPSS 15.0 (SPSS, Inc).

Results

Progressive deregulation of the mTOR pathway along the stepwise development of multiple myeloma

We analyzed the expression levels of mTOR pathway–related genes using a publicly available microarray dataset (33) with healthy donors (n = 15) and
samples from patients in different stages of the disease. The dataset included patients with MGUS (n = 22), smoldering multiple myeloma (SMM; n = 24), and multiple myeloma (n = 101). The expression level of several genes of interest was analyzed in those plasma cells. A progressive increase of the expression of S6, a common downstream substrate of mTORC1 (6), was observed among plasma cells from healthy donors, MGUS, SMM, and multiple myeloma (Fig. 1A), indicating a steady lesion of the regulation of S6 during the evolution of myeloma. A similar tendency in the V-ATPase ATP6V1G1, transcriptionally regulated by mTORC1 (35), was also observed. Besides, a decrease of Grb10, a recently discovered negative regulator of mTORC1 (35), was also observed. A statistical significant increase in multiple myeloma as compared with healthy donors was observed for DEPTOR, whose overexpression leads to activation of Akt in myeloma (12), and S6K2, which is described to be upstream of S6 protein and regulated by mTORC1 (8). No major changes in the mTORC2 pathway components were detected (data not shown).

**Activation of mTORC1 and mTORC2 in malignant plasma cells**

To evaluate the activation status of mTORC1 and mTORC2 in multiple myeloma, we used a flow cytometry approach to detect the phosphorylation of proteins used as surrogate markers of the activity of both mTOR branches. Such an approach was necessary due to the relative low number of plasma cells in the bone marrow. We initially optimized the use of monoclonal antibodies recognizing phosphorylated S6 and Akt in the myeloma cell line OPM-2. Using peptide competition, we confirmed the validity and specificity of the antibodies (Supplementary Fig. S1A). The workflow of the data analysis (Supplementary Fig. S1B) and an example of a positive sample for each marker (Supplementary Fig. S1C) are shown. In most of the patients with multiple myeloma, only a fraction of the malignant plasma cells were positively stained for the phospho-proteins when compared with the control of peptide competition. The mean percentage of plasma cells that positively stained for pS6 and pAkt was 7.50% and 1.44%, respectively. We considered positive staining if a subpopulation of the malignant plasma cells was stained for the marker independently of the percentage.

When each marker was individually analyzed, we found a higher frequency of S6 activation in patients with multiple myeloma than in patients with MGUS (67% vs. 38% positive cases; Fig. 1B). Similarly, increased activation was observed for Akt (38% vs. 25%) in myeloma plasma cells with respect to MGUS plasma cells. However, no statistical significance is reached between the two groups (P > 0.05 for both cases, Fisher exact test), which could be due to the low case number for MGUS. Unavailability of SMM samples prevented us from...
evaluating the levels of these phosphoproteins in this group of patients. When both mTORC1 and mTORC2 substrates were considered together, we found that 71% of the patients harbored activation of the mTOR pathway, as indicated by the positive staining of phospho-S6 or phospho–Akt (Supplementary Fig. S1D). mTORC1 activation was more frequent, as indicated by the presence of the phosphorylation of S6 when compared with mTORC2 activation, measured by the presence of Akt phosphorylation (62% vs. 33%; \( P = 0.016 \) by the Fisher exact test; Supplementary Fig. S1D). No correlation between positivity of Akt and pS6 was observed, suggesting the nonredundant function of mTORC1 and mTORC2 routes.

In the bone marrow samples from some patients with multiple myeloma, the residual normal plasma cells identified by CD19\(^+\)/CD38\(^+\) staining were detectable, allowing comparison of the activation status of mTORC1 and mTORC2 among normal and malignant plasma cells from the same individual. In normal plasma cells from 17 patients, the frequency of positive staining of pS6 was much lower than in their malignant counterparts (12% vs. 65%; \( P = 0.004 \) by the Fisher exact test; Fig. 1C). No activation of Akt was observed in normal plasma cells (data not shown). These data suggest that the activation of the mTOR pathway is more restricted to malignant plasma cells.

**The mTOR pathway controls the proliferation of myeloma cells**

The finding that the mTOR pathway was active in a substantial number of patients with multiple myeloma prompted us to analyze the importance of this route in multiple myeloma. Western blot analyses of the different components of the mTORC1 and mTORC2 pathways indicated that they were all expressed in multiple myeloma cell lines (Fig. 2A). To analyze the activation of mTORC1 and mTORC2 in the different cell lines, we evaluated S6 and Akt phosphorylation as readouts of cell proliferation (Fig. 2B, C). The proliferation of the indicated cells after the knockdown of raptor, rictor, mTOR, or raptor and rictor was analyzed at the indicated time points by cell counting. Graph shows an experiment repeated at least three times. *, nonspecific bands.

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**Figure 2.** Status and role of the mTOR pathway in myeloma cell lines. A, expression levels of several key components of the mTOR complexes and phosphorylation status of their substrates S6 and Akt in the indicated multiple myeloma cell lines determined by Western blotting. B, the levels of raptor, rictor, mTOR, or raptor and rictor proteins were modulated in MM1S or OPM-2 cells by RNAi techniques. The effects of the knockdown on the different components and their downstream substrates were detected by Western blotting. C, the proliferation of the indicated cells after the knockdown of raptor, rictor, mTOR, or raptor and rictor was analyzed at the indicated time points by cell counting. Graph shows an experiment repeated at least three times. *: nonspecific bands.
mTORC1 and mTORC2 activities, respectively. Phosphorylation of S6 was more frequently observed than that of Akt, corroborating what we observed in patient plasma cells (Fig. 2A).

To analyze the importance of the mTOR pathway in myeloma cells, we used RNA interference (RNAi) against mTOR in two different multiple myeloma cell lines. We knocked down the mTORC1-specific component raptor and the mTORC2-specific one rictor to determine the role of each complex in the regulation of the proliferation of myeloma cells. In addition, we also knocked down mTOR, expecting to reduce both mTORC1 as well as mTORC2 outputs. The efficiency and specificity of knockdown was confirmed by Western blot analysis of the corresponding components (Fig. 2B). Raptor knockdown caused a decrease in the phosphorylation of S6 and 4E-BP1, but increased the amount of pAkt. This may result from the negative feedback effect exerted by mTORC1 over mTORC2. Rictor knockdown decreased pAkt. mTOR knockdown mainly decreased the phosphorylation of S6 and 4E-BP1. Proliferation assays demonstrated that mTOR knockdown strongly affected proliferation of MM1S or OPM-2 cells (Fig. 2C). Raptor knockdown also resulted in a decrease in cell proliferation although to a lesser extent than mTOR knockdown in MM1S, whereas its knockdown had almost similar effect as mTOR knockdown in OPM-2. Rictor diminution also reduced proliferation. Combined knockdown of raptor and rictor had a higher antiproliferative effect than the individual knockdowns, especially in the OPM-2 cell lines. In this latter cell line, the combined rictor and raptor knockdown had a strong antiproliferative effect, of a magnitude analogous to the mTOR knockdown. Similar results were obtained by using a second set of shRNAs (data not shown).

Figure 3. Action of mTOR inhibitors on myeloma cells. A, the effect of CC214-1 on different multiple myeloma cell lines was determined by a MTT incorporation assay. Cells were treated with CC214-1 for 3 days and the dose–response curves were plotted. B, comparison of the action of CC214-1 and rapamycin (Rapa) on MM1S and OPM-2 cells. Cells were treated with the indicated concentration of CC214-1 (open symbols) or rapamycin (full symbols) and the results are shown together. C, effect of CC214-1 on an in vitro coculture model. Myeloma cells were grown on the stromal cell line HS-5, treated with the indicated concentrations of CC214-1 for 2 days and stained with Annexin V-APC/7-AAD. The stromal cells were previously stained with CSFE to distinguish them from the myeloma ones. D, the changes in the phosphorylation of the mTOR pathway downstream substrates pS6, p4E-BP1, and pAkt after CC214-1 (left) or rapamycin (right) treatment were analyzed by Western blotting in MM1S (top) or OPM-2 (bottom) cells as indicated.
Allosteric and active-site mTOR inhibitors differentially affect myeloma cell growth

The above data indicated that mTOR signaling plays a relevant role in the control of multiple myeloma cell number. Moreover, the knockdown data suggested that mTOR targeting may be more efficient as an antimyeloma therapy than exclusively acting on mTORC1. To further study this possibility, we tested the effect of mTOR inhibition on myeloma preclinical models using both allosteric and active-site mTOR inhibitors. We focused on a new-generation active-site mTOR inhibitor, CC214-1. This inhibitor exerts selectivity for mTOR over other related kinases such as phosphoinositide 3-kinase (PI3Kα; ref. 37).

We also used rapamycin, a classic allosteric mTORC1 inhibitor, whose action was compared with that of CC214-1. CC214-1 substantially decreased MTT metabolization in all the myeloma cell lines tested, although more efficiently in MM1S, MM1R, OPM-2, NCI-H929, and SJR (Fig. 3A). We also compared the efficacy of CC214-1 with rapamycin in MM1S and OPM-2 cells. Very low concentrations of rapamycin had a clear effect on cell growth, but it reached a plateau for cell growth inhibition, leaving more than 30% of cells unaffected (Fig. 3B). However, CC214-1 almost completely inhibited the cell growth in these two cell lines, although at higher concentrations.
CC214-1 inhibits proliferation of multiple myeloma cells in coculture with bone marrow stromal cells

The microenvironment plays a pivotal role in the control of the survival and proliferation of malignant plasma cells in patients with myeloma, and contributes to drug resistance (38). Therefore, we tested the effect of CC214-1 in an in vitro coculture model that mimicked the bone marrow microenvironment. The effect of CC214-1 on MM1S or OPM-2 cell lines cultured on HS-5 stromal cells was similar to the above described MTT data (Fig. 3C), indicating that CC214-1 overcomes the proliferative/pro- tective advantage induced by stromal cells. Moreover, no cytotoxic effect was observed on HS-5 cells even at high concentrations, suggesting specificity of this drug for the myeloma cells.

mTOR pathway inhibition in myeloma cells provokes cell-cycle arrest

Subsequently, we compared the effect of those mTOR inhibitors (rapamycin and CC214-1) on the mTOR pathway. CC214-1 completely inhibited the phosphorylation of S6, 4E-BP1, and Akt in MM1S or OPM-2; however, rapamycin inhibited the phosphorylation of S6 but not that of 4E-BP1 (Fig. 3D). In contrast, rapamycin treatment caused an increase in pAkt. In addition, we observed that CC214-1 did not affect the mitogen—activated protein kinase (MAPK) pathway, another major route controlling cell growth and proliferation (data not shown).

To further explore the mechanism of action of mTOR inhibition on myeloma cell survival and proliferation, the cell-cycle profile of cells treated with CC214-1 was analyzed. A clear G0–G1 arrest was observed in the cell lines tested at a concentration of CC214-1 up to 1 μmol/L (Fig. 4A). At higher concentrations, CC214-1 caused an increase in the sub-G0 population. Myeloma cells treated with rapamycin showed a similar cell-cycle arrest (Fig. 4B), confirming previous studies and suggesting a role of mTORC1 in the control of cell-cycle progression (39). We also analyzed actively cycling cells using BrdUrd incorporation assays. There was no incorporation of BrdUrd after CC214-1 treatment in the cell lines tested (Fig. 4C). In contrast, BrdUrd incorporation was only partially inhibited by rapamycin treatment in the same cell lines.

We also tested the effect of CC214-1 and rapamycin on cell proliferation in an ex vivo model using freshly isolated bone marrow cells from patients with myeloma. The percentage of plasma cells from patients that incorporated BrdUrd was very low, as previously reported (40); however, a substantial decrease in BrdUrd-incorporating plasma cells after being treated with CC214-1 was observed in all the patient samples tested (Fig. 4D). In general, rapamycin also blocked the incorporation of BrdUrd in plasma cells, but to a lesser extent than CC214-1 treatment. These results indicated that both agents acted on the cell cycle; however, CC214-1 blocked cell-cycle progression more efficiently than rapamycin.

At the molecular level, we found an increase in p27 protein after CC214-1 treatment in all the cell lines tested (Fig. 5A). Besides, coimmunoprecipitation experiments indicated an increased association of CDK2, cyclin E, and cyclin A to p27 in CC214-1–treated cells (Fig. 5B). Moreover, Retinoblastoma (Rb), a key cell-cycle ‘gatekeeper’ in the G1–S transition, which is hyperphosphorylated by the CDK2–cyclin E complex, was dephosphorylated as a result of CC214-1 treatment (Fig. 5A).

CC214-1–induced apoptosis in multiple myeloma cells

In addition to the G0–G1 arrest, the PI staining experiments indicated accumulation of cells in sub-G0, especially in the MM1S cell line, suggesting that CC214-1 might induce apoptosis (Fig. 4A). Staining with Annexin V confirmed the induction of apoptosis after treatment with CC214-1 in MM1S and OPM-2 cells (Fig. 6A). In contrast, treatment with rapamycin failed to induce clear apoptosis in both cell lines (Fig. 6B). We also tested the ability of CC214-1 to induce apoptosis on plasma cells from freshly isolated bone marrow samples from patients with myeloma. Although the response to CC214-1 was variable, we
did observe a dose-dependent induction of apoptosis in most of these patient samples (Fig. 6C).

Mitochondria play a relevant role in apoptotic processes. Several apoptotic stimuli provoke permeabilization of the mitochondrial outer membrane, which allows the escape of proapoptotic proteins from the intermembrane space to the cytosol (41). In addition to cytochrome c, which is involved in caspase-dependent apoptosis, apoptosis inducing factor (AIF), and endonuclease G may also be released, and these latter two proteins contribute to caspase-independent cell death (42). Derangement of the mitochondrial outer membrane by CC214-1 was indicated by the analysis of the mitochondrial membrane potential ($\Delta \psi_{m}$) using TMRE dye (Fig. 7A). Moreover, translocation of cytochrome c, Smac/Diablo and AIF was observed after CC214-1 treatment, although that of AIF, to a lesser extent (Fig. 7B). In addition, cleavage of caspase-3 and PARP was evidenced in MM1S and OPM-2 cells treated with CC214-1 (Fig. 7C). Using the pan-caspase inhibitor Z-VAD-fmk, we confirmed the involvement of caspase-dependent apoptosis in the action of CC214-1, as there was a protective effect from apoptosis in the presence of this inhibitor (Fig. 7D). However, the partial inhibition of apoptosis by Z-VAD-fmk indicated the possible existence of caspase-independent apoptotic mechanisms. On the other hand, we confirmed the absence of apoptosis in myeloma cells treated with rapamycin by Western blot analysis in which no cleavage of PARP was detected (data not shown).

Discussion

This study analyzed the role of the mTOR pathway and its two branches mTORC1 and mTORC2 in the control of multiple myeloma cell number. Moreover, we explored the value of targeting mTOR with respect to exclusively acting on either of its two signaling branches. This is relevant because some anti-mTOR therapies under clinical evaluation use rapalogs, which are expected to partially target signal outputs from the mTOR route, as they preferentially act on mTORC1. Gene expression data found deregulation of genes involved in the mTOR pathway, especially with an increased expression of S6 protein and decreased Grb10. Moreover, a high frequency of activation of the mTOR pathway in plasma cells from patients with myeloma was detected by flow cytometry techniques. Of note, 71% of the cases had activation of this pathway indicated by the phosphorylation of S6 or Akt. Because it has previously been demonstrated that aberrant activation of the mTOR pathway favors tumorigenesis (6) and drug resistance (43), these myeloma patient cells harboring mTOR activation might hold more tumor formation capacity and, at the same time, could be effectively targeted in therapy. Interestingly, mTOR activation was more frequent in patients with multiple myeloma than in the premalignant stage MGUS, indicating the importance of this route in multiple myeloma progression and, according to previous reports, pointing to increased activation of Akt along disease evolution (11). Activation of the mTOR pathway was only detected in malignant plasma cells, and not in their normal counterparts. However, the percentage of malignant plasma cells bearing phosphorylated forms of proteins that serve as readouts of mTOR activity was low. This indicates that the population of malignant plasma cells is heterogeneous, at least with respect to these markers. It is possible that the subpopulation of plasma cells with active mTOR may correspond to a population with stem-like properties, as has been reported in other malignant tissues (44). It would be
interesting to test this possibility when adequate markers for myeloma stem cells will be available (45).

The mTOR pathway was also frequently activated in multiple myeloma cell lines. In them, silencing rictor, raptor, or mTOR allowed us to evaluate the potential relevance of targeting mTORC1, mTORC2, or both for myeloma therapy. Reducing the activity of the mTORC1 route had major antiproliferative effect than inhibiting the mTORC2 pathway. This is interesting, as it indicates that mTORC1 predominates over mTORC2 in the control of the proliferation of multiple myeloma cells. A recent report also confirmed the value of targeting mTORC1 or mTORC2 in myeloma (46). However, that study suggested that mTORC2 targeting may be more relevant than targeting mTORC1. Our knockdown data indicate predominance of mTORC1 over mTORC2 in the regulation of myeloma cell proliferation. Moreover, rapamycin treatment reduced approximately 60% proliferation of these cells, indicating that rapamycin-sensitive mTOR outputs are largely responsible for the effects of mTOR on the proliferation of myeloma cells. That mTORC1 might have a more important role than mTORC2 in the control of proliferation of cancer cells, has also been suggested in ovarian cancer (18) and triple-negative breast cancer (47).

In any case, our results indicate that mTOR knockdown had a superior antiproliferative effect than acting on either of the two mTOR branches individually. This finding reveals the importance of mTOR targeting instead of only acting on each of the multiprotein complexes to which mTOR belongs. This conclusion was also supported by pharmacologic studies using two different types of mTOR inhibitors, rapamycin and CC214-1. Rapamycin, an allosteric mTOR inhibitor that disrupts the mTOR complex formation, has been used in cancer therapy (14). Moreover, former studies have indicated that the mTORC1 inhibitor rapamycin showed antmyeloma effect (39). However, recent studies demonstrated that this drug is unable to completely block the mTOR pathway due to the existence of rapamycin-insensitive components (21, 23). Because of this limitation, new-generation, active-site mTOR inhibitors have been investigated for potential clinical utility, as is the case of CC214-1. Rapamycin and CC214-1 decreased

Figure 7. Mechanism of apoptosis induction in CC214-1–treated cells. A, cells were plated, treated for 48 hours with CC214-1 as indicated, and stained with TMRE. The percentage of cells losing membrane potential (TMRE−) is shown. B, cell fractionation of MM1S or OPM-2 cell lines after CC214-1 treatment was performed as described and the localization of several key proteins was analyzed by Western blotting. C, cleavage of caspase-3 and PARP in myeloma cells treated with CC214-1 was determined by Western blotting. D, cells were plated, preincubated with 50 μmol/L of Z-VAD-fmk for 1 hour when indicated, and then treated for 48 hours with 2.5 μmol/L of CC214-1. Once harvested, cells were stained with Annexin V/PI as described. The percentage of cells alive (Annexin V−/PI−) is shown.
MTT metabolism, indicative of inhibition of proliferation. However, although rapamycin was more potent than CC214-1, it was less efficient. In fact, rapamycin only partially inhibited cell growth. Biochemically, CC214-1 efficiently inhibited both mTORC1 and mTORC2 routes in myeloma cells. In contrast, rapamycin failed to inhibit the phosphorylation of 4E-BP1 even though it was efficient inhibiting S6 phosphorylation in myeloma cells. These results are in agreement with studies in other cell types, which reported that rapamycin inhibited S6 phosphorylation but failed to affect the phosphorylation of 4E-BP1 (23, 48). Moreover, rapamycin treatment provoked an increase in pAkt. This effect may be due to the negative feedback action of mTORC1 exereted over mTORC2, and which may involve the adapter proteins IRS-1 or Grb2 (19, 36). Therefore, signaling through these rapamycin-insensitive mTOR-derived pathways may explain the escape of a number of cells to the inhibition by rapamycin. Importantly, this fraction of cells was still attacked by CC214-1, indicating the superior potential anticancer activity of active-site inhibitors such as CC214-1 as compared with rapamycin.

Mechanistically, CC214-1 induced cell-cycle arrest and apoptosis, whereas rapamycin mainly produced cell-cycle arrest, suggesting a role of mTORC2 complex or rapamycin-insensitive mTORC1 actions in survival and programmed cell death inhibition. CC214-1 was more efficient than rapamycin in inhibiting cell-cycle progression, as measured by the BrdUrd incorporation assay. Furthermore, we confirmed that CC214-1 inhibited more efficiently than rapamycin BrdUrd incorporation in malignant plasma cells ex vivo. Concerning the cell-cycle arrest induced by CC214-1, we have detected an increase of p27 and dephosphorylation of Rb as well as an enhanced association of CDK2, cyclin E, and cyclin A with p27 after treatment with CC214-1. Thus, we hypothesize that CC214-1-induced cell-cycle arrest may occur through the upregulation of p27, which in turn binds and inactivates CDK2–cyclin complexes, that finally results in Rb dephosphorylation (49, 50). A substantial difference among the actions of CC214-1 and rapamycin were observed when analyzing their effect on cell death. CC214-1 seemed to induce apoptosis in plasma cells, whereas rapamycin failed to do so. This proapoptotic effect of CC214-1 was observed both on cell lines and ex vivo, on patient plasma cells. The mechanisms of apoptotic cell death triggered by CC214-1 may involve caspase-dependent and -independent actions. The activation of caspases after treatment with CC214-1 was evidenced by cleavage of PARP and caspase-3. The partial inhibition of cell death by the pan-caspase inhibitor Z-VAD-fmk in cells treated with CC214-1 suggested the involvement of both caspase-dependent and -independent mechanisms. In line with the latter, is the release of AIF from the mitochondria to the cytosol. Several conclusions, some of them with therapeutic relevance, may be extracted from our study. Our experiments indicated that mTOR kinase targeting is more relevant, as an antiproliferative strategy, than just targeting mTORC1 or mTORC2 exclusively. This conclusion is supported by the knockdown genetic experiments as well as by the pharmacologic data, which demonstrated the antitumoral advantage of the active-site mTOR inhibitor CC214-1 over the mTOR allosteric inhibitor rapamycin. Moreover, the data we obtained challenge the hypothesis of rapamycin as a bona fide mTOR inhibitor, and suggest that active-site mTOR inhibitors may be more appropriate to block mTOR function. It will be important to address whether mTOR targeting will be a more efficient anti-multiple myeloma therapy than using rapalogs in clinical settings. Finally, although therapies using these mTOR inhibitors seem to be promising in preclinical multiple myeloma studies, it will, however, be necessary to evaluate their degree of toxicity in the clinic.

**Disclosure of Potential Conflicts of Interest**

E.M. Ocío is a consultant/advisory board member for Celgene. B. Paiva has honoraria from Millennium, Celgene, Janssen, and The Binding Site and is consultant/advisory board member for Millennium. A. Pandiella received a commercial research grant from and is a consultant/advisory board member for Celgene. No potential conflicts of interest were disclosed by the other authors.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** X. Chen, E. Díaz-Rodríguez, E.M. Ocío, A. Pandiella

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**Other:** Disclosed/identified/made mTOR kinase inhibitor, D.S. Mortensen

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