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Cancer Therapy: Preclinical

Clinical Cancer Research

Mechanisms of Activity of the TORC1 Inhibitor Everolimus in Waldenstrom Macroglobulinemia

Aldo M. Roccaro, Antonio Sacco, Xiaojing Jia, Ranjit Banwait, Patricia Maiso, Feda Azab, Ludmila Flores, Salomon Manier, Abdel Kareem Azab, and Irene M. Ghobrial

Abstract

Purpose: The TORC1 inhibitor everolimus has previously shown significant activity as a single agent in hematologic malignancies, with reported responses of 30% to 70% in Waldenstrom macroglobulinemia. However, the specific mechanisms by which this class of mTOR inhibitors exerts anti–Waldenstrom macroglobulinemia activity have not been fully investigated. We therefore sought to dissect the mechanisms of everolimus-dependent modulation of Waldenstrom macroglobulinemia cell survival.

Experimental Design: We confirmed that everolimus targets mTOR in patients treated with everolimus and responding to therapy. We evaluated the effect of everolimus on proliferation and survival of primary Waldenstrom macroglobulinemia cells, as well as of other IgM-secreting lymphoma cell lines. Everolimus-dependent mechanisms of induced apoptosis and its effect on Waldenstrom macroglobulinemia cells in the context of bone marrow microenvironment have been also evaluated. miRNA-155 loss-of-function studies were conducted. Moreover, the combinatory effect of bortezomib and rituximab has been tested.

Results: We showed that everolimus targeted mTOR downstream signaling pathways, *ex vivo*, in patients responding to everolimus treatment. Everolimus induced toxicity in primary Waldenstrom macroglobulinemia cells, as well as in other IgM-secreting lymphoma cells, supported by cell-cycle arrest and caspase-dependent and -independent induction of apoptosis. Importantly, everolimus targeted Waldenstrom macroglobulinemia cells even in the context of bone marrow milieu, where it affected migration, adhesion, and angiogenesis. Everolimus-dependent anti–Waldenstrom macroglobulinemia activity was partially driven by miRNA-155. Moreover, everolimus synergized with bortezomib and rituximab in targeting Waldenstrom macroglobulinemia cells, as shown by synergistic inhibition of p65/ and p50/NF-κB activities.

Conclusions: These findings provide a better understanding of the mechanisms that are responsible for everolimus-induced anti–Waldenstrom macroglobulinemia activity. *Clin Cancer Res; 18(24); 6609–22. ©2012 AACR.*

Introduction

Tumorigenesis results from synergistic interactions of a complex of signal transduction processes, including multiple oncoproteins and tumor suppressors such as Ras, Myc, PKB/Akt, Her-2/Neu, p53, and PTEN (1–3). PTEN, specifically, acts as negative regulator of the PI3K/Akt/mTOR pathway; and PTEN mutations have been shown to regulate constitutive activation of the PI3K/Akt pathway (4–9). Importantly, overexpression of Akt and mTOR signaling cascades plays an important role on the initiation and progression of malignancies. Indeed the PI3K/mTOR pathway is important in enhancing cell survival by stimulating cell proliferation and inhibiting apoptosis.

mTOR kinase exists in at least 2 multiple protein complexes, TORC1 and TORC2. TORC1 is composed of the mTOR catalytic subunit and 3 associated proteins, Raptor, PRAS40, and mLST8/GβL, whereas TORC2 also contains mTOR and mLST8/GβL, but instead of Raptor and PRAS40, contains the proteins Rictor, mSin1, and Protor. TORC1 is activated by growth factor stimulation via the canonical PI3K/Akt/mTOR pathway and is partially sensitive to rapamycin (10). TORC1 also connects nutrient, stress, and hormone signaling via tuberous sclerosis complex (TSC) and AMPK signaling components (11–13).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

We have evaluated the antitumor effect of the TORC1 inhibitor everolimus, *ex vivo* and *in vitro*, in Waldenstrom macroglobulinemia, either as single agent or in combinatory strategies using everolimus with proteasome inhibitor and/or anti-CD20 monoclonal antibody. Our findings enhance our understanding of the mechanisms responsible for everolimus-induced antitumor effect in Waldenstrom macroglobulinemia and provide the rationale for testing everolimus in combination with bortezomib or rituximab, in clinical trials, for patients with Waldenstrom macroglobulinemia who present with refractory disease to everolimus used as single agent.

Everolimus is a TORC1 inhibitor that has shown significant activity in tumors that are dependent on mTOR activity such as neurofibromatosis and tuberous sclerosis with specific mutations that are targeted by this inhibitor. Neurofibromatosis type 1 and TSC) are autosomal-dominant genetic disorders that result from dysregulation of the PI3K/AKT/mTOR pathway. For example, TSC is caused by mutations in either the TSC1 (chromosome 9q34) or TSC2 (chromosome 16p.13.3) genes. Their protein products, hamartin and tuberin, respectively, form a dimer that acts via the GAP protein Rheb (Ras homolog enhanced in brain) to directly inhibit mTOR: their absence will therefore result in mTOR upregulation. In addition, everolimus has been successfully tested in patients with metastatic renal cell carcinoma who progressed on other targeted therapies, showing prolonged progression-free survival (14).

Everolimus has also shown significant activity in lymphomas, including low-grade lymphomas such as Waldenstrom macroglobulinemia (15). However, these lymphomas do not have specific mutations that induce activation of this pathway. Specifically, there have been no reported mutations in the PI3K/mTOR pathway in Waldenstrom macroglobulinemia that would account for the activity of everolimus in this lymphoplasmacytic lymphoma. In a phase II study, everolimus exerted significant anti–Waldenstrom macroglobulinemia activity with more than 42% partial responses and 70% minimal responses or better in patients with relapsed/refractory Waldenstrom macroglobulinemia (reference). Interestingly, patients with other plasma cell dyscrasias do not exert such a high response rate to single-agent TORC1 inhibitors (15).

We therefore sought to dissect the mechanisms of everolimus-dependent anti–Waldenstrom macroglobulinemia activity both *in vitro* and *ex vivo*, hypothesizing that everolimus may exert antitumor effects in Waldenstrom macroglobulinemia, either by directly targeting Waldenstrom macroglobulinemia clonal cells, in terms of mTOR downstream targeted proteins, or by indirectly affecting the surrounding bone marrow milieu, known to play a supportive role in the pathogenesis of B-cell malignancies.

Materials and Methods

Cells

Primary Waldenstrom macroglobulinemia cells were obtained from bone marrow (BM) samples of previously treated patients with Waldenstrom macroglobulinemia using $CD19^+$ microbead selection (Miltenyi Biotec) with more than 90% purity, as confirmed by flow cytometric analysis with monoclonal antibody reactive to human CD20-PE (BD-Bioscience). The Waldenstrom macroglobulinemia and the IgM-secreting lymphoma cell lines (BCWM.1, MEC.1, and RL) were used in this study (16). Peripheral blood mononuclear cells (PBMC) were obtained from healthy subjects by Ficoll-Hypaque density sedimentation, and $CD19^+$ selection was conducted as described above. All cells were cultured at 37°C in RPMI-1640 containing 10% FBS (Sigma Chemical), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Approval for these studies was obtained from the Dana-Farber Cancer Institute Institutional Review Board (Boston, MA). Informed consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki protocol.

Reagents

Everolimus was provided by Novartis and diluted in dimethyl sulfoxide (DMSO) and stored at 4°C until use, then diluted in culture medium immediately before use. The maximum final concentration of DMSO (<0.1%) did not affect cell proliferation and did not induce cytotoxicity on the cell lines and primary cells tested (data not shown). Bortezomib and rituximab were obtained from Hospital Pharmacy.

Growth inhibition assay

The inhibitory effect of everolimus on the growth of Waldenstrom macroglobulinemia cells, IgM-secreting cell lines, and primary cells was assessed by measuring MTT (Chemicon International) dye absorbance, as previously described (17).

DNA synthesis

DNA synthesis was measured by [^{3}H]-thymidine ([^{3}H]-TdR; Perkin Elmer) uptake, as previously described (17).

DNA fragmentation

Cell Death Detection ELISA (Roche Applied Science) was used to quantify DNA fragmentation, as per the manufacturer's instructions, and as previously described (18).

Immunoblotting

Waldenstrom macroglobulinemia and IgM-secreting cell lines were harvested and lysed using lysis buffer (Cell Signaling Technology) reconstituted with 5 mmol/L NaF, 2 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 5 μg/mL leupeptin, and 5 μg/mL aprotinin. Whole-cell lysates (50 μg/lane) were subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories). The antibodies used for

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immunoblotting included anti-caspase3-, -caspase-9, -PARP, phospho (p)-mTOR, p-p70S6K, p-4EBP1, p-FAK, -cyclinD1, -cyclinD2, -$p27^{Kip1}$, -$p21^{Cip1}$, p-Akt, -Akt, pERK, -ERK, p-GSK3, -p-STAT3, -p-p65, -p-p50, -p-IkB, -nucleolin, and -α-tubulin antibodies (Cell Signaling,).

Caspase activation

Waldenstrom macroglobulinemia cell lines have been exposed to everolimus (0.1–10 nmol/L) for 24 hours, and caspase activation has been assessed using Caspase 3/7 Glo Assay (Promega), according to manufacturer's procedure.

Effect of everolimus on paracrine Waldenstrom macroglobulinemia cell growth in the bone marrow

To evaluate growth stimulation and signaling in Waldenstrom macroglobulinemia cells adherent to bone marrow stromal cells (BMSC), 3×10^4 BCWM.1 cells were cultured in BMSC-coated 96-well plates for 48 hours in the presence or absence of everolimus. DNA synthesis was measured as described (16).

Transwell migration assay

We conducted Transwell migration assay (Costar; Corning) using BCWM.1 cells in the presence or absence of 30 nmol/L SDF-1, as described (19).

Cell-cycle analysis

BCWM.1 cells were stained with propidium iodine (PI; Sigma Chemical) and cell cycle was determined using an Epics (Coulter Immunology) flow cytometry, as previously described (20).

Antibody-dependent cell-mediated cytotoxicity assay

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay was conducted as previously described (21). Briefly, interleukin-2 (IL-2; R&D)-activated PBMCs were used as effector cells, and calcein-AM–labeled BCWM.1 cell line as targets. PBMCs were separated from leukopheresis products from healthy donors over Ficoll-Hypaque solution after informed consent. Effector cells were used immediately at 37°C in RPMI complete media after being activated with IL-2 (100 UI/mL for 36 hours). Target cells were labeled with calcein-AM for 1 hour at 37°C, washed 3 times, and plated in triplicate in 96-well plates (5,000 cells per well). ADCC was conducted in the presence of rituximab (10 μg/mL) or human control IgG1 (2 μg/mL) at various effector/target (E:T) ratios (10:1, 20:1, and 40:1). The 96-well plates were centrifuged at $30 \times g$ for 2 minutes at room temperature, followed by a 4-hour incubation at 37°C. Culture supernatants were transferred to a Black ViewPlate-96 plate and read on Wallac VICTOR2 using 492/520 nm filter set (Perkin Elmer). Combinatory studies using everolimus (1 nmol/L), bortezomib (5 nmol/L), and rituximab (10 μg/mL) were also conducted. This assay was valid only if (mean maximum release − medium control release)/(mean spontaneous release − medium control release) was more than 7. Spontaneous release is the count per minute (CPM) in the supernatant from wells containing target cells alone. Maximum release is the supernatants of wells containing target cells and Triton X-100. Experimental release is obtained from the supernatant of wells containing effector cells, target cells, and antibody. Calculation of percentage specific lysis from triplicate experiments was done using the following equation: % specific lysis = 100 × [(mean experimental release − mean spontaneous release)/(mean maximum release − mean spontaneous release)].

NF-κB activity

NF-κB activity was investigated using the Active Motif TransAM kits, a DNA-binding ELISA-based assay (Active Motif North America). Briefly, BCWM.1 cells were treated with everolimus (1 nmol/L) or bortezomib (5 nmol/L) alone or in combination for 4 hours and stimulated with TNF-α (10 ng/mL) during the last 20 minutes of culture. NF-κBp65 transcription factor binding to its consensus sequence on the plate-bound oligonucleotide was studied from nuclear extracts, following the manufacturer's procedure (16).

Morphogenesis assay on Matrigel

Unpolymerized Matrigel (17 mg/mL; Becton Dickinson) was placed (50 μL/well) in a 96-well microtiter plate (0.32 cm^2/well) and polymerized for 1 hour at 37°C. Human umbilical vein endothelial cells (HUVEC) and mouse mammary epithelial cells (MMEC; 5×10^3 per well) in 200 μL of Dulbecco's Modified Eagle Medium (DMEM)/10% fetal calf serum (FCS; positive control), serum-free medium (negative control), as well as in the presence or absence of everolimus (0, 0.01, 0.1, 1, 10 nmol/L) were layered onto the Matrigel surface. After 6 hours of incubation in a 5% CO_2 humidified atmosphere at 37°C, cell growth and tridimensional organization were observed using a reverted phase-contrast light microscope, as described (22).

Immunofluorescence

The effect of everolimus in combination with bortezomib on TNF-α–induced nuclear translocation of p65 was examined by an immunocytochemical method. Briefly, BCWM.1 cells were cultured in presence or absence of everolimus (1 nmol/L) and bortezomib (5 nmol/L) for 4 hours and then stimulated with TNF-α (10 ng/mL) during the last 20 minutes of culture. Immunocytochemical analysis was conducted using an epifluorescence microscope (Nikon Eclipse E800; Nikon) and a Photometrics Coolsnap CF color camera (Nikon), as previously described. Similarly, evaluation of p-S6R has been evaluated by using immunofluorescence on Waldenstrom macroglobulinemia cells treated with everolimus (1 nmol/L), bortezomib (5 nmol/L), rituximab (10 μg/mL), either alone or in combination (16).

Immunohistochemistry

Bone marrow biopsies from patients with Waldenstrom macroglobulinemia at pretreatment and at the end of therapy were fixed in Zenker formalin, embedded in paraffin blocks, and sectioned. Sections were stained for p-4EBP1 and p-S6R (Cell Signaling Technology, Inc.). Signal quantification for

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each target has been obtained from 4 different areas, on matched pre- and post-everolimus treatment of 4 patients presenting with partial response; and on matched pre- and post-everolimus treatment of 3 patients presenting with stable disease. Number of positive cells has been obtained on 4 different fields of the bone marrow biopsy and average and SD provided, as previously described (23).

Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (qRT-PCR) for miRNA-155 (TaqMan microRNA Assays, Applied Biosystems) was carried out on an Applied Biosystems AB7500 Real Time PCR system. All PCR reactions were run in triplicate and miRNA expression, relative to *RNU6B*, was calculated using the $2^{-\Delta\Delta C_t}$ method (17).

miRNA transfection

Waldenstrom macroglobulinemia cells (BCWM.1 and MWCL.1) were transfected with either miRNA-155 LNA knockdown probe (Exiqon) or control knockdown probe (Exiqon), at final concentration of 40 nmol/L, using Lipofectamine 2000 (Invitrogen) following manufacturer's instruction, and as previously described (17).

Statistical analysis

Statistical significance of differences in drug-treated versus control cultures was determined using the Student *t* test. The minimal level of significance was $P < 0.05$. Drug synergism was analyzed by isobologram analysis using the CalcuSyn software program (Biosoft), as described (16). Experiments have been repeated in triplicates. Error bars reported in the figures represent SDs.

Results

Everolimus targets Waldenstrom macroglobulinemia cells *ex vivo*: mechanisms of everolimus-dependent anti–Waldenstrom macroglobulinemia activity

A recently conducted phase II trial of the oral mammalian target of rapamycin inhibitor everolimus (RAD001) in patients with relapsed or refractory Waldenstrom macroglobulinemia showed an overall response rate of 70%, with a partial response (PR) of 42% and 28% minor response (15). In the present study, we evaluated bone marrow biopsies of patients who presented with PR and showed an inhibition of phosphorylation of S6R and 4EBP1 (Fig. 1A and B), indicating the ability of everolimus to specifically downmodulate the activity of 2 mTOR downstream targeted proteins. In contrast, we did not observe everolimus-dependent modulation of p-S6R and p-4EBP1 in those patients with stable disease (Fig. 1C). Quantification of p-S6R and p-4EBP1 expression has been obtained as described (23) and included in Supplementary Fig. S1. These findings show the ability of everolimus to target mTOR in patients with Waldenstrom macroglobulinemia, resulting in antitumor activity.

We next therefore sought to determine the underlying mechanisms that led to everolimus-dependent antitumor effect in Waldenstrom macroglobulinemia. The activity of

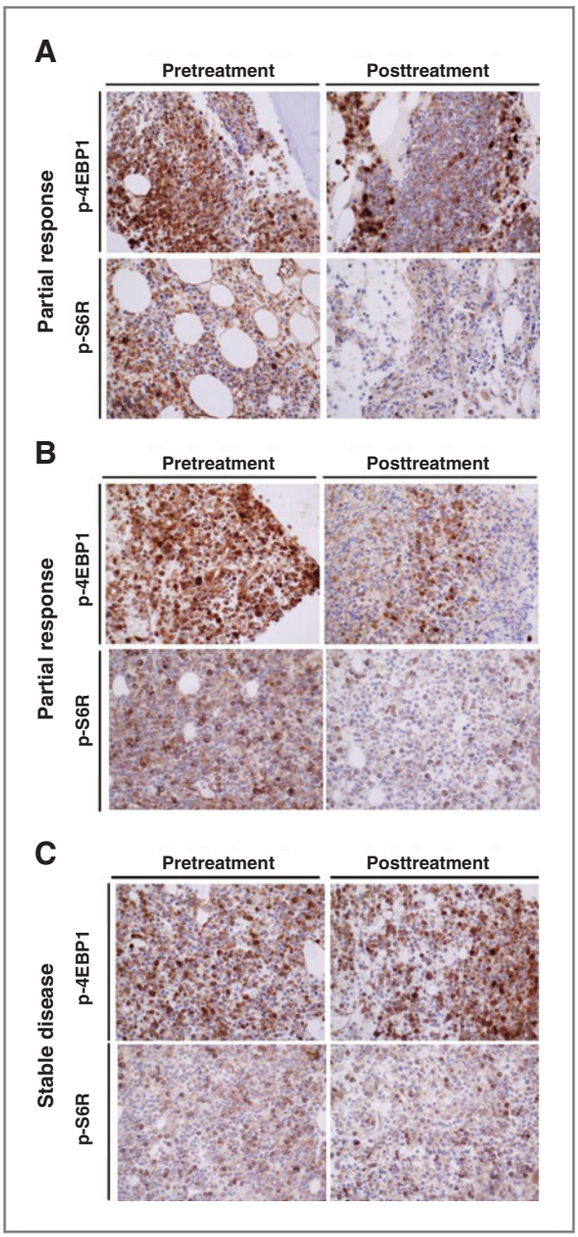

Figure 1. Everolimus targets mTOR downstream targeted proteins in patients with Waldenstrom macroglobulinemia. Immunohistochemistry of p-4EBP1 and p-S6R in pre- and post-everolimus treated patients with Waldenstrom macroglobulinemia that presented either a partial response (A and B) or stable disease (C).

everolimus in targeting Waldenstrom macroglobulinemia cells was tested in primary Waldenstrom macroglobulinemia $CD19^+$ cells and Waldenstrom macroglobulinemia and IgM-secreting lymphoma cell lines (BCWM.1, MWCL.1, and RL), as well as in normal PBMC-derived $CD19^+$ cells. We first evaluated the cytotoxic effect of everolimus (0.1–10 nmol/L) on primary Waldenstrom macroglobulinemia bone marrow–derived $CD19^+$ cells, isolated from 4 patients with relapsed/refractory Waldenstrom

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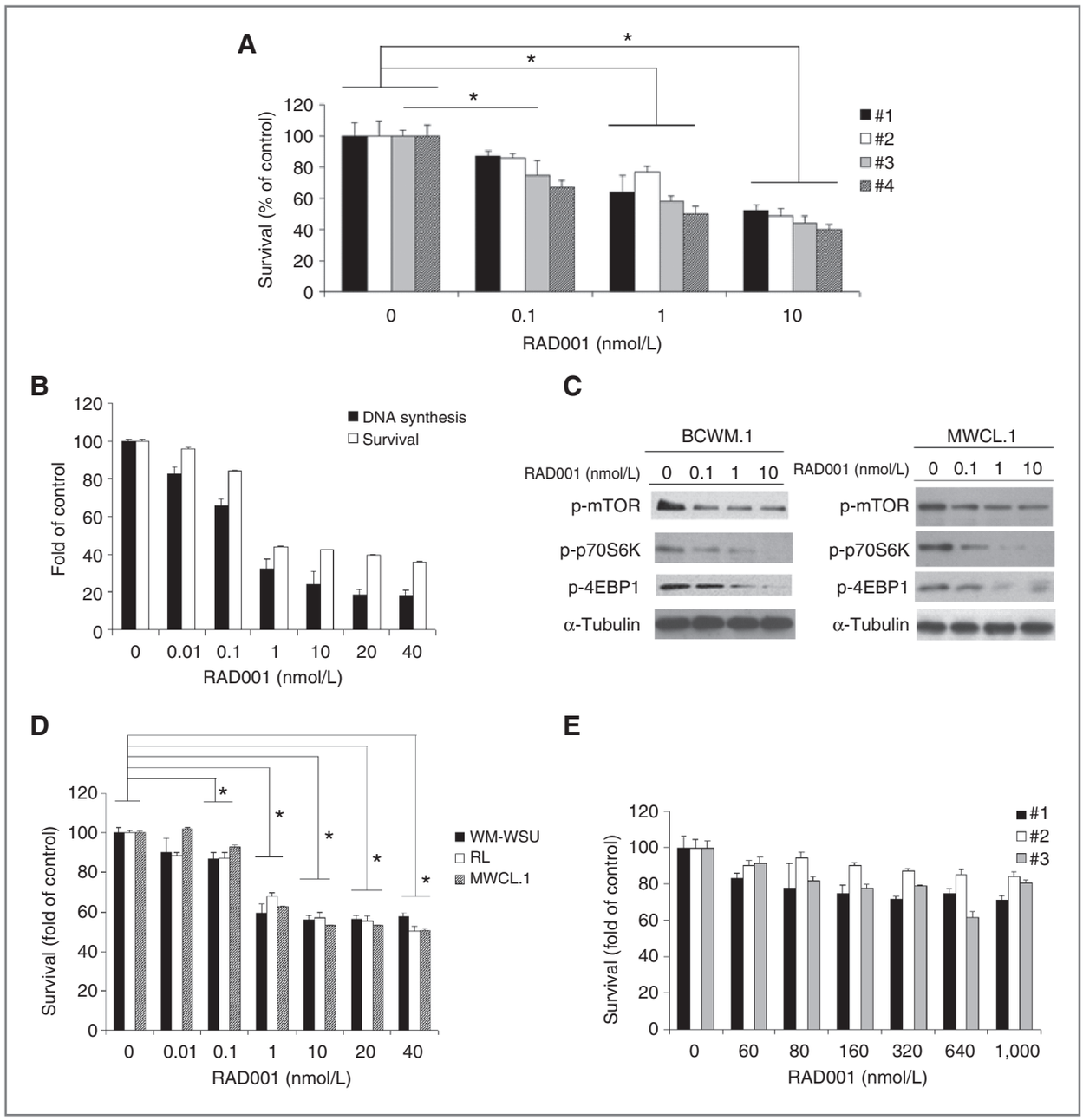

Figure 2. Everolimus targets Waldenstrom macroglobulinemia cells as well as other low-grade lymphoma IgM-secreting cells. A, cytotoxicity was assessed by MTT assay in primary Waldenstrom macroglobulinemia bone marrow–derived $CD19^+$ cells isolated from 4 patients with relapsed/refractory Waldenstrom macroglobulinemia (everolimus, 0.1–10 nmol/L; 48 hours). B, BCWM.1 cells were cultured with everolimus (0.01–40 nmol/L) for 48 hours: cytotoxicity and cell proliferation have been tested by MTT assay and thymidine uptake assay, respectively. C, BCWM.1 and MWCL.1 cells were treated with everolimus (0.1–10 nmol/L) for 6 hours. Whole-cell lysates were subjected to Western blotting using anti-p-mTOR, -p-p70S6K, -p-4EBP1 and -tubulin antibodies. D, WM-WSU, RL, and MWCL.1 cells were cultured with everolimus (0.01–40 nmol/L) for 48 hours: cytotoxicity has been tested by MTT assay. E, cytotoxicity was assessed by MTT in freshly isolated primary peripheral blood–derived $CD19^+$ cells from 3 healthy donors, treated with everolimus (60–1,000 nmol/L), for 48 hours. Bars represent SD.

macroglobulinemia and found that everolimus significantly induced toxicity in all the 4 samples evaluated (Fig. 2A; $P < 0.05$). We subsequently validated everolimus-induced toxicity in Waldenstrom macroglobulinemia and IgM-secreting lymphoma cell lines. Everolimus targeted Waldenstrom macroglobulinemia cells by inhibiting cell proliferation and by inducing cytotoxicity in a dose-dependent manner (Fig. 2B), supported by inhibition of p-mTOR, and mTOR downstream targeted proteins, such as p-p70S6K and p-4EBP1 (Fig. 2C). Similarly, everolimus-dependent induction of cytotoxicity was validated on IgM low-grade lymphoma cells (Fig. 2D). Importantly, everolimus did not

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show cytotoxicity on primary peripheral blood–derived $CD19^+$ cells isolated from healthy individuals (Fig. 2E).

It is known that mTOR regulates cell-cycle progression (24); we therefore examined the effect of everolimus in modulating Waldenstrom macroglobulinemia cell-cycle progression and found that everolimus-treated cells presented with cell-cycle arrest. Specifically, everolimus increased G_1 phase population in BCWM.1 cells: G_1 phase BCWM.1 cells increased from 52.52% in control to 61.25% and 70.98% after treatment with 0.1 and 1 nmol/L everolimus, respectively. Also, inhibition of the S-phase was observed, as indicated by reduction of cells in S-phase from 36.29% in control to 29.14% and 21.71% after treatment with 0.1 and 1 nmol/L everolimus (Fig. 3A). To determine

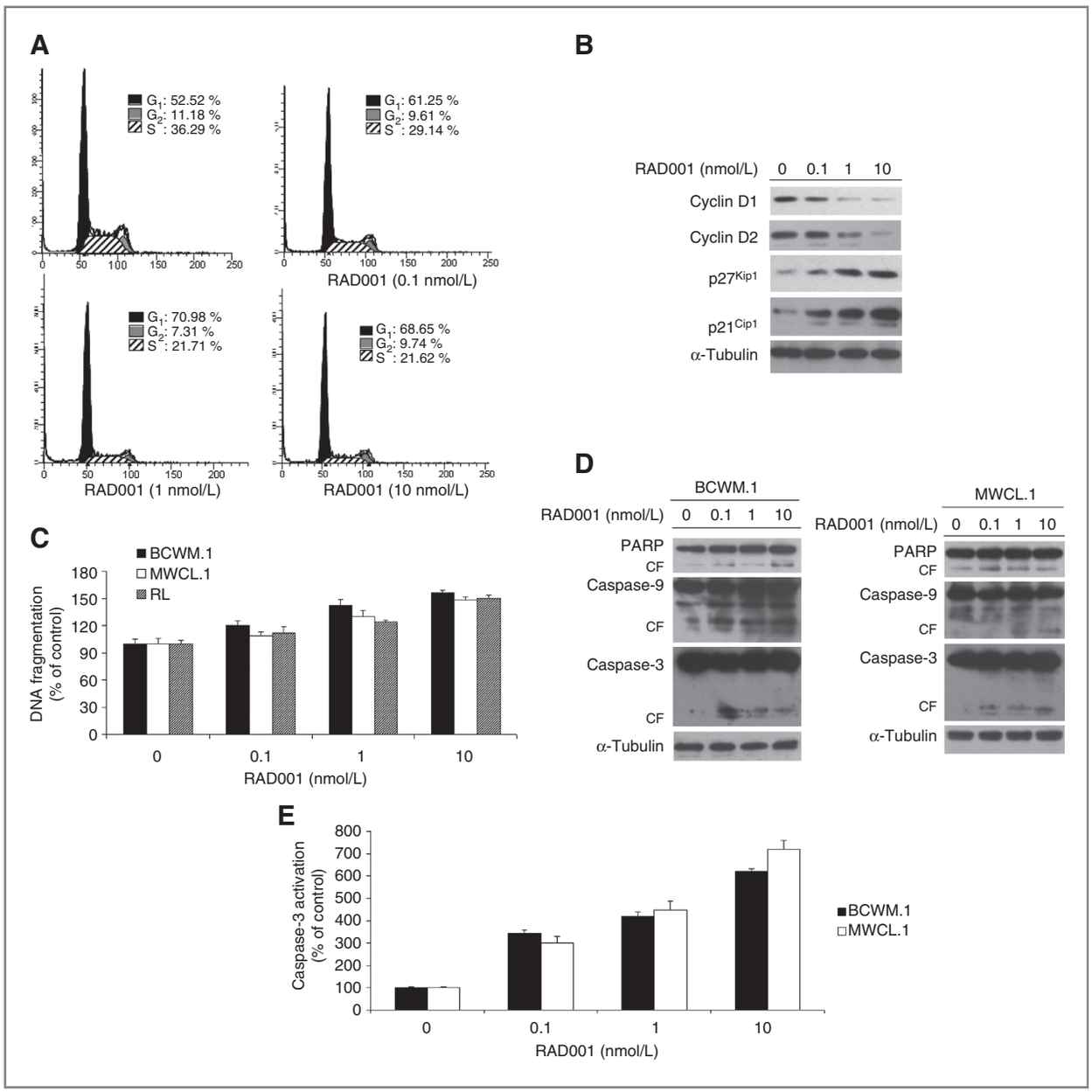

Figure 3. Everolimus modulates cell-cycle progression and induces apoptosis in Waldenstrom macroglobulinemia and low-grade lymphoma IgM-secreting cells. A, BCWM.1 cells were cultured with everolimus (0.1–10 nmol/L) for 12 hours, and cell-cycle was assessed by PI staining and flow cytometric analysis. B, BCWM.1 cells were treated with everolimus (0.1–10 nmol/L) for 12 hours. Whole-cell lysates were subjected to Western blotting using anti-cyclin D1, -cyclin D2, -$p27^{Kip1}$, -$p21^{Cip1}$, and -α tubulin antibodies. C, Waldenstrom macroglobulinemia and low-grade lymphoma IgM-secreting cells (BCWM.1, MWCL.1, and RL) were treated with everolimus (0.1–10 nmol/L) for 24 hours, and apoptosis has been tested by DNA fragmentation. Bars represent SD. D, BCWM.1 and MWCL.1 cells were treated with everolimus (0.1–10 nmol/L) for 24 hours. Whole-cell lysates were subjected to Western blotting using anti-PARP, -caspase-9, -caspase-3, and -α tubulin antibodies. E, BCWM.1 and MWCL.1 cells were treated with everolimus (0.1–10 nmol/L) for 24 hours, and caspase-3 activation has been tested by using Caspase-Glo assay.

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the mechanism of everolimus-induced cell-cycle arrest, we investigated the effect of the compound on Waldenstrom macroglobulinemia cells using immunoblotting and found that everolimus induced upregulation of cyclin kinase inhibitor protein $p27^{Kip1}$; $p21^{Cip1}$ together with downregulation of cyclins D1 and D2 (Fig. 3B).

We next confirmed that everolimus also induced apoptosis in a dose-dependent manner, in Waldenstrom macroglobulinemia and IgM low-grade lymphoma cells, as assessed by DNA fragmentation (Fig. 3C). We next examined the molecular mechanisms whereby everolimus induces cytotoxicity in Waldenstrom macroglobulinemia cells and found that everolimus induced caspase-9, -3, and PARP cleavage in a dose-dependent manner (Fig. 3D and E).

We have previously reported that primary Waldenstrom macroglobulinemia cells present with an increased expression of miRNA-155 compared with the normal cellular counterpart (17); and it has been also well documented that miRNA155 targets SHIP1 which acts as negative regulator of the PI3K/Akt and mTOR pathway (25). We therefore hypothesized that everolimus-dependent anti–Waldenstrom macroglobulinemia activity may be related, at least in part, to its ability to target miRNA-155 in tumor cells. We therefore sought to determine whether everolimus-dependent mTOR inhibition could be related to miRNA-155 expression level in Waldenstrom macroglobulinemia cells. We first found that everolimus inhibited miRNA-155 level in a dose-dependent manner (Fig. 4A). We have previously shown that miRNA-155 loss-of-function leads to inhibition of Waldenstrom macroglobulinemia cell proliferation, without inducing Waldenstrom macroglobulinemia cytotoxicity (17). We therefore conducted miRNA-155 loss-of-function studies and found that everolimus-dependent induction of toxicity in Waldenstrom macroglobulinemia cells was partially inhibited by knocking down miRNA-155 (Fig. 4B). Importantly, the effect of miRNA-155 knockdown

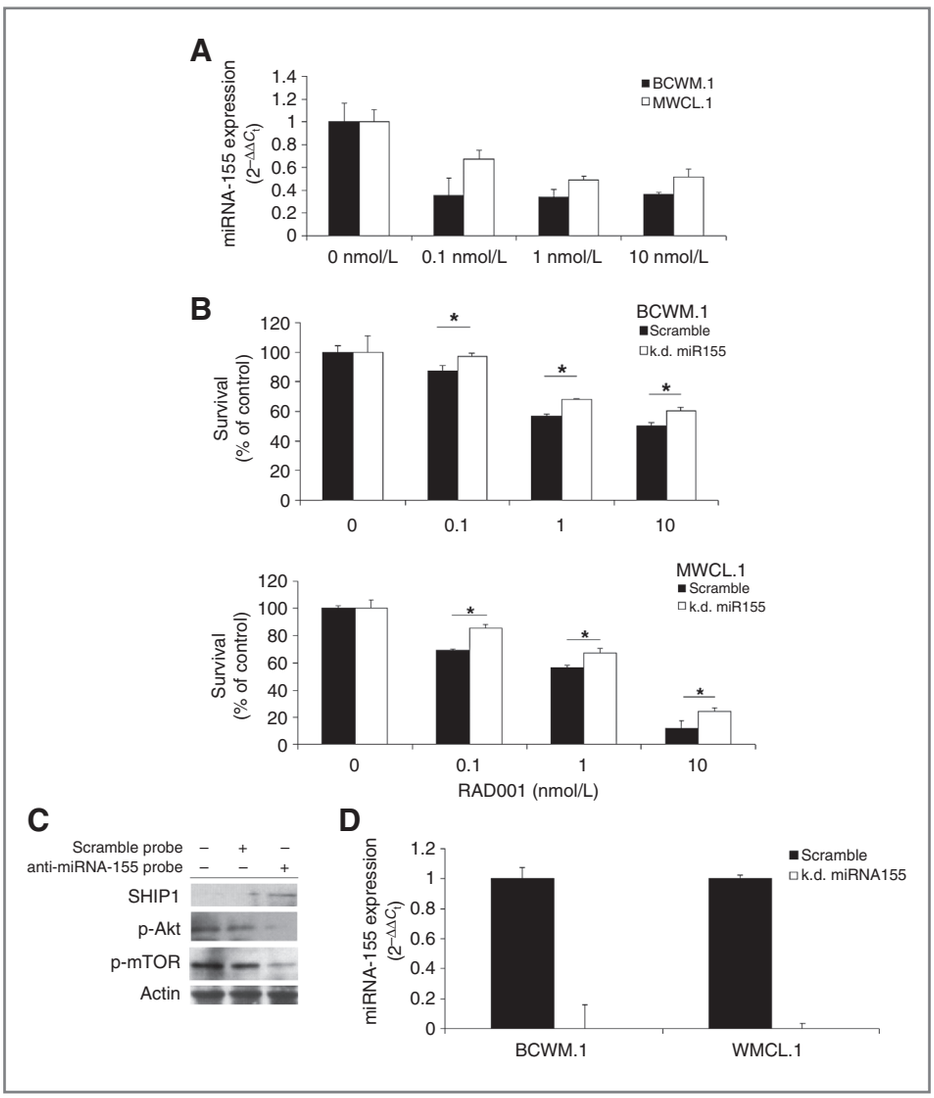

Figure 4. miRNA-155 contributes to everolimus-dependent anti–Waldenstrom macroglobulinemia activity. A, Waldenstrom macroglobulinemia cell lines (BCWM.1 and MWCL.1) have been treated with everolimus (0.1, 1, and 10 nmol/L) for 12 hours. miRNA-155 expression level has been evaluated by qRT-PCR, using the $\Delta\Delta C_t$ method. B, Waldenstrom macroglobulinemia cell lines (BCWM.1 and MWCL.1) were transfected with either miRNA-155 knockdown probe or scramble probe and exposed to everolimus (0.1, 1, and 10 nmol/L) for 48 hours. Cytotoxicity was assessed by MTT assay. C, BCWM.1 cells were transfected with either scramble probe or anti-miRNA-155 probe and harvested at 8 hours after transfection. Whole-cell lysates were subjected to Western blotting using anti-SHIP, -p-Akt, -p-mTOR, and -actin antibodies. D, efficiency of transfection: Waldenstrom macroglobulinemia cell lines (miRNA-155 knockdown probe-, scramble probe–transfected BCWM.1 and MWCL.1 cells) were harvested 48 hours after transfection. miRNA-155 expression level has been evaluated by qRT-PCR, using the $\Delta\Delta C_t$ method.

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on its direct target SHIP has been shown on anti-miRNA-155–transfected cells (Fig. 4C). miRNA-155 silencing in Waldenstrom macroglobulinemia cells have been validated by qRT-PCR (Fig. 4D). These findings indicate that everolimus exerts anti–Waldenstrom macroglobulinemia activity, at least in part, by targeting miRNA-155 in Waldenstrom macroglobulinemia cells.

Everolimus targets Waldenstrom macroglobulinemia cells in the context of bone marrow microenvironment

It has been widely accepted that bone marrow microenvironment confers growth and induces drug resistance in malignant cells (26). We therefore sought to determine the antitumor activity of everolimus in the presence of the bone marrow microenvironment. We first investigated whether everolimus inhibits Waldenstrom macroglobulinemia cell growth in the context of the bone marrow milieu. BCWM.1 cells were exposed to everolimus (0.1–10 nmol/L) in the presence or absence of BMSCs for 48 hours. The viability of BMSCs assessed by MTT was not affected by everolimus treatment (data not shown). Using [^{3}H]-TdR uptake assay, the adherence of BCWM.1 cells to BMSCs triggered a significant increase in proliferation, which was inhibited by everolimus in a dose-dependent manner (Fig. 5A). We next evaluated the efficacy of everolimus in affecting migration and adhesion of Waldenstrom macroglobulinemia cells. We first showed that stromal-derived factor-1 (SDF-1)–induced migration in Waldenstrom macroglobulinemia cells at 30 nmol/L SDF-1. To study the effect of everolimus on the migration of Waldenstrom macroglobulinemia cells, BCWM.1 cells were incubated with everolimus (0.01–1 nmol/L) for 4 hours. These doses and duration of incubation did not induce cytotoxicity in Waldenstrom macroglobulinemia cells as confirmed by MTT (data not shown). Cells were then subjected to migration as previously reported (20). We found that everolimus inhibited Waldenstrom macroglobulinemia cell migration toward SDF-1, in a dose-dependent manner. Similar findings were also confirmed with IgM low-grade lymphoma cells (Fig. 5B; $P < 0.05$). We next tested the effect of everolimus on the adhesion of Waldenstrom macroglobulinemia and IgM low-grade lymphoma cell lines to primary Waldenstrom macroglobulinemia BMSCs and found that everolimus inhibited Waldenstrom macroglobulinemia cell adhesion in a dose-dependent manner (Fig. 5C; $P < 0.05$).

It has been widely showed that neo-angiogenesis supports the pathogenesis of B-cell malignancies, including multiple myeloma and Waldenstrom macroglobulinemia (27). We therefore investigated the role of endothelial cells in supporting Waldenstrom macroglobulinemia cells growth and showed that endothelial cells increase the proliferative rate of Waldenstrom macroglobulinemia cells by 32% (Fig. 5D), supported by upregulation of pro-survival signaling pathways, in Waldenstrom macroglobulinemia cells exposed to endothelial cells, such as such as p-Akt, p-GSK3, -pS6R, p-ERK, and p-STAT3 (Fig. 5E). We therefore evaluated the efficacy of everolimus in targeting Waldenstrom macroglobulinemia cells even in presence of endothelial cells and showed that everolimus reduced Waldenstrom macroglobulinemia cell proliferation, despite the presence of endothelial cells (Fig. 5D). We next sought to determine the effect of everolimus on modulating vessel formation. To examine the anti-vascular activity of everolimus, we conducted capillary tubule formation assays with the use of HUVECs and found that everolimus inhibited endothelial cell morphogenesis on Matrigel in a dose-

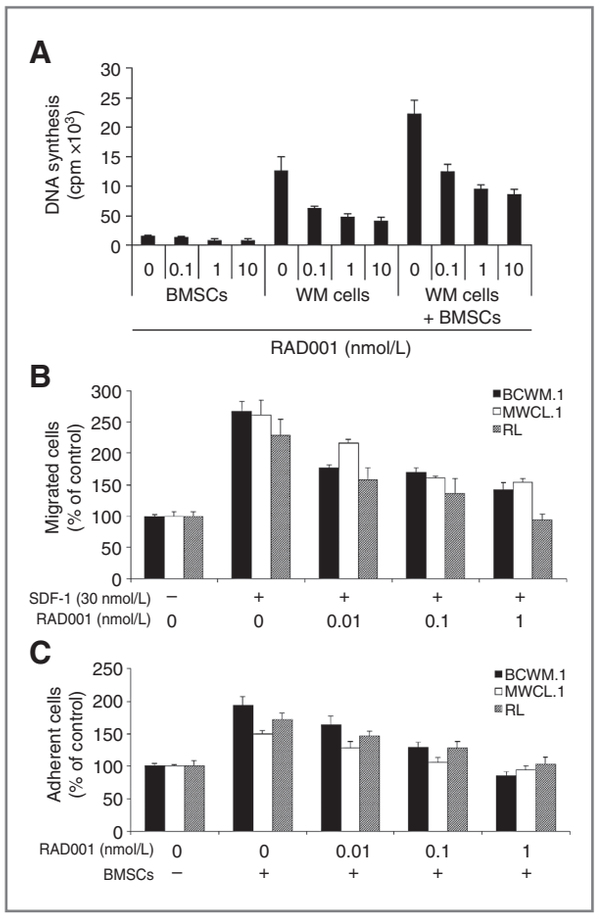

Figure 5. Everolimus exerts anti–Waldenstrom macroglobulinemia (WM) activity in the context of bone marrow microenvironment, and its anti–Waldenstrom macroglobulinemia effect is partially due to its antiangiogenic properties. A, BCWM.1 cells were cultured with control media and with everolimus (0.1–10 nmol/L), for 48 hours, in the presence or absence of primary Waldenstrom macroglobulinemia BMSCs. Cell proliferation was assessed using [^{3}H]-thymidine uptake assay. All data represent mean (±SD) of triplicate experiments. B, Transwell migration assay of Waldenstrom macroglobulinemia and low-grade lymphoma IgM-secreting cells. SDF-1 (30 nmol/L) was placed in the lower chambers, and migration was determined after 3 hours. Transwell migration assay showing inhibition of migration of BCWM.1, MWCL.1, and RL in the presence of everolimus (0.01–1 nmol/L). SDF-1 (30 nmol/L) was placed in the lower chambers and induced migration as compared with control with no SDF-1. Everolimus inhibited SDF-1–induced migration in Waldenstrom macroglobulinemia cells ($P < 0.05$). Bars represent SD. C, adhesion assay with BCWM.1, MWCL.1, and RL in the presence or absence of everolimus (0.01–1 nmol/L). Waldenstrom macroglobulinemia cells showed increased adhesion in fibronectin-coated wells as compared with control. Everolimus inhibited adhesion in fibronectin-coated wells in a dose-dependent manner ($P < 0.05$). All data represent mean (±SD) of triplicate experiments.

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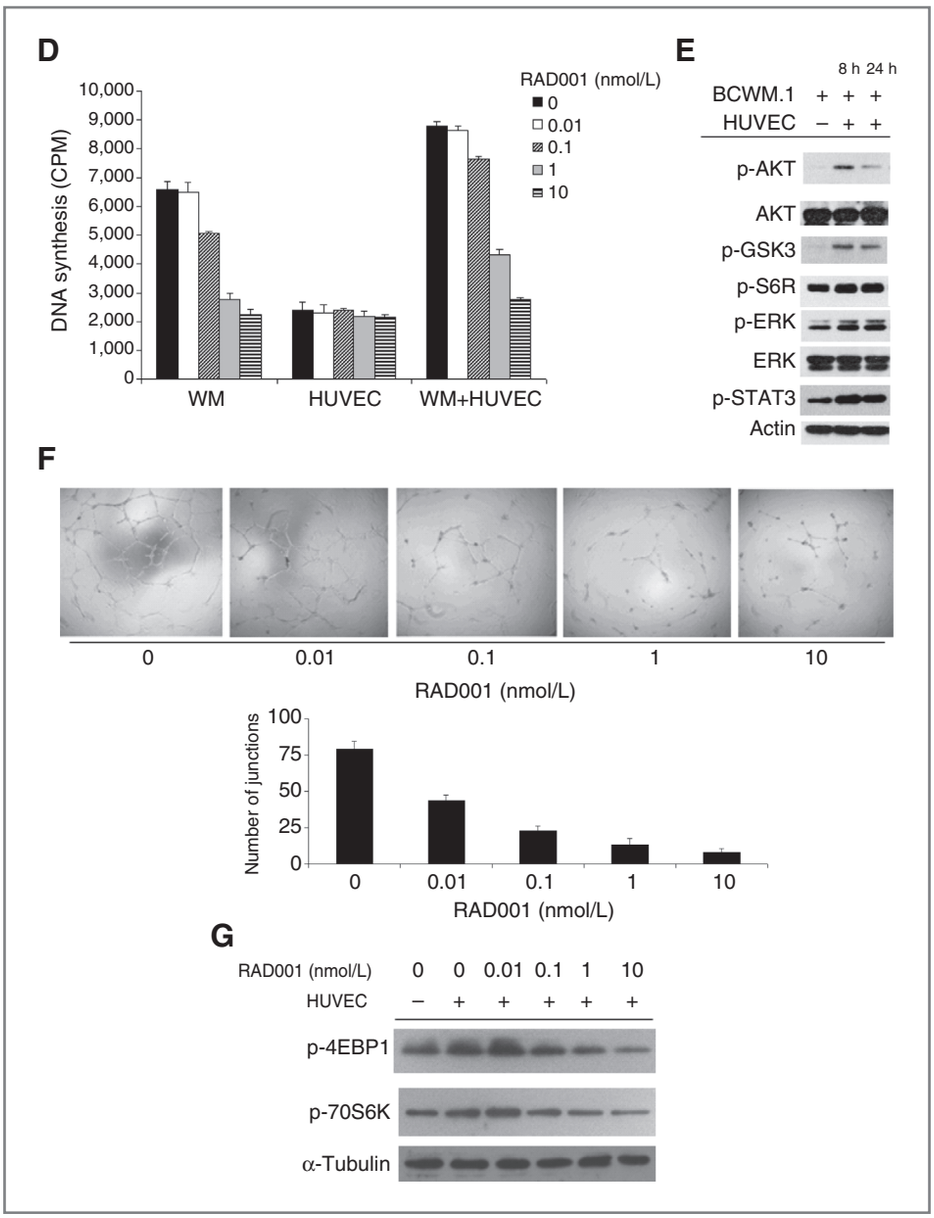

Figure 5. (*Continued*) D, BCWM.1 cells were cultured with control media and with everolimus (0.01–10 nmol/L), for 48 hours, in the presence or absence of endothelial cells (HUVECs). Cell proliferation was assessed using [^{3}H]-thymidine uptake assay. All data represent mean (±SD) of triplicate experiments. E, BCWM.1 cells were cultured in presence or absence of endothelial cells (HUVECs) for 8 and 24 hours. Whole-cell lysates isolated from Waldenstrom macroglobulinemia cells were subjected to Western blotting using anti-p-Akt, -AKT, p-GSK3, -pS6R, -p-ERK, -ERK, p-STAT3, -p-70S6K, and -actin antibodies. F, HUVECs were treated with everolimus (0.01–10 nmol/L) for 4 hours and assessed for *in vitro* vascularization with the use of Matrigel capillary-like tube structure formation assays. Quantification of number of junctions has been obtained by using ImageJ software. G, BCWM.1 cells were cultured in presence or absence of endothelial cells (HUVECs) and exposed or not to increasing concentration of everolimus (0.01–10 nmol/L) for 6 hours. Whole-cell lysates isolated from Waldenstrom macroglobulinemia cells were subjected to Western blotting using anti-p-4EBP1, -p-70S6K, and -α tubulin antibodies.

dependent manner. In the absence of everolimus, MMECs arranged in branching tubes forming a closely knit capillary-like plexus; at increasing concentrations of drug, tube formation was blocked with almost complete inhibition of capillary formation at 1 nmol/L of everolimus treatment (Fig. 5F). The impact of everolimus to target Waldenstrom macroglobulinemia cells in presence of endothelial cells was next evaluated at signaling level. Everolimus inhibited endothelial cell–dependent phosphorylation of 4EBP1 and 70SK, in Waldenstrom macroglobulinemia cells, in a dose-dependent manner (Fig. 5G).

Taken together, these findings indicate the efficacy of everolimus in targeting Waldenstrom macroglobulinemia cells, even in the context of bone marrow milieu where it overcomes BMSC-dependent proliferation, as well as adhesion and migration *in vitro*.

Everolimus acts synergistically with bortezomib and rituximab in targeting Waldenstrom macroglobulinemia cells

The lack of complete responses with everolimus in patients with relapsed or relapsed/refractory Waldenstrom macroglobulinemia suggests that some of the lymphoplasmacytic cells might be resistant to mTOR inhibition, and this led us to hypothesize that the use of everolimus in combination with other therapeutical agents could be beneficial in patients with Waldenstrom macroglobulinemia (15).

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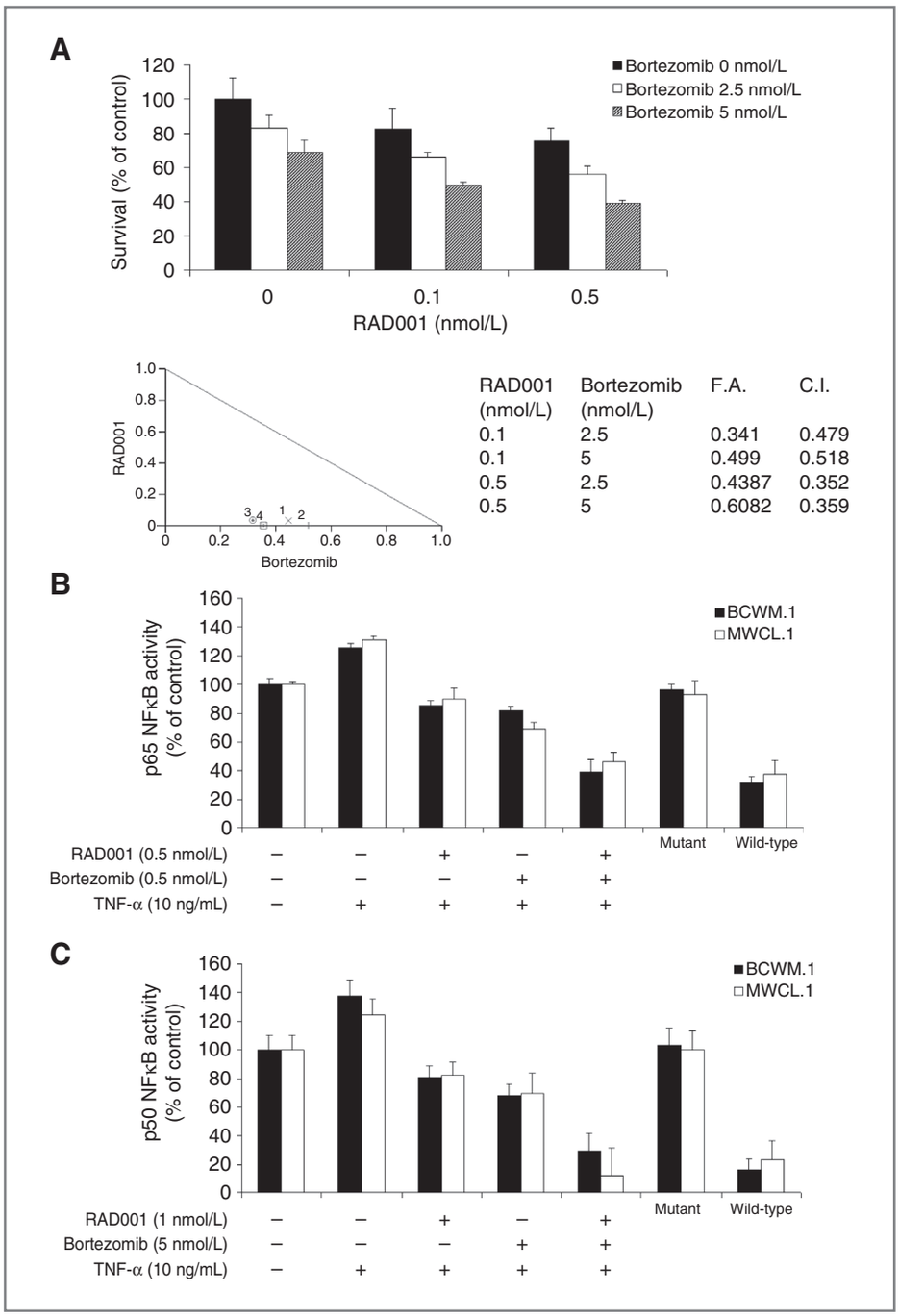

Figure 6. Everolimus synergizes with bortezomib and rituximab in targeting Waldenstrom macroglobulinemia cells. A, BCWM.1 cells were treated with everolimus (0.1–1 nmol/L) in presence or absence of bortezomib (5–10 nmol/L) for 48 hours; and effects on cell survival was assessed by MTT assay. CalcuSyn software: isobologram, CI, and fractions affected (FA) of the combination of everolimus and bortezomib are shown. All experiments were repeated in triplicate. B and C, BCWM.1 and MWCL.1 cells were cultured with either everolimus (1 nmol/L), bortezomib (5 nmol/L), or the combination for 4 hours and then TNF-α (10 ng/mL) was added for the last 20 minutes. NF-κBp65/p50 transcription factor binding to its consensus sequence on the plate-bound oligonucleotide was studied from nuclear extracts. Wild-type and mutant are wild-type and mutated consensus competitor oligonucleotides, respectively. All results represent means (±SD) of triplicate experiments.

BCWM.1 cells were next cultured with everolimus (0.1–0.5 nmol/L) for 48 hours, in the presence or absence of bortezomib (2.5–5 nmol/L). Everolimus showed significant cytotoxic effects when combined with bortezomib, as showed by using MTT assays at 48 hours (Fig. 6A). Everolimus (0.5 nmol/L) induced cytotoxicity in 24% of BCWM.1 cells, which was increased to 43% and 60% in the presence of bortezomib at 2.5 nmol/L [combination index (CI), 0.47] and 5 nmol/L (CI, 0.51), respectively, indicating synergistic activity. Similar results were obtained using bortezomib with 0.1 nmol/L everolimus. Isobologram analysis, fractions affected, and the combination indexes for each of these combinations are indicated (Fig. 6A).

We have previously reported that primary Waldenstrom macroglobulinemia cells present with a constitutive activation of the NF-κB pathway, thus resulting in increased tumor cell growth and disease progression (21).

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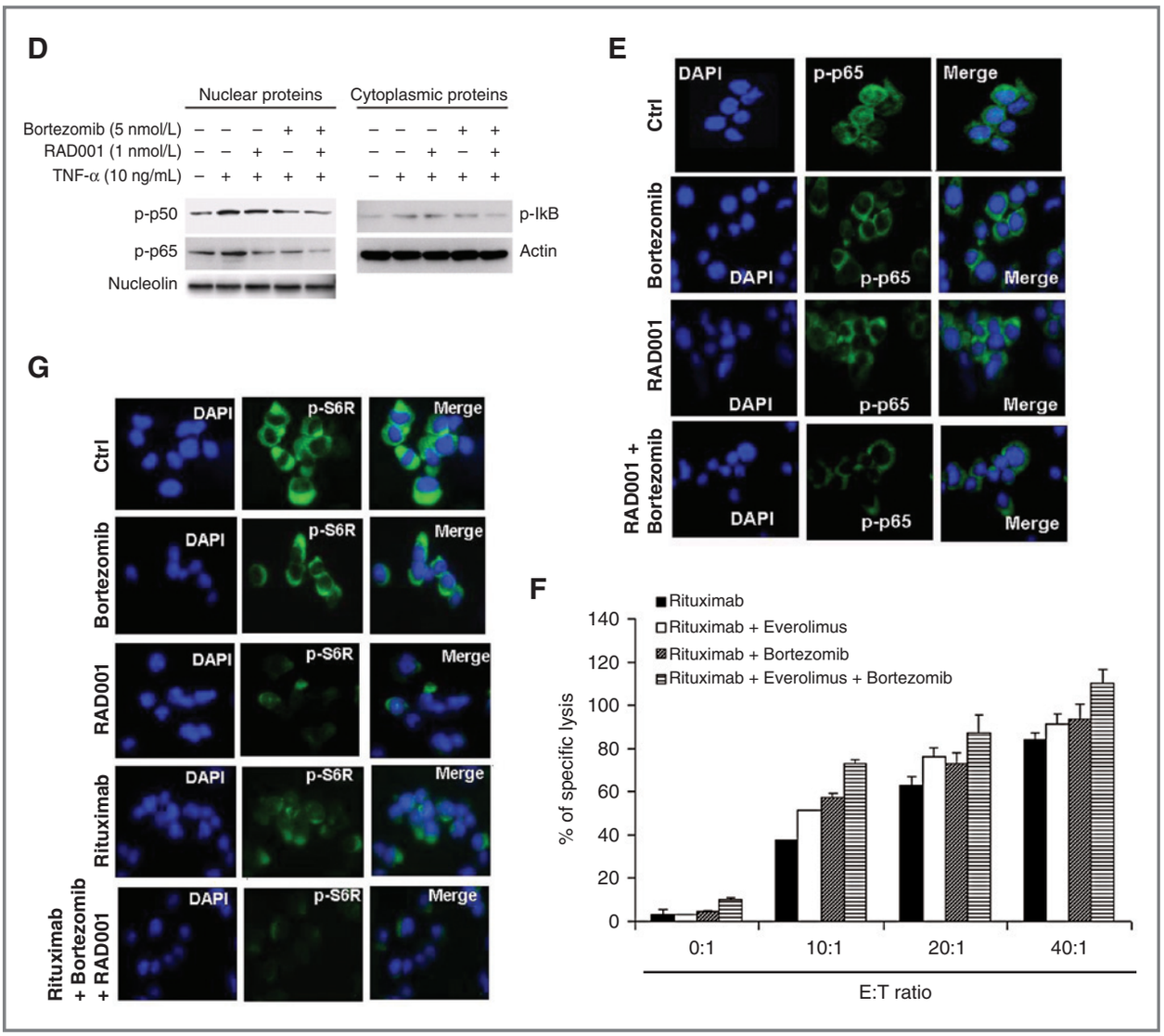

Figure 6. (*Continued*) D, BCWM.1 cells were cultured with either everolimus (1 nmol/L), bortezomib (5 nmol/L), or the combination for 4 hours, and TNF-α (10 ng/mL) was added for the last 20 minutes. Cytoplasmic and nuclear extracts were subjected to Western blotting using anti–p-NF-κBp65, –NF-κBp50, -nucleolin, -p-IkB, and -actin antibodies. E, BCWM.1 cells were cultured with everolimus (1 nmol/L), bortezomib (5 nmol/L), or the combination for 4 hours, and TNF-α (10 ng/mL) was added for the last 20 minutes. Immunocytochemical analysis was assessed using anti-pospho-NF-κBp65 antibody. DAPI (4′,6-diamidino-2-phenylindole) was used to stain nuclei. F, ADCC realized on BCWM.1 cells treated with everolimus (1 nmol/L), bortezomib (5 nmol/L), rituximab (10 μg/mL), and the combination in absence and presence of activated effector cells. Results are reported in terms of mean percentage of specific lysis characterized by measurement of release of calcein-AM upon different effector/target ratios (E:T ratio). The following controls showed minimum released of calcein-AM and were not added to the figure: medium alone, BCWM.1 or activated effector cells alone, BCWM.1 incubated with activated effector cells in absence of rituximab, activated effector cells with rituximab in absence of target cells. G, BCWM.1 cells were cultured with everolimus (1 nmol/L), bortezomib (5 nmol/L), rituximab (10 μg/mL), or the triple combination for 4 hours, and TNF-α (10 ng/mL) was added for the last 20 minutes. Immunocytochemical analysis was assessed using anti-phospho-NF-κB/p65 antibody. DAPI was used to stain nuclei.

We therefore investigated whether everolimus-induced Waldenstrom macroglobulinemia cytotoxicity may be due, at least in part, to its ability to modulate NF-κB–related proteins and whether everolimus would lead to a synergist inhibition of this pathway, when used with bortezomib, in a combinatory regimen. We first evaluated the effect of everolimus, either alone or in combination with bortezomib, on the NF-κB/p65 and NF-κB/p50 binding activities, studying nuclear extracts from treated cells using the Active Motif Assay. We found that TNF-α treatment induced NF-κB recruitment to the nucleus in BCWM.1 and MWCL.1 cells, which was similarly inhibited by either everolimus or bortezomib, used as single agents. Importantly, a significant inhibition of NF-κB/p65 DNA-binding activity (84%) was documented in Waldenstrom macroglobulinemia cells exposed to the

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combination of everolimus and bortezomib (Fig. 6B). Similar findings were observed by testing the effect of everolimus on NF-κB/p65 binding activity (Fig. 6C). These findings were further corroborated at protein level, by studying nuclear protein lysates: TNF-α–dependent nuclear translocation of p-p65 and p-p50 was more significantly inhibited in Waldenstrom macroglobulinemia cells exposed to the combination of everolimus and bortezomib, compared with each agent used as single drug. This was also supported by a synergistic inhibition of p-IkB at cytoplasmic level, when everolimus and bortezomib were used in a combinatory regimen (Fig. 6D). We further confirmed that p-p65 translocation from the cytoplasmic compartment to the nucleus was inhibited by the combination of everolimus and bortezomib, resulting in a significant increase in p-p65 expression in the cytoplasmic compartment as shown by immunofluorescence (Fig. 6E).

The monoclonal anti-CD20 antibody represents one of the main therapeutic approaches in patients with Waldenstrom macroglobulinemia. We therefore evaluated the effect of the combination of everolimus with rituximab, assessed by ADCC. A modest increase in specific lysis has been observed in Waldenstrom macroglobulinemia cells exposed to rituximab and bortezomib, compared with Rituximab used as single agent: indeed, the specific lysis increased from 37.7% with rituximab alone to 51.4% when 10 μg/mL rituximab was combined with everolimus (1 nmol/L) with an E:T ratio of 10:1. Importantly, specific lysis increased to 73.1% when Waldenstrom macroglobulinemia cells were exposed to the combination of everolimus, rituximab, and bortezomib (E:T ratio of 10:1). Increased specific lysis with the combination was observed at all E:T ratios studied (Fig. 6F). These findings were next confirmed by immunofluorescence, where the everolimus + rituximab + bortezomib combination led to significant inhibition of phosphorylation of S6R, mTOR downstream targeted protein (Fig. 6G).

Discussion

Prior studies have shown that everolimus is active in many B-cell lymphomas but had a significantly high response rate of 70% in Waldenstrom macroglobulinemia (15). To better understand everolimus-dependent anti–Waldenstrom macroglobulinemia activity, we investigated the putative mechanisms that may explain how everolimus may target Waldenstrom macroglobulinemia clonal cells. We first examined bone marrow specimens of patients with Waldenstrom macroglobulinemia pre- and post-everolimus treatment and showed that those patients who achieved a partial response presented with a significant inhibition of phosphorylation of 4EBP1 and S6R, known to be mTOR downstream-targeted proteins. In contrast, patients who did not respond to treatment presented with unmodified phosphorylated levels of 4EBP1 and S6R within the bone marrow. In the present studies, we have not dissected the mechanisms leading to absence of mTORC1 inhibition in non-responder patients, due to the absence of statistical power. These findings indicate that everolimus specifically targets mTOR signaling cascade *in vivo* and that lack of response could depend on tumor clones where silencing of mTOR pathway was not achieved. These markers could therefore be used as dependable pharmacodynamic markers of response in patients with Waldenstrom macroglobulinemia and other types of lymphomas.

We previously showed that miR155 is highly upregulated in Waldenstrom macroglobulinemia (17). miR155 acts an oncomiR in many lymphomas and was found to induce the development of lymphomas in transgenic mouse models (28, 29). In this study, we showed that everolimus can specifically target miR155. Importantly, everolimus-dependent anti–Waldenstrom macroglobulinemia effect was related to its ability to target miRNA-155: indeed, by miRNA-155 loss-of-function studies in Waldenstrom macroglobulinemia cells, we showed that tumor cells can only partially overcome everolimus-dependent cytotoxicity, indicating that miRNA-155 may represent, at least in part, one of the targets responsible for everolimus-induced anti–Waldenstrom macroglobulinemia effect. Given that miR155 is elevated in many patients with low-grade lymphomas including Waldenstrom macroglobulinemia, we believe that miR155 could potentially be tested in the future as a biomarker to personalize therapy for patients with low-grade lymphoma, where patients with high levels of miR-155 may respond better to everolimus than those with lower levels of miR155.

The clinical studies of everolimus show responses in the range of 30% to 70% in lymphomas. In this study, we examined potential mechanisms by which everolimus induces cell death in lymphoma cells. We showed that everolimus induces direct cell cytotoxicity with induction of caspase cleavage and cell-cycle arrest. In addition, everolimus inhibited angiogenesis and induced cytotoxicity even in the presence of BMSCs. These studies indicate that TORC1 inhibitors alone may be sufficient in targeting tumor cells.

It has been widely shown the importance of the bone marrow milieu in supporting the pathogenesis and of several B-cell malignancies, including Waldenstrom macroglobulinemia (26).

Everolimus inhibited migration and adhesion of Waldenstrom macroglobulinemia cells to BMSCs. This *in vitro* effect may explain the effect of everolimus in vivo on the mobilization of tumor cells from lymph nodes into the circulation. Indeed, this effect was observed in a phase II clinical trial of chronic lymphocytic leukemia (CLL) where an increase in absolute lymphocyte count (ALC) associated with a decrease in lymphadenopathy in 8 (36%) patients. ALC increased a median of 4.8-fold (range, 1.9- to 25.1-fold), and the clinically measurable lymphadenopathy decreased a median of 75.5% (range, 38%–93%) compared with baseline measurements (30).

As we have reported in the phase II clinical trial of mTOR inhibitor in relapsed or refractory patients with Waldenstrom macroglobulinemia, there was a lack of complete

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remissions, suggesting the presence of tumor cells resistant to anti-mTOR treatment (15). This observation led us to hypothesize that mTOR inhibition may have a beneficial impact in the treatment of refractory patients, by combining mTOR with other novel agents, such as proteasome inhibitor or anti-CD20 monoclonal antibody. We therefore conducted further studies to provide the preclinical evidence for combining the mTOR inhibitor with other agents and found that everolimus exerted a synergistic effect in inducing cytotoxicity in Waldenstrom macroglobulinemia cells, when used in combination with bortezomib and rituximab. This was further supported by a stronger inhibition of NF-κB/p65 activity in Waldenstrom macroglobulinemia cells when exposed to the combinatory regimen of everolimus + bortezomib. Moreover, everolimus used in combination with rituximab, enhanced rituximab-dependent ADCC in Waldenstrom macroglobulinemia cells, which led to a more significant inhibition of p-S6R when used in combination, compared with single-agent treatment. Finally, increased rate of ADCC in Waldenstrom macroglobulinemia cells was observed upon treatment of everolimus, rituximab, and bortezomib.

These findings provide a better understanding of the molecular mechanisms responsible for everolimus-based antitumor effect in Waldenstrom macroglobulinemia and define a preclinical rational for combinatory regimen of everolimus with bortezomib or rituximab, that may result in a better response rate, in those patients refractory to single-agent everolimus.

Disclosure of Potential Conflicts of Interest

I.M. Ghobrial is a consultant/advisory board member of Novartis, Millennium, Onyx, and BMS. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: A.M. Roccaro, A. Sacco, F. Azab, F. Ludmila, I.M. Ghobrial
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Roccaro, A. Sacco, X. Jia, R. Banwit, S. Manier, A.K. Azab, I.M. Ghobrial
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Roccaro, P. Maiso, A.K. Azab, I.M. Ghobrial
Writing, review, and/or revision of the manuscript: A.M. Roccaro, A. Sacco, I.M. Ghobrial
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Sacco
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Conducted research: A. Sacco, X. Jia, R. Banwit, P. Maiso, F. Azab, F. Ludmila, S. Manier, A.K. Azab

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