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Preclinical Development

Molecular Cancer Therapeutics

RITA Inhibits Multiple Myeloma Cell Growth through Induction of p53-Mediated Caspase-Dependent Apoptosis and Synergistically Enhances Nutlin-Induced Cytotoxic Responses

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Abstract

Mutations or deletions of p53 are relatively rare in multiple myeloma (MM), at least in newly diagnosed patients. Thus, restoration of p53 tumor suppressor function in MM by blocking the inhibitory role of murine double minute 2 (MDM2) is a promising and applicable therapeutic strategy. RITA and nutlin are two new classes of small molecule MDM2 inhibitors that prevent the p53-MDM2 interaction. Earlier reports showed p53-dependent activity of RITA in solid tumors as well as in leukemias. We and others recently described nutlin-induced apoptosis in MM cells, but it remains unclear whether RITA exerts antimyeloma activity. Here, we found that RITA activates the p53 pathway and induces apoptosis in MM cell lines and primary MM samples, preferentially killing myeloma cells. The activation of p53 induced by RITA was mediated through modulation of multiple apoptotic regulatory proteins, including upregulation of a proapoptotic protein (NOXA), downregulation of an antiapoptotic protein, Mcl-1, and activation of caspases through extrinsic pathways. Moreover, a number of key p53-mediated apoptotic target genes were identified by gene expression profiling and further validated by quantitative real-time PCR. Importantly, the combination of RITA with nutlin displayed a strong synergism on growth inhibition with the combination index ranging from 0.56 to 0.82 in MM cells. Our data support further clinical evaluation of RITA as a potential novel therapeutic intervention in MM. *Mol Cancer Ther; 9(11); 3041–51. ©2010 AACR.*

Introduction

Multiple myeloma (MM) is a plasma cell malignancy of terminally differentiated B cells. MM remains an incurable disease because of its high resistance to apoptosis as well as both intrinsic and acquired drug resistance (1–4). Therefore, new therapeutic strategies are needed to improve patient outcome.

The p53 tumor suppressor is a multifunctional transcription factor that regulates cellular processes affecting proliferation and apoptosis (5). Inactivation of p53 by mutations and/or deletions occurs in half of human tumors (6). However, p53 mutations and/or deletions are rare in MM with the incidence of ~10% in newly diagnosed patients (7–9). Inactivation of wild-type p53 is often acquired largely through deregulation of the human homologue of murine double minute 2 (MDM2) protein (10). MDM2 is the principal endogenous negative regulator of p53 and is an E3 ligase, which binds to the amino terminal p53 and facilitates proteasomal degradation of p53 (11, 12). Due to the importance of the p53-MDM2 interaction, pharmacologic activation of the p53 pathway by inhibition of MDM2 binding has been shown to be an alternative therapeutic strategy in various cancers, including hematologic malignancies (13, 14).

Accumulated experimental data suggest that p53 reactivation might be a widely applicable and efficient antimyeloma strategy (15–18). Presumably, nongenotoxic small molecule MDM2 inhibitors, such as nutlin, induce apoptosis in malignant cells but leave normal nonmalignant cells intact (15, 19). Nutlin binds to the p53 binding pocket of MDM2, blocks the p53-MDM2 interaction, and prevents MDM2-mediated degradation of p53 (19). Recently, another small molecule inhibitor of the p53-MDM2 interaction, RITA (reactivation of p53 and induction of tumor cell apoptosis), which restores the apoptosis inducing function of p53 in tumor cells has been identified (20). In contrast to nutlin, RITA binds to the

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NH_2 terminal site of p53 and induces its active conformation that has lower affinity for its negative regulators such as MDM2. Thus, RITA prevents the p53-MDM2 interaction and induces accumulation of p53 in tumor cells as a result of its increased half-life (20). The chemical structures of RITA and nutlin are shown in Fig. 1A.

We and others have shown that inhibition of the p53-MDM2 interaction by nutlin stabilizes p53 and induces apoptosis in MM cells through regulation of p53 downstream targets (15–17). In addition, we recently showed that nutlin synergizes with the proteasome inhibitor velcade in the induction of apoptosis of MM cells (18). Nutlin may induce cell cycle arrest and/or apoptosis in leukemia, lymphoma, or myeloma cells harboring wild-type p53 (15–18, 21–24). RITA, in contrast, preferentially promotes apoptosis in tumor cells (19, 25–28). Significantly, both nutlin and RITA has been shown to execute their functions in a p53-dependent manner. RITA has been shown to have efficacy in various solid tumors such as colon, breast, cervical, and lung carcinoma, osteosarcoma, and renal cell carcinoma (20, 26–30). There has been only one report describing antileukemic activity of RITA in acute myelogenous leukemia and chronic lymphocytic leukemia, which was limited to the study of the expression of p53 only (31). The role of RITA in MM has not been addressed, and little is known about the downstream effectors of the p53/MDM2 pathway in response to RITA in hematologic malignancies.

In this study, we have assessed the antimyeloma activity of RITA alone or in combination with nutlin and explored the effects on p53 signaling pathways in MM. We have shown that RITA efficiently induced cytotoxicity through stabilization and activation of p53 and regulation of p53 downstream targets in both MM cell lines and primary MM samples. Importantly, nutlin plus RITA in combination displays a synergistic antimyeloma activity. Our study provides the preclinical framework for the therapeutic intervention of MM using

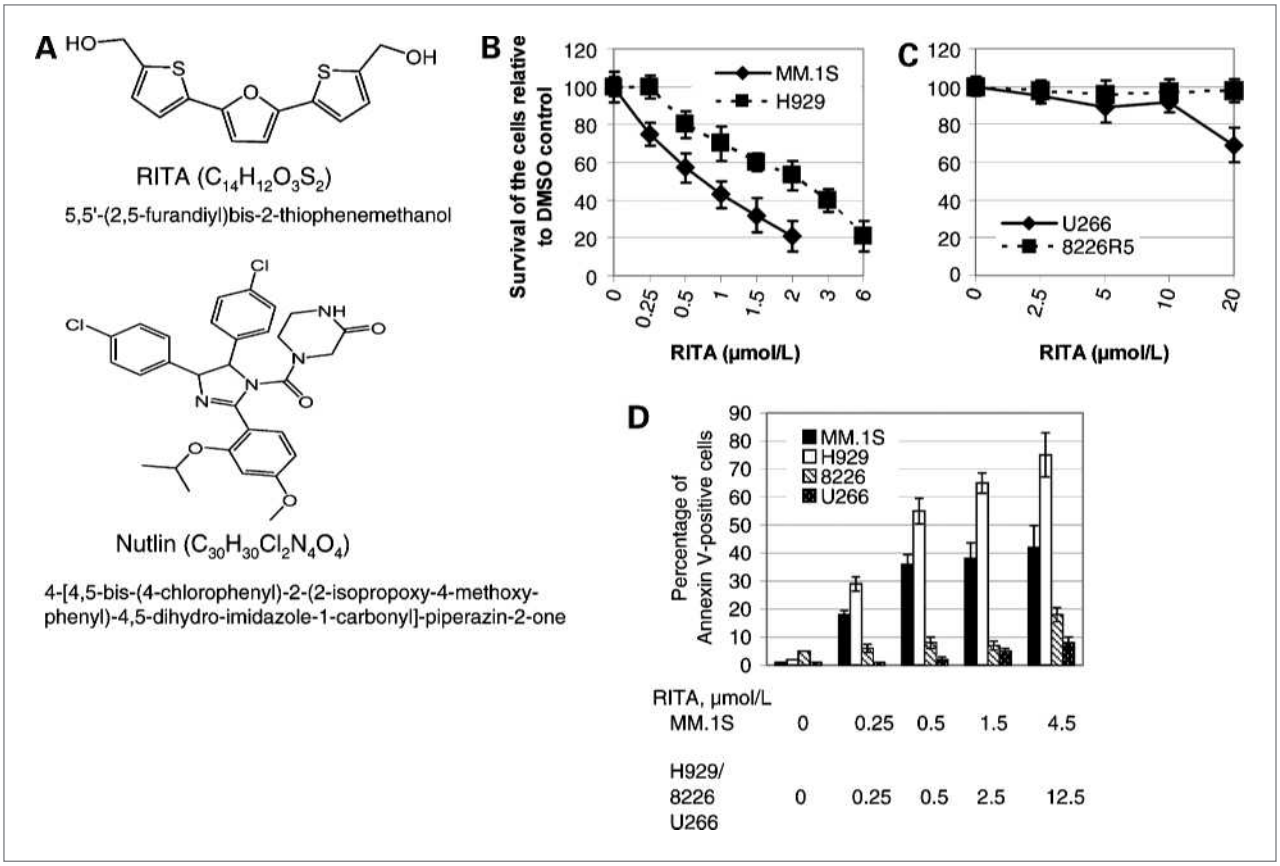

Figure 1. A, structural formula of RITA [2,5-bis(5-hydroxymethyl-2-thienyl)furan] and nutlin [4-(4,5-bis-(4-chlorophenyl)-2-(2-isopropoxy-4-methoxy-phenyl)-4,5-dihydro-imidazole-1-carbonyl)-piperazin-2-one]. B, C, and D, RITA induces growth arrest and apoptosis of MM cells harboring wild-type p53. MM cells were plated in 96-well plates and cultured either with or without different concentrations of RITA (0.25–20 μmol/L). After 48 h, the cells were treated with MTT for 4 h at 37°C, and MTT activity was measured. Survival of the cells were expressed as percentage of the DMSO-treated control in cells harboring wild-type p53 (B) or mutant p53 (U266) or null p53 (8266R5) (C). Results represent the mean ± SD of three independent experiments done in triplicate. D, MM cells were treated with various concentrations of RITA (0.25–12.5 μmol/L) for 48 h. The cells were subsequently stained with FITC-Annexin V and propidium iodide and analyzed by flow cytometry. Each column represents the mean of apoptotic cells (Annexin V and propidium iodide positive) of three independent experiments after normalizing the data with vehicle treatment, and each bar represents SD.

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these small molecule inhibitors targeting the p53-MDM2 interaction.

Materials and Methods

Myeloma cell lines and primary MM samples

MM.1S and H929 cell lines harboring wild-type p53; LP1 and U266 cell lines expressing mutant p53 were obtained from American Type Culture Collection (with no further authentication in our laboratory). 8226R5 cell lines that do not express p53 (17) was kindly provided by Dr. R. Buzzeo (32). All cell lines were grown in standard culture medium (Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 50 units penicillin, and 50 μg/mL streptomycin) at 37°C in a 5% CO_2 incubator.

Bone marrow (BM) samples from patients with MM were cultured and maintained as described previously (18). Peripheral blood mononuclear cells (PBMNC) and BM mononuclear cells (BMMNC) were separated by Ficoll density gradient centrifugation. For the stimulation of PBMNCs, 1% phytohemagglutinin (M Form; Invitrogen) was added to the culture 3 hours before treating the cells with RITA. This study was approved by the research ethics committee of University Health Network, Toronto, in accordance with the declaration of Helsinki.

Antibodies

Antibodies against the following proteins were used in this study: mouse monoclonal antibodies to p53 (DO-7), p21, and β-actin purchased from Santa Cruz Biotechnology; MDM2 from Calbiochem; NOXA from Abcam; β-tubulin from Sigma; rabbit polyclonal antibodies to Bax and Mcl-1 and mouse monoclonal antibodies to caspase-3 and poly (ADP-ribose) polymerase (PARP) from Cell Signaling Technology; rabbit polyclonal antibody to caspase-8 purchased from BD Biosciences; and caspase-9 from Santa Cruz Biotechnology. Peroxidase-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Cell Signaling and Santa Cruz Biotechnology, respectively.

Drug treatment

RITA and nutlin (nutlin-3) were purchased from Cayman Chemical, dissolved in DMSO to create a 50 mmol/L stock solution, and stored at −20°C until use. Cell lines were harvested in log-phase growth and exposed to RITA or nutlin for different time periods. In each experiment, the final DMSO concentration was kept constant and did not exceed 0.1% (v/v). In some experiments, cells were simultaneously exposed to RITA and nutlin. After drug treatment, cells were harvested and subjected to further analysis as described below. A pan-caspase inhibitor (Z-VAD-FMK) and an inhibitor of caspase-8 (Z-IETD-FMK) were purchased from Biovision, Inc. and MBL International Corporation), respectively. Cells were pretreated with the specific inhibitors 2 hours before treatment with RITA.

Cell viability assay

Cell viability was assessed by MTT colorimetric assay. MM cell lines or $CD138^+$ myeloma cells purified from BM aspirates of patients with MM or PBMNCs and BMMNCs obtained from healthy donors were seeded in 96-well plates (Sarstedt, Inc.) in 100-μL complete medium at a density of 20×10^4 cells per well. MM cells were incubated with various concentrations of drugs (0.25–20 μmol/L) for 48 hours. After incubation, MTT (0.5 mg/mL) was added, and the cells were further incubated for an additional 4 hours. This was followed by the addition of acidified isopropanol to the wells and overnight incubation at 37°C. Following incubation, the absorbance of the cells was read with a microplate reader set at a test wave length of 570 nm and a reference wavelength of 650 nm. Each experiment was made in triplicate, and the mean value was calculated.

Assessment of apoptosis

To examine apoptotic cell death, MM cells were treated with various concentrations of RITA for 48 hours and stained for flow cytometry with Annexin V-FITC (Abcam) and propidium iodide (Sigma). Stained cells were analyzed using a FACScan (Becton Dickinson) flow cytometer. Data were analyzed using FlowJo software. The extent of apoptosis was quantified as percentage of Annexin V–positive cells, and the extent of drug-specific apoptosis was assessed by the formula: percentage of Annexin V–positive cells = (test − control) × 100/(100 − control).

Protein extraction and Western blot analysis

Whole-cell lysates were prepared by extraction of cell pellets, which were lysed for 10 minutes on ice in a buffer composed of 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 1% (v/v) Nonidet P-40, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, and 25 μg/mL leupeptin. Protein concentrations were measured using a Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific). Equal amounts of protein extracts were resolved using 10% or 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Perkin-Elmer, Inc.). After blocking for 1 hour at room temperature with PBS containing either 5% skim milk or 3% bovine serum albumin, depending on the antibodies used for probing the blots, the membranes were probed with primary antibody. The filter was washed and incubated with a horseradish peroxidase–labeled secondary antibody for 1 hour, and the blots were developed using a chemiluminescent detection system (ECL, Perkin-Elmer).

Gene expression analysis

Total RNA was isolated using TRIzol reagent (Invitrogen), and the gene expression profile was evaluated using Illumina RNA analysis Beadchips (Illumina, Inc.) representing ~48,000 human genes (human HT12) as described earlier (17). Expression of key genes in

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RITA-induced MM.1S cells involved in cell proliferation, cell cycle arrest, or apoptosis was analyzed.

Quantitative real-time PCR

To quantify and validate the expression of p53 target genes of interest at their mRNA level, quantitative real-time PCR (qRT-PCR) assays using glyceraldehyde-3-phosphate dehydrogenase as a reference gene were done. Total RNA was isolated using the same method as described above for the gene expression analysis by microarray. cDNA was synthesized using the SuperScript III first-strand synthesis supermix for qRT-PCR (Invitrogen). Samples for the qRT-PCR were prepared using Platinum SYBR Green qPCR SuperMix-UDG with Rox (Invitrogen) and run on the StepOnePlus Real-Time PCR System (Applied Biosystems) using a thermal profile of an initial 2-minute UDG incubation step at 50°C and 2-minute melting step at 95°C, followed by 40 cycles at 95°C for 20 seconds and 55°C for 40 seconds. The primers used for analysis by qRT-PCR are listed in Supplementary Table S1. To verify the presence of only one amplicon, a melting curve was processed after each run. After normalization with glyceraldehyde-3-phosphate dehydrogenase expression, regulation was calculated between treated and untreated cells. All reactions were carried out at least twice in triplicate.

Statistical analysis

Synergism, additive effects, or antagonism was assessed using the Chou-Talalay method as described previously (18). The combination index (CI) for each experimental combination was calculated according to the following equation: $CI = (D)_1/(D_x)_1 + (D)_2/(Dx)_2 + (D)_1(D)_2/(D_x)_1(Dx)_2$, where $(D)_1$ and $(D)_2$ are the doses of drug 1 and drug 2 that have x effect when used in combination and $(Dx)_1$ and $(Dx)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone. The combination is additive when CI = 1, synergistic when CI < 1.0, and antagonistic when CI > 1.0.

Statistical significance levels were determined by two-tailed *t* test analysis. *P* values of <0.05 were considered significant.

Results

RITA induces growth inhibition of MM cells harboring wild-type p53

We first did a dose-dependent killing of MM cells to determine the optimal concentration of RITA needed to achieve maximal apoptosis in different cell lines treated with RITA for 48 hours. The results obtained from four different cell lines of different p53 status are depicted in Fig. 1. A decrease in cell proliferation of MM cells was observed at doses starting from 0.25 μmol/L in MM.1S and 0.5 μmol/L in H929 cells (Fig. 1B). MM.1S cells were sensitive to low doses of RITA, reaching >50% inhibition of growth at 1.0 μmol/L, whereas a similar concentration caused 30% inhibition of growth of H929 cells. Thus, cell lines harboring wild-type p53 were clearly sensitive to MDM2 inhibition. In contrast, cell lines harboring mutant p53 or null p53 hardly responded to RITA (Fig. 1C).

RITA induces apoptosis in MM cells harboring wild-type p53

Having shown the dose-dependent decline in the survival of wild-type p53-expressing MM cells, we next examined whether the inhibition of survival is due to apoptosis induced by RITA. MM cells were treated with various concentrations of RITA. After 48 hours, apoptosis was assessed by Annexin V flow cytometry. Both MM.1S and H929 cells harboring wild-type p53 showed a dose-dependent induction of apoptosis induced by RITA. Interestingly, RITA showed relatively stronger inhibition of cell survival in MM.1S cells than in H929; however, it induced more apoptosis in H929 cells than in MM.1S cells as measured by Annexin V binding. Importantly, there was only a modest induction of apoptosis by RITA in the cells that do not express p53 (8226R5) or express mutant p53 (U266; Fig. 1D).

RITA induces activation of the p53 pathway in MM cells in a time- and dose-dependent manner

To examine RITA-induced cell signaling pathways, Western blot analysis was done using total cell lysates derived from MM.1S and H929 cells treated with various concentrations of RITA for various time periods. p53 levels began to increase after 4 hours of RITA treatment, remained high up to 12 hours, and then declining by 24 hours, indicating p53 protein is upregulated following sustained p53 activation and subsequently undergoes degradation.

Our data indicated that apoptosis was induced by activation of the p53 pathway. Next, we examined the effects of RITA in regulating the expression of downstream effectors of p53 including MDM2, p21, NOXA, Mcl-1, and Bcl-2. Although the expression of p53 was upregulated, p21 and MDM2 was downregulated over time. The level of MDM2 and p21 decreased rapidly after treatment, between 6 and 8 hours of treatment with RITA (Fig. 2A and B). Exposure to RITA of MM.1S cells (Fig. 2A) or H929 cells (Fig. 2B) caused an early increase in NOXA levels after 4 to 6 hours of exposure, which further increased in a time-dependent manner of up to 24 hours poststimulation. RITA also induced downregulations of antiapoptotic proteins such as Mcl-1 and Bcl-2; however, the downregulation of Mcl-1 was evident earlier than that of Bcl-2. For example, Mcl-1 protein levels started to decline as early as 6 hours, whereas the decrease in Bcl-2 was clearly evident at 12 to 24 hours. No significant changes in the Bcl-2 expression were found at 6 hours poststimulation. Consistent with the upregulation of p53, cleavage of caspase-8, caspase-3, and PARP occurred in both MM.1S (Fig. 2A) and H929 (Fig. 2B) cell lines within 6 to 12 hours after treatment with RITA. These data suggest that p53 induced by RITA was functionally active.

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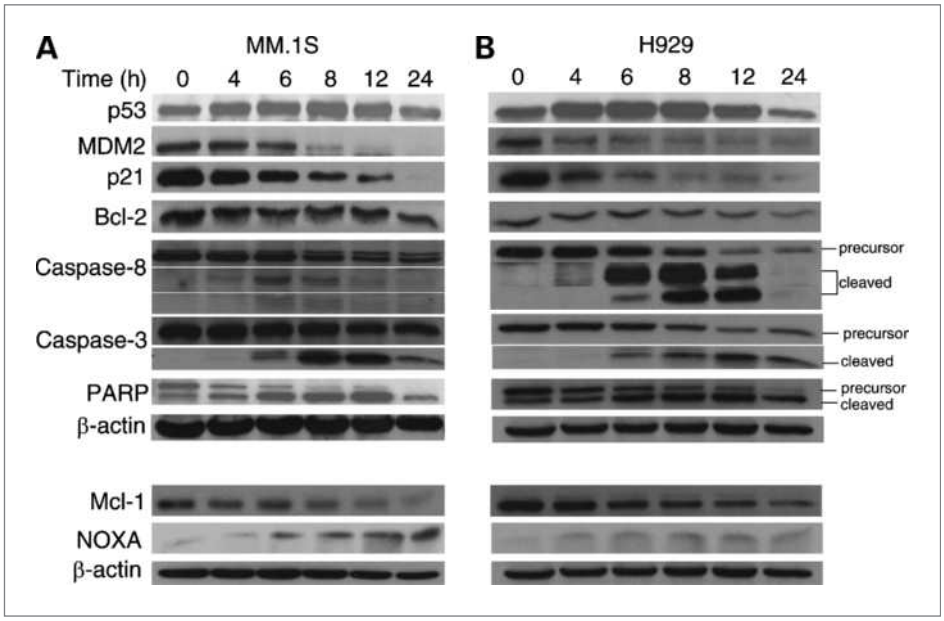

Figure 2. RITA induces activation of the p53 pathway in MM cells in a time-dependent manner. MM.1S (A) and H929 (B) cells were treated with 0.5 and 2.5 μmol/L RITA, respectively, at different time periods (4–24 h). Total cell lysates were prepared at the indicated hours after treatment of the cells with RITA and analyzed by Western blot for the indicated proteins. Data shown are representative of three independent experiments.

Having shown that RITA activated the p53 pathway in MM cells as early as 6 hours, we further examined the dose responses for the activation of p53 in MM cells. Depending on the IC_{50} observed in the cytotoxic response to RITA in various MM cell lines, cells were treated with various concentrations of RITA. After 6 hours, cells were lysed, and Western immunoblotting of these cells was done. The combined results obtained from 8266R5 (p53 null), U266 (mutant p53), and the wild-type p53-expressing MM.1S and H929 cells are presented in Fig. 3. RITA induced dose-dependent increases in the level of p53 and NOXA and a decrease in p21, MDM2, and Mcl-1 levels in MM.1S and H929 cells (Fig. 3A and B). In contrast, treatment of p53 null or mutant cell lines with RITA did not show any changes in the expression of p53, p21, or MDM2 proteins or cleavage of PARP (Fig. 3C and D).

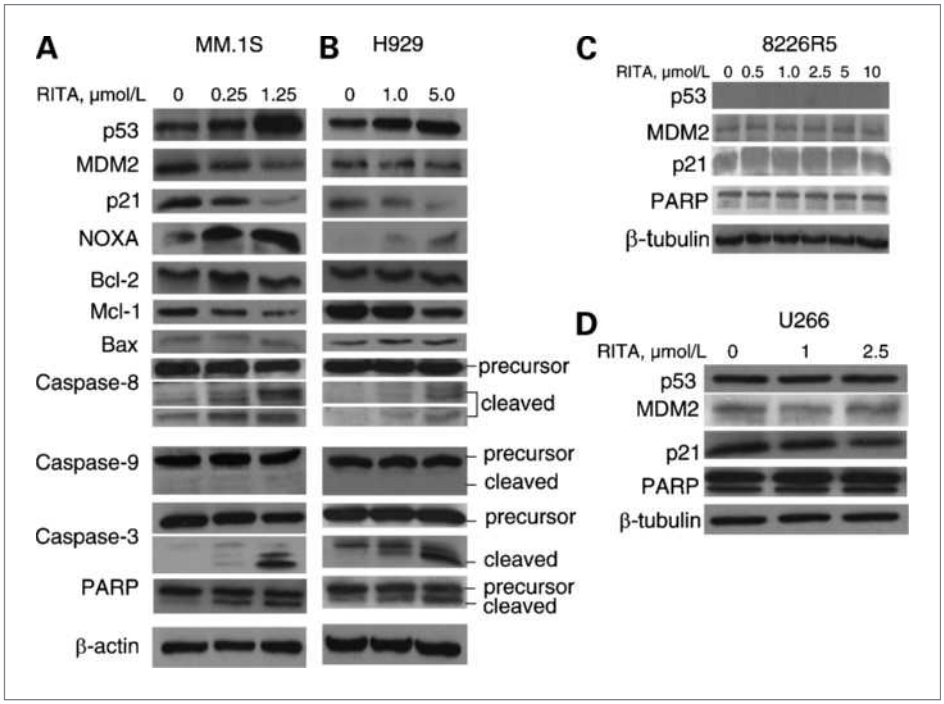

Figure 3. RITA induces p53-dependent activation of the p53 pathway in MM cells in a dose-dependent manner. MM.1S (A), H929 (B), 8226R5 (C), and U266 (D) cells were treated with RITA for 6 hours at various concentrations (0.25–10 μmol/L). Total cell lysates were prepared and analyzed by Western blot for the expression of p53 and its transcriptional targets as well as apoptosis-associated proteins. Data shown are representative of three independent experiments.

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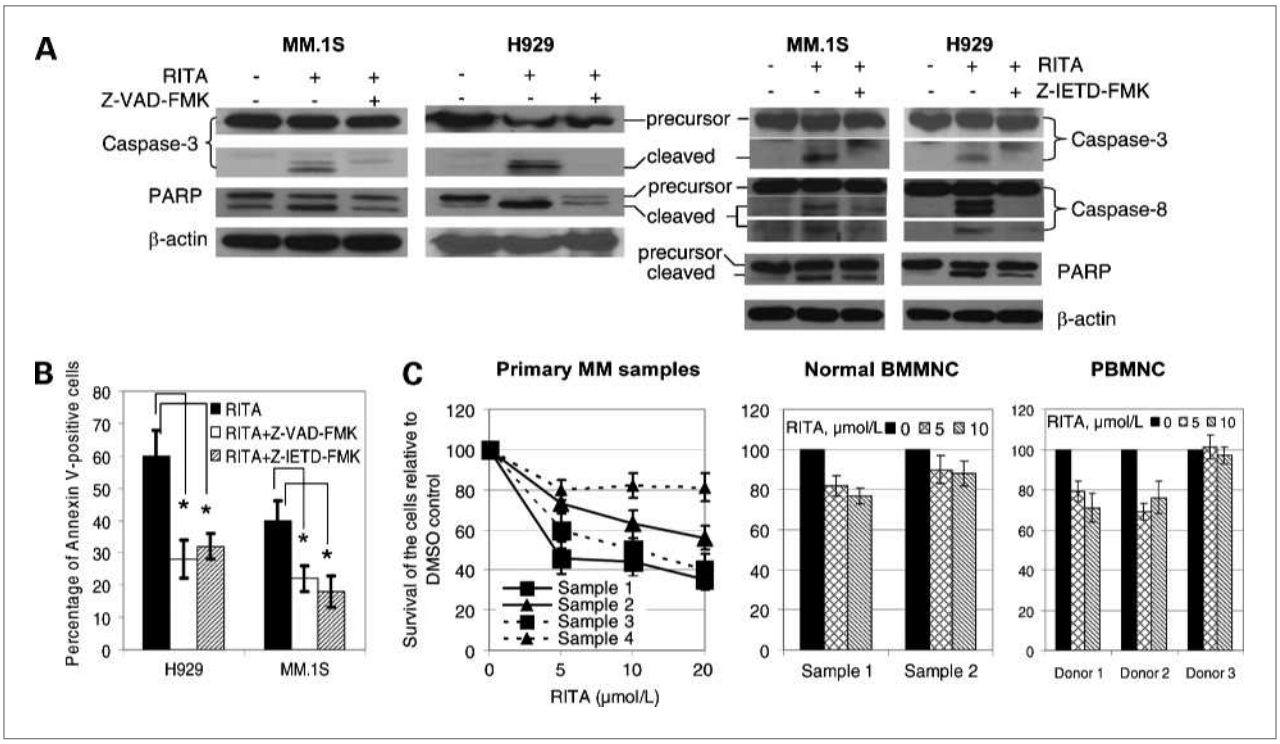

Figure 4. RITA induces caspase-dependent apoptosis in MM cells harboring wild-type p53. MM.1S or H929 cells were treated with 20 μmol/L pan-caspase inhibitor, Z-VAD-FMK, or 100 μmol/L caspase-8 inhibitor, Z-IETD-FMK, before treating the cells with RITA. A, after 6 h, cells were lysed and analyzed by Western blot for the expression of caspase-3 and/or caspase-8 and PARP. B, apoptosis was measured by flow cytometry in MM.1S and H929 cells treated with RITA in the presence or absence of Z-VAD-FMK or Z-IETD-FMK. Each column represents the mean of apoptotic cells (Annexin V–positive cells) of three independent experiments after normalizing the data with vehicle treatment, and each bar represents SD. *, significance at $P < 0.02$ by two-tailed t test. C, primary MM samples from four MM patients or BMMNC from two healthy volunteers were grown in normal growth medium. PBMNCs from three healthy volunteers were grown in the presence of 1% phytohemagglutinin. Cells were treated with escalating doses of RITA (0–20 μmol/L), and after 48 h, cell viability was measured by MTT assay.

RITA induces myeloma cell apoptosis through an extrinsic signaling pathway

To gain insight into the apoptotic mechanism of RITA-induced apoptosis in MM cells, we further tested whether RITA had an effect on caspase activation. Because caspases are proteolytically cleaved and activated during stress-induced apoptosis in MM cells, we attempted to discern which caspases are activated in apoptotic signaling. Monitoring cleavage of caspases by Western blot analysis revealed that RITA enhanced processing of caspase-3, caspase-8, and PARP in both MM.1S and H929 cells in a time- and dose-dependent manner (Figs. 2 and 3). In cells exposed to the solvent alone (DMSO), these caspases were present predominantly as their zymogens. Following exposure to RITA, caspase-8 was processed to its p40/p23 fragments between 6 and 8 hours posttreatment. Cleavage of caspase-3 to its p17 fragment started as early as 6 hours and continued to increase up to 12 hours (Fig. 2A and B).

Exposure to RITA also resulted in a dose-dependent processing of caspase-8 and caspase-3, but not caspase-9, at 6 hours poststimulation (Fig. 3A and B). Importantly, although at 6 hours of treatment there were significant differences in the expression of cleaved caspase-8 and caspase-3, there were no noticeable differences in the Bcl-2 and Bax expression in these cells between RITA-treated and nontreated samples at this time. These results suggest RITA-induced apoptosis in MM cells may occur by extrinsic pathways.

The caspase-dependent mechanism of apoptosis and an association of death receptor pathway were further confirmed by treating MM cells with a pan-caspase inhibitor, Z-VAD-FMK, or a specific inhibitor of caspase-8, Z-IETD-FMK, before treating the cells with RITA. Pretreatment of the cells with Z-VAD-FMK or Z-IETD-FMK significantly inhibited caspase-3 or caspase-8 cleavage, respectively (Fig. 4A). In addition, cleavage of caspase-3 was also inhibited by Z-IETD-FMK. Importantly, apoptosis induction by RITA in MM.1S and H929 cells was inhibited by these inhibitors, which was observed by inhibition of cleavage of PARP as evident by Western blot analysis (Fig. 4A) and decreased amount of Annexin V–positive cells as evident by flow cytometry (Fig. 4B). These results indicated that RITA-mediated apoptosis was a downstream event following caspase activation, and the apoptosis is mediated mainly

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by death receptor extrinsic pathway through activation of caspase-8.

RITA induces preferential killing of MM cells

We also assessed the effects of RITA on the viability of primary MM cells and normal hematopoietic cells. MM cells obtained from patients with MM and normal mononuclear cells obtained from BM aspirates or peripheral blood of healthy volunteers were treated with RITA and then assayed for survival. RITA elicited a significant inhibitory effect on the growth of the cells in three of four primary MM samples in a dose-dependent manner. In contrast, RITA had only a weak inhibitory effect on the survival of the BMMNCs or PBMNCs (Fig. 4C). Thus, inhibition of the p53-MDM2 interaction by RITA is preferentially cytotoxic to malignant cells.

MDM2 inhibition by RITA and nutlin induces synergistic killing of MM cells and primary MM samples

We and others previously showed that nutlin induced growth inhibition of MM.1S and H929 cells over a dose range of 1 to 10 μmol/L. In this study we hypothesized that inhibition of the growth and subsequent induction of apoptosis can be further increased by combining two small molecule MDM2 inhibitors, nutlin and RITA. We evaluated the synergistic effect of nutlin (2–6 μmol/L) and RITA (0.25–6 μmol/L) in MM cell lines that were sensitive to both drugs. In addition, we further evaluated effects of this combination in primary MM samples. The results are depicted in Fig. 5. Treatment of MM.1S and H929 cells with RITA alone or nutlin alone for 48 hours resulted in 60%–70% inhibition of cell growth at the highest concentrations of both drugs (6 μmol/L nutlin and 1 μmol/L RITA for MM.1S or 6 μmol/L RITA for H929). In contrast, the combination of the two drugs was clearly synergistic in all but one combination tested in these two cell lines, with a maximal growth inhibition obtained of 90% (Fig. 5A). The CI value calculated from the value of percentage growth inhibition confirmed the synergistic responses of these two drugs. For example, in MM.1S cells, the CI value obtained for nutlin plus RITA combination of doses (2 + 0.25), (4 + 0.25), and (4 + 0.5) are 0.56, 0.79, and 0.79, respectively (Fig. 5A). The CI value obtained in H929 cells for nutlin + RITA combination of doses in (2 + 1.5), (4 + 3), and (6 + 6) are 0.73, 0.82, and 0.79, respectively (Fig. 5A). Similar results were obtained for one of the two primary MM samples, shown in Fig. 5B, with 90% growth inhibition with the combination of both drugs, compared with 10% and 50% inhibition with nutlin (5 μmol/L) alone or RITA (10 μmol/L) alone. Thus, even in relatively resistant primary MM cells, the combination of nutlin with RITA showed synergism with an improved ability to inhibit growth.

The combination of RITA and nutlin was at least additive in the induction of apoptosis in both MM.1S and H929 cells (Fig. 5C). Following 48 hours of treatment of H929 cells, a mean percentage apoptosis 52% ± 8.5% was obtained for the combination of RITA (1.5 μmol/L) plus nutlin (4 μmol/L), whereas a mean of 18% ± 3.8% and 35% ± 7% was obtained for RITA alone and nutlin alone, respectively. Similar results were obtained for MM.1S cells with 45% ± 7.6% apoptosis with both drugs following 48 hours treatment, compared with a maximum of 28% ± 7% and 28% ± 5.8% apoptosis with RITA (0.5 μmol/L) or nutlin (4 μmol/L) alone, respectively.

Gene expression profiling by microarray and validation by qRT-PCR

In an attempt to identify the differentially expressed genes between RITA-treated and nontreated MM cells, we did genome-wide expression profiling, which was

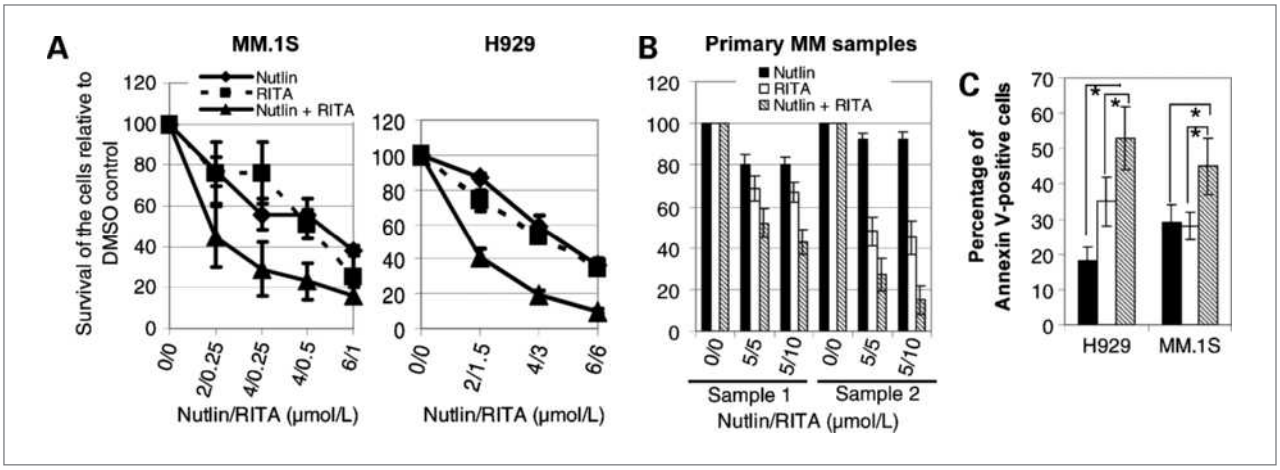

Figure 5. RITA and nutlin display synergistic cytotoxic effect in MM cell lines (A) and in primary MM samples (B). MM cells were seeded at 20 × 10^4/mL and were treated simultaneously with escalating doses of RITA and nutlin. After 48 h, cell viability was measured by MTT assay. CI value was calculated as described in Materials and Methods. C, a combination of RITA plus nutlin enhances killing of MM cells at 48 h as measured by flow cytometry. Graph represents data from three independent experiments. *, significance at $P < 0.05$ by two-tailed t test. Filled, empty, and hatched columns represent the percentage of apoptosis in nultin-, RITA-, or nutlin plus RITA–treated cells, respectively.

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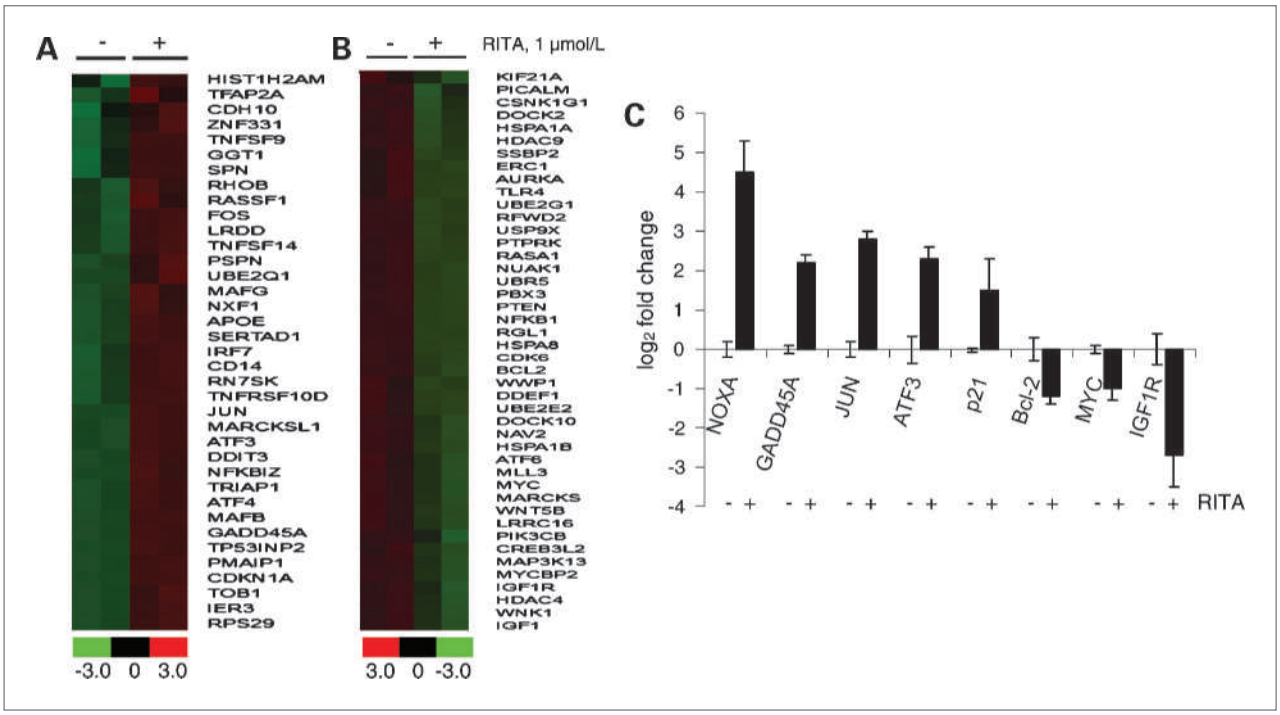

Figure 6. RITA induces transcriptional regulation of a set of p53 target genes. A and B, microarray analysis of gene expression in MM.1S cells after 6 h treatment with RITA. The heat map of genes differentially expressed in MM cells. Vertical rows indicate separate arrays, and horizontal rows indicate genes. Values are normalized to untreated control. Green indicates low expression; red indicates high expression. C, mRNA levels of oncogenes were detected by qRT-PCR in MM.1S cells 6 h after treatment with 1 μmol/L RITA. Values were normalized to those obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as $\log_2$-fold induction over DMSO-treated cells (mean ± SD).

further validated by qRT-PCR. In agreement with the antiproliferative effect of RITA, transcription of inhibitory genes such as GADD45A (growth arrest and DNA damage–inducible α) and CDKN1A/p21 (cyclin-dependent kinase inhibitor 1A) was increased (Fig. 6A). In addition to the regulation of genes involved in proliferation control, we also observed regulation of apoptosis-linked genes in a proapoptotic manner, indicating that RITA could sensitize cells for apoptotic events. An increase in apoptotic events would also contribute to the decrease in cellular proliferation observed. In cells treated with RITA the transcript of the antiapoptotic target Bcl-2 was downregulated (Fig. 6B), whereas genes coding for apoptosis-inducing and -supporting products (PMAIP1/NOXA and TP53INP2) were increased (Fig. 6A). GADD45A and p21 are also connected with apoptosis in addition to their role in proliferation or cell cycle control. In addition, upregulation of genes considered transcriptional factors, such as JUN, activating transcription factor 3 (ATF3), ATF4, and DNA damage–inducible transcript 3 (DDIT3 = CHOP = GADD153), and downregulation of MYC and insulin-like growth factor-I receptor (IGF-IR) molecules involved in proliferative cell signaling was also observed (Fig. 6B).

For validation of certain key findings in the microarray data, RT-PCR was done for eight selected genes that may play significant roles in p53-mediated apoptosis of MM cells (Fig. 6C). qRT-PCR confirmed the transcriptional induction of NOXA, GADD45A, JUN, ATF3, and p21 and transcriptional repression of Bcl-2, MYC, and IGF-IR. These results paralleled the mRNA expression levels acquired via microarray analysis. For example, NOXA expression was generally highest among the upregulated genes, whereas IGF-IR showed maximum downregulation (Fig. 6C). Our results suggest that pharmacologically reactivated p53 acts as a potent repressor of a number of oncogenic and survival factors and functions as a powerful trigger of proapoptotic proteins.

Discussion

In this study, we have shown that RITA exerts an antiproliferative and apoptosis-inducing effect on MM cell lines at nanomolar levels, along with associated molecular changes in the p53 activation pathway. Consistent with our findings, RITA inhibited cell growth and induced cell death in solid tumors (20, 26, 27) and leukemic cells from patients with acute myelogenous leukemia and chronic lymphocytic leukemia (31) in a p53-dependent manner. This might be due to an increased half-life of p53 (20, 33). Potent cytotoxic activity of RITA was observed by MTT assay in some primary MM cells, suggesting that freshly isolated myeloma cells could also be sensitive to RITA. Importantly, RITA showed only a minimal inhibitory effect on survival of normal BMMNCs or phytohemagglutinin-stimulated, proliferating healthy peripheral blood mononuclear cells compared

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with MM cells, suggesting a preferential cytotoxic killing of tumor cells by RITA.

Our time course analysis done on RITA treatment showed that induction of p53 was associated with a decrease in MDM2 and p21 expression. Increased levels of p53 in response to RITA correlated with the induction of apoptosis reported earlier (31). Activation of the p53 apoptotic pathway in MM cells was further documented by the upregulation of NOXA and GADD45A, which was confirmed and verified by microarray and qRT-PCR. RITA-activated p53 unleashes the transcriptional induction of proapoptotic proteins such as NOXA and transcriptional repression of the antiapoptotic proteins Mcl-1 and Bcl-2. These results suggest that, following exposure to RITA, induction of NOXA and repression of Mcl-1 were dependent on transcription. Similar to our results, an important role of NOXA and Mcl-1 in p53-mediated apoptosis of leukemic and myeloma cells was described in two recent studies (34–36). Mcl-1 is one of the key proteins in regulating MM cell survival. Using antisense approaches, it has been shown that only Mcl-1 but not Bcl-2 antisense triggered a rapid induction of apoptosis. Thus, the role of Mcl-1 is distinguished from that of the other antiapoptotic members such as Bcl-2 (37) in MM.

In line with our results on the differential regulation of p21 transcription by RITA and nutlin, we found that p21 protein levels were regulated differently by these two p53 activators. In MM cells treated with nutlin, the immediate p53 downstream target p21 was upregulated both at the mRNA and protein levels (17, 18). Whereas in the current study, induction of p21 was only found at its mRNA level, the protein levels of p21 decreased on RITA treatment. A possible explanation is that p21 is a transcriptionally and posttranscriptionally regulated gene; therefore, the induction of gene expression presented in Fig. 6C did not correlate with its protein level (Figs. 2A and B and 3A and B). Of note, a previous report on RITA-induced acute myelogenous leukemia/chronic lymphocytic leukemia cells (31) did not study changes in p21 and MDM2 expression. Nevertheless, the downregulation of p21 and MDM2 in myeloma cells in the current study is consistent with the results by Rinaldo et al. (25) and Enge et al. (26), in which p21 and MDM2 protein levels in breast and colon carcinoma and sarcoma cells were decreased on RITA treatment. This may be explained by the fact that while binding with p53 RITA frees MDM2 that promotes p21 degradation (26). In addition, the downregulation of p21 on RITA treatment has been shown to play a critical role in regulating the switch from cell cycle arrest to apoptosis (26). Further studies are required to verify and elucidate the mechanisms of RITA-induced MDM2-mediated degradation of p21 in MM cells.

p53-mediated apoptosis can be examined for multiple molecular mechanisms that may engage the extrinsic/receptor-mediated and/or the intrinsic/mitchondria-associated pathways, depending on the functionality of p53 in MM cells. We recently reported that nongenotoxic small molecule inhibitors, such as nutlin, can engage both the intrinsic and extrinsic apoptotic pathways (17, 18). Here, we showed that activation of caspase-8 and caspase-3 was significantly inhibited by the pan-caspase inhibitor Z-VAD-FMK and the specific caspase-8 inhibitor Z-IETD-FMK. Therefore, RITA-induced apoptosis in MM cells is mediated predominantly by extrinsic apoptotic signaling. The functional linkage between RITA-induced caspase activation and apoptosis was shown in our study by the ability of the caspase inhibitors to significantly diminish cleavage/activation of caspases and PARP and subsequently inhibit apoptosis in RITA-treated MM cells.

It has been shown that both RITA and nutlin block the MDM2-mediated ubiquitination and proteasomal degradation of p53 (38). We recently showed that nutlin and velcade displayed synergistic cytotoxic activity in MM cells (18). A synergistic effect is expected if two agents target the same pathway through different but complementary mechanisms. Here, we showed that RITA plus nutlin combination displays a synergistic cytotoxic response in MM cells. The synergistic activity might be due to the strong inhibition of the p53-MDM2 interaction when it is blocked by binding of nutlin with MDM2 in its p53 binding pocket combined with direct binding of RITA to p53. Combination of nutlin and RITA resulted in a further increase in the upregulation of NOXA in both MM.1S and H929 cells, which was consistent with the cyto toxic or apoptotic responses of these two drugs. However, because nutlin upregulates p21 and MDM2, whereas RITA downregulates these two proteins, as expected, the expression levels of p21 and MDM2 were not further altered when the cells were treated with nutlin and RITA in combination (data not shown). Recently, an additive response of nutlin and RITA was described by Rinaldo et al. (25) in sarcoma cell lines in which the response of nutlin and RITA were dependent on the sequential addition of nutlin and RITA. Our results showed that the synergistic effect of nutlin and RITA can be obtained by simultaneous addition of lower concentrations of RITA and nutlin, which may decrease the nonspecific toxicity of the drugs. Further work aimed at detailed characterization of molecular events on p53 activation might help to guide rational development of more efficient and less toxic drug combinations.

Using global gene expression profiling, we identified a significant number of p53 target genes, which were induced or repressed on RITA treatment. The changes in the mRNA expression of some of the selected genes were further validated by qRT-PCR. Similar to our findings, RITA-induced upregulation of NOXA, GADD45A, and p21 and downregulation of Myc, Bcl-2, and IGF-IR have been reported in colon and breast carcinoma cells (27). In addition, we identified some p53 target genes, which may provide the clue to determine the pathways followed by RITA-induced apoptosis. For example, upregulation of DDIT3, ATF3, and ATF4 is associated with endoplasmic reticulum stress–induced apoptosis (39, 40).

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Therefore, RITA-induced upregulation of DDIT3, ATF3, JUN, and NOXA may be a consequence of endoplasmic reticulum stress induction and activation of p53, but this remains to be further investigated.

In conclusion, we have provided evidence that RITA induces cytotoxic activity against MM through activation of the p53 pathway. Furthermore, RITA and nutlin in combination display a synergistic response for killing of MM cells. The fact that freshly isolated myeloma cells have increased susceptibility to the combination of nutlin and RITA suggests that our findings are likely to be clinically relevant and that these two drugs may work in a similar way *in vivo*. Given the low toxicity of nutlin and RITA as well as the synergy observed between these two drugs, our data provide the rationale for the clinical evaluation of RITA, both as a single agent and in combination with other available novel targeted therapeutics such as nutlin in the treatment of MM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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