Characterizing the Individual and Combined Effect of Rapamycin and MS-275 on U266, a Multiple Myeloma Cell Line

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Multiple myeloma, an incurable clonal B-cell malignancy, is the second most common hematologic malignancy in the United States, afflicting close to 20,000 new people per year. It results in lytic bone disease, renal insufficiency, and immunodeficiency; symptoms include fatigue, bone pain, and recurrent infections. As in all cancers, tumor progression is the result of genetic mutations, epigenetic modifications, and allelic variation. With these considerations in mind, and given the current use of mammalian target of rapamycin (mTOR) and histone deacetylase (HDAC) inhibitors in clinical trials, we attempted to evaluate the anti-cancer properties of the mTOR inhibitor rapamycin and the HDAC inhibitor MS-275, alone and in combination. We treated U266, a multiple myeloma cell line, with graded concentrations of these drugs for 24 and 48 hours and examined the effects on cell proliferation, cell cycle progression, apoptosis, and gene expression. In the cellular proliferation studies, rapamycin had a cytostatic effect over a broad range of concentrations (0.001-1.0 μM) while MS-275 had a dose-dependent effect over a higher range of concentrations (0.5-2.0 μM). Fifty percent inhibition of cell growth was achieved at nanomolar concentrations (<0.1 μM) of rapamycin and micromolar concentrations (1.0-2.0 μM) of MS-275; combining the two drugs yielded a synergistic effect. Results from the cell cycle analysis show an increase in G_0/G_1 arrest after treatment with both drugs, with a high degree of G_0/G_1 arrest at nanomolar concentrations of rapamycin. Individual and combination treatments with the drugs induced apoptosis in as much as 37% of the cells over a 48-hour period. Quantitative real-time PCR data suggests that MS-275 increases p21 expression while decreasing cyclin D1 expression; importantly, rapamycin yielded opposing results by decreasing p21 expression while increasing cyclin D1 expression. Western blotting shows that MS-275 increases acetylation of histones H3 and H4 while rapamycin significantly reduces phosphorylation of the S6 protein. Together, these data suggest that the drugs work synergistically through distinct mechanisms. As both rapamycin and MS-275 show powerful anti-cancer properties, these experiments should provide a framework for future *in vitro* and *in vivo* studies.

INTRODUCTION

The mammalian target of rapamycin (mTOR) plays a crucial role in regulating critical aspects of cell growth, including cell cycle progression, membrane trafficking, protein degradation, and protein kinase C signaling and transcription[1]. Rapamycin, an mTOR inhibitor that has been widely tested *in vitro* and *in vivo*, is a macrocylic lactone taken from *Streptomyces hygroscopius*, a soil bacterium from Rapa Nui (Easter Island); rapamycin is known for its fungicidal, immunosuppressive, and antiproliferative properties[2]. Rapamycin functions by binding to FK506-binding protein (FKBP12), which is ubiquitously expressed; the rapamycin-FKBP12 complex then binds to the FKBP12-rapamycin-binding (FRB) binding domain near the kinase domain of mTOR, subsequently inhibiting mTOR's function[3,4]. As inhibition of mTOR could lead to the subsequent inhibition of growth, it is easy to see the potential therapeutic power of this drug.

Preclinical studies indicate that rapamycin can potentially be used as treatment for multiple myeloma (as well as a considerable number of other human cancer cell lines,

including those derived from osteosarcoma, neuroblastoma, breast cancer, glioblastoma, small-cell lung cancer, Ewing sarcoma, pancreatic cancer, leukemia, and prostate cancer[5]). Multiple myeloma cell lines have been shown to exhibit both dose-dependent and cytostatic responses to treatment with rapamycin. Experimentation also confirms that growth inhibition works in an expected and specific manner, as rapamycin treatment does not affect Akt phosphorylation but can completely inhibit $P70^{S6K}$ phosphorylation[6]. Other studies have shown that rapamycin induces G_0/G_1 cell cycle arrest in some cells by delaying the accumulation of cyclin D1 mRNA during progression through G_1 and by accelerated degradation of synthesized cyclin D1 protein[7]. Three of rapamycin's analogues (CCI-779, RAD001, and AP23573), which have more favorable pharmacokinetic properties due to increased water solubility and stability in solution[8,9], are currently in varying phases (I-III) of clinical trials, and are being tested on a range of malignancies, including advanced solid cancers, mantle cell lymphoma, and advanced sarcomas. Results are promising; for example, AP23573 has shown clinical efficacy (e.g., 56% clinical improvement) and low toxicity profiles in patients[10].

Histone acetylation affects transcriptional activity and repression through hyperacetylation and hypoacetylation, respectively. These states are controlled by the balance of activity between histone acetyltransferases (HATs) and histone deacetylases (HDACs)[11,12,13]. Ultimately, histone acetylation by HATs leads to the neutralization of the positive charge of lysine, decreasing the interaction between the histone and the DNA, a conformational change which gives molecules like RNA polymerase, transcription factors, and regulatory complexes greater access to the DNA[14,15]. This leads to an increase in transcription of a specific set of genes.

Since carcinogenesis is frequently associated with the repression of tumor suppression genes, HDAC inhibitors can de-repress these genes and allow growth inhibition and antitumor activity[16,17]. Other biological consequences of HDAC inhibition include cell differentiation, cell cycle arrest, apoptosis, cytoskeletal alterations, and angiogenesis[18]. Microarray analyses suggest that HDAC inhibition works rather specifically on cancer cell lines, affecting as few as 1-2% of genes regulated in both normal and cancer cells within the initial hours of treatment. These analyses suggest that a core set of genes affected by HDAC inhibitors specifically affect cell cycle progression, DNA synthesis, and apoptosis[19].

MS-275, an active benzamide derivative[20], is one such HDAC inhibitor, and is currently in clinical trials[21]. Previous studies indicate that MS-275 induces a dose-dependent response in cancer cells[22]. At lower concentrations (1 μM), MS-275 showed potent antiproliferative activity, partially through the induction of p21-mediated growth arrest, increased levels of hypoposphorylated retinoblastoma protein, and downregulated levels of proteins associated with the cell cycle, including cyclin D1. At higher concentrations (5 μM), MS-275 induced cell death in approximately 70% of cells within 48 hours[23].

While limited experiments have utilized an mTOR and HDAC inhibitor in combination, one such study found that temsirolimus combined with vorinostat have synergistic antiproliferative activity in mantle cell lymphoma cells by targeting apoptosis and autophagy[24]. Thus, further experimentation is needed to increase understanding of the mechanisms by which these drugs work, both alone and in combination.

MATERIALS AND METHODS

Cell lines and cell culture.

The human multiple myeloma cell line U266 was kindly provided by Dr. W. Michael Kuehl (Genetics Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen Corporation, Carlsbad, CA, USA) in a humid environment with 5% CO_2 at 37°C.

MS-275 and rapamycin.

MS-275 was obtained from Sigma Chemical Corporation (St. Louis, MO, USA) and rapamycin was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD, USA). The drugs were dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Corporation) at a concentration of 10 mM and were stored at -20°C until use; in the culture medium, final concentrations of the drugs ranged from 0.1-5.0 μM for MS-275 and 0.001-2.0 μM for rapamycin with a maximum of 0.2% DMSO.

In vitro cell proliferation assay.

50,000 U266 cells per 200 μl of media per well were cultured in a 96-well plate. Following the addition of varying concentrations of rapamycin and MS-275, alone or in combination, the cells were incubated for 24 or 48 hours. Cell proliferation was measured by adding 20 μl of the Cell Proliferation Reagent WST-1 (Roche Diagnostics, Mannheim, Germany) to the 200 μl of cell suspension. The absorbance was measured at 450 nm using a VERSAmax^{TM} tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA) and Softmax Pro software (Molecular Devices). Each measurement was made in quadruplicate and the mean value was determined.

Cell cycle analysis and detection of apoptosis.

U266 cells (2×10^6) were cultured in 6 mL of media in a 6-well plate. Following the addition of varying concentrations of rapamycin and MS-275, alone or in combination, the cells were incubated for 24 or 48 hours. Cells were harvested, washed three times with ice-cold phosphate buffered saline (PBS), and fixed with 70% ethanol overnight at -20°C. The cells were washed again with PBS and stained with propidium iodide (PI; BD Biosciences Pharminogen, San Diego, CA, USA). Cell cycle fractions were determined after incubation at room temperature for 30 minutes. Sample analysis was performed with a Becton Dickinson FACSCalibur machine and data were collected with CellQuest Pro (BD Biosciences) and further analyzed using Mod-Fit LT (Verity Software House, Inc., Topsham, ME, USA).

In addition to observing sub-G_0/G_1 cells by cell cycle analysis, as described above, apoptosis was confirmed with the Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen^{TM}/BD Biosciences). U266 cells (2×10^6) were cultured in 6 mL of media in a 6-well plate. Following the addition of varying concentrations of rapamycin and MS-275, alone or in combination, the cells were incubated for 24 or 48 hours. Cells were harvested, washed three times with ice-cold PBS, aliquoted into separate tubes, and

resuspended in BD Pharmingen Annexin V Binding Buffer. Cells were stained with Annexin V-PE and/or 7-AAD, incubated for 15 minutes in the dark, and analyzed with a Becton Dickinson FACSCalibur machine. Data were collected with CellQuest Pro (BD Biosciences). Viable cells were characterized as Annexin V and 7-AAD negative; cells in early apoptosis were characterized as Annexin V positive and 7-AAD negative; cells in late apoptosis (or already dead) were characterized as positive for both Annexin V and 7-AAD.

Quantitative RT-PCR.

We performed real-time quantitative RT-PCR for p21, cyclin D1, and β-actin using the Applied Biosystems 7500 Real Time PCR System (Foster City, CA, USA). U266 cells (2×10^6) were cultured in 6 mL of media in a 6-well plate. Following the addition of varying concentrations of rapamycin and MS-275, alone or in combination, the cells were incubated for 24 or 48 hours. Cells were harvested and washed three times with ice-cold PBS. Total RNA was extracted using Trizol and phenol:chloroform (Invitrogen); RNA concentration was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). cDNA was synthesized from 1 μg of total RNA for each sample by reverse transcription PCR using the TaqMan Reverse Transcription Reagents (Applied Biosystems) in a final volume of 25 μl. The reaction profile involved sequential incubation at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. Quantification of mRNA by quantitative real-time PCR was done using the SYBR Green PCR Master Mix (Applied Biosystems) and primers designed with Primer Express Software (Applied Biosystems). Data were collected with Applied Biosystems Sequence Detection Software.

Western blot analysis.

U266 cells (2×10^6) were cultured in 6 mL of media in a 6-well plate. Following the addition of varying concentrations of rapamycin and MS-275, alone or in combination, the cells were incubated for 24 or 48 hours. Cells were harvested and washed three times with ice-cold PBS. Whole-cell protein extracts were prepared by incubating the cells in RIPA buffer (Pierce Chemical, Rockford, IL, USA) for 60 minutes on ice and then centrifuging to remove cellular debris. The protein in the resulting supernatant was quantified by the bicinchoninic acid method (Pierce Chemical), diluted in SDS loading buffer, and heated for 3 minutes at 95°C. A total of 20 μg of protein was loaded onto 4-20% Tris-Glycine Gels (Invitrogen), transferred to a nitrocellulose transfer membrane (Invitrogen), immunoblotted with antibodies, and detected using SuperSignal West Dura Extended Duration Substrate (Pierce Chemical). Primary antibodies to β-actin, Phospho-S6 (Ser240/244), Acetyl-Histone H3 (Lys9/Lys14), and Acetyl-Histone H4 (Lys8) were obtained from Cell Signaling Technology (Beverly, MA, USA).

Calculation for synergism between drugs.

To determine whether the antiproliferative effect of the drugs in combination was additive or synergistic, we calculated the combination index (CI) of the drugs based on the following equation: $CI = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2$, where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have x effect 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; CI < 0.1 indicates very strong synergism; CI = 0.1 to 0.3 indicates strong synergism; and CI = 0.3 to 0.85 indicates synergism[25,26,27].

RESULTS

Rapamycin, MS-275, and combination treatments inhibit U266 proliferation.

We studied the antiproliferative effect of rapamycin and MS-275 on U266 cells after 24 hours and 48 hours of treatment (Figures 1A-B). Rapamycin demonstrated a cytostatic effect over a broad range of concentrations (0.001-1.0 μM) while MS-275 had a dose-dependent effect over a higher range of concentrations (0.1-5.0 μM). Fifty percent inhibition of cell growth (IC_{50}) was achieved after 48 hours at nanomolar concentrations of rapamycin (<0.1 μM rapamycin) and micromolar concentrations of MS-275 (1.0-2.0 μM).

Because these drugs work via distinct mechanisms, we assessed their potential for use in combination (Figure 1C). When examining the antiproliferative effect of these drugs, approximately 70% growth inhibition was seen at concentrations of 0.001 μM rapamycin and 0.5 μM MS-275. This level of growth inhibition was much higher than what we saw when either of the drugs were used alone. Our calculations indicate that these drugs have a synergistic effect (CI = 0.375) when used together.

Rapamycin and MS-275 induce cell cycle arrest on U266 cells.

To further characterize the effects of rapamycin and MS-275, alone and in combination, we performed a cell cycle analysis on the U266 cells after treating them for 24 and 48 hours (Figures 2A-B). Both rapamycin and MS-275 induced G_0/G_1 cell cycle arrest and decreased the percentage of cells in the S and G_2/M phases. In fact, after 48 hours of treatment, the percentage of cells in the G_0/G_1 phase went from 58.09% to as high as 85.18% with 0.01 μM rapamycin treatment, 89.86% with 1.0 μM MS-275 treatment, and 92.41% with a combination treatment of 0.01 μM rapamycin and 0.5 μM MS-275.

After observing a large population of sub-G_1 cells, we used Annexin V-PE/7-AAD staining to gauge the effect of rapamycin and MS-275 on apoptosis (Figures 3A-B). With a 0.01 μM rapamycin treatment, we saw the percentage of late-stage apoptotic cells increase from 0.66% to 9.25% after 48 hours. With a 5.0 μM MS-275 treatment, we saw the percentage of apoptotic cells increase from 0.27% to 37.42% after 48 hours. Though we did not extensively investigate the mechanism by which apoptosis was induced, we did notice increasing amounts of PARP cleavage in our Western blot analysis (data not shown).

Rapamycin decreases p21 expression while MS-275 increases p21 expression and decreases cyclin D1 expression.

We tested the mRNA expression of p21 and cyclin D1 using quantitative real-time PCR. To compare the relative mRNA expression between samples, we divided the amount of mRNA of the gene of interest by the amount of mRNA of β-actin expressed in the same sample. Our results indicate that rapamycin decreases p21 expression by less than a two-fold change (0.001 μM treatment of rapamycin) while MS-275 increases p21 expression by as much as a nine-fold change (2.0 μM treatment of MS-275) (Figure 3C).

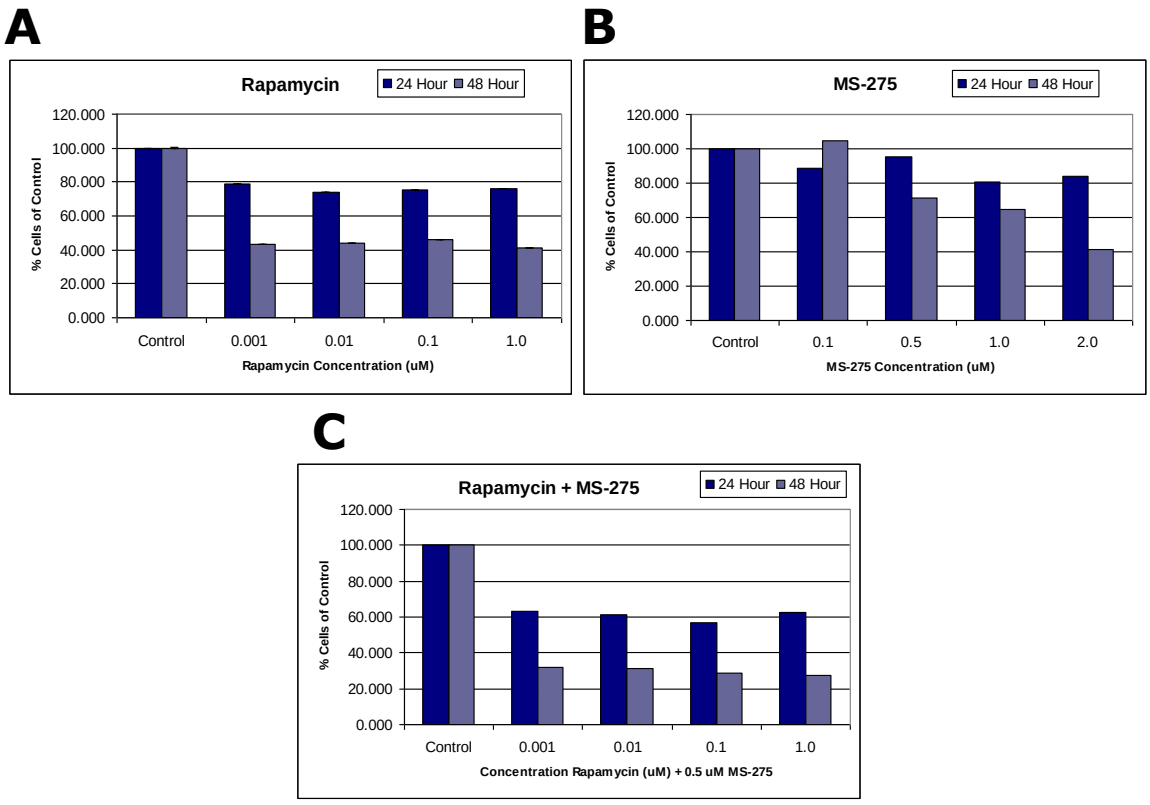

Figure 1. We treated U266 cells with rapamycin, MS-275, and combination for 24 or 48 hours. **(A)** Rapamycin had a cytostatic effect across a range of concentrations (0.001-0.01 μM) with a greater effect after 48 hours of treatment. **(B)** MS-275 had a dose-dependent effect across a higher range of concentrations (0.1-2.0 μM) with a greater effect after 48 hours of treatment. **(C)** We observed synergy (CI = 0.375) after combining a range of concentrations of rapamycin (0.001-0.01 μM) with the lowest effective dose of MS-275 (0.5 μM).

Finally, we found that increasing concentrations of MS-275 decreased the relative expression of cyclin D1, while rapamycin increased the relative expression of cyclin D1 (Figure 3D).

Rapamycin reduces S6 phosphorylation and MS-275 increases acetylation of histone H3 and histone H4.

We performed a western blot analysis to confirm that rapamycin and MS-275 work via their proposed mechanisms. Typically, mTOR phosphorylates $p70^{s6k}$, which subsequently phosphorylates S6. To assess mTOR function after inhibition with rapamycin, we probed for the phospho-S6 protein (Figure 4) and noted that the presence of phospho-S6 was dramatically reduced after treatment with rapamycin (alone and in combination).

Since MS-275 works by inhibiting histone deacetylases, we tested for changes in histone acetylation among MS-275 treated cells (Figure 4). We observed that increasing concentrations of both of these drugs resulted in an increase in acetylation of histone H3 and histone H4. Acetylation was most prominent with a treatment of 2.0 μM MS-275.

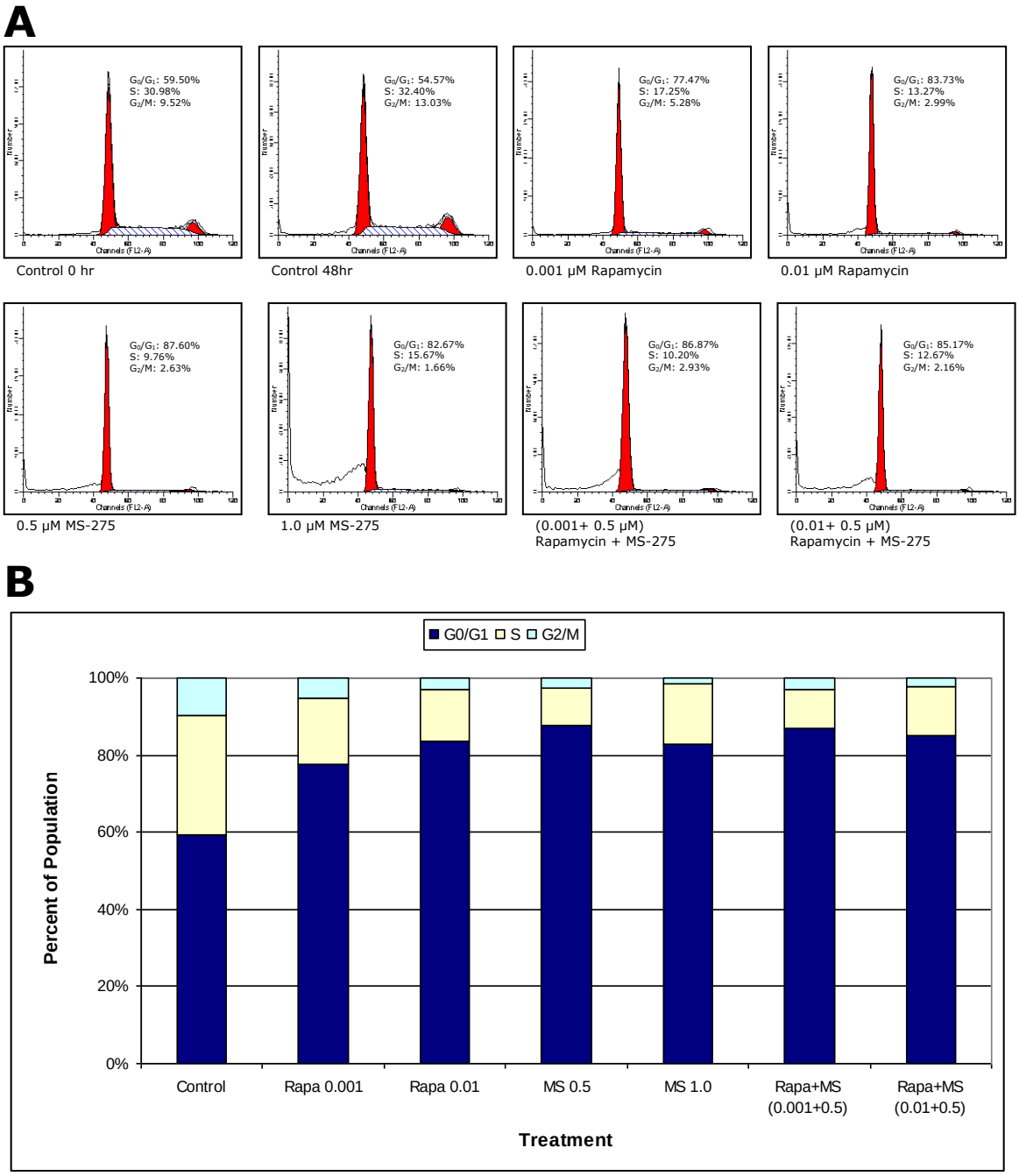

Figure 2. Treating cells with rapamycin and MS-275 led to G_0/G_1 arrest. **(A)** We incubated U266 cells with rapamycin, MS-275, and combination treatments for 48 hours before cell cycle fractions were determined by propidium iodide staining. Rapamycin and MS-275 both induced cell cycle arrest and apoptosis (sub-G_1 fraction). **(B)** The cell cycle fractions from a single experiment represented in graphical form. Rapamycin (Rapa), MS-275 (MS), and combination (Rapa+MS) treatments all increased the fraction of cells in G_0/G_1 while decreasing the S-phase and G_2/M fractions. Note that a treatment of 0.5 µM MS-275 induced the highest degree of cell cycle arrest.

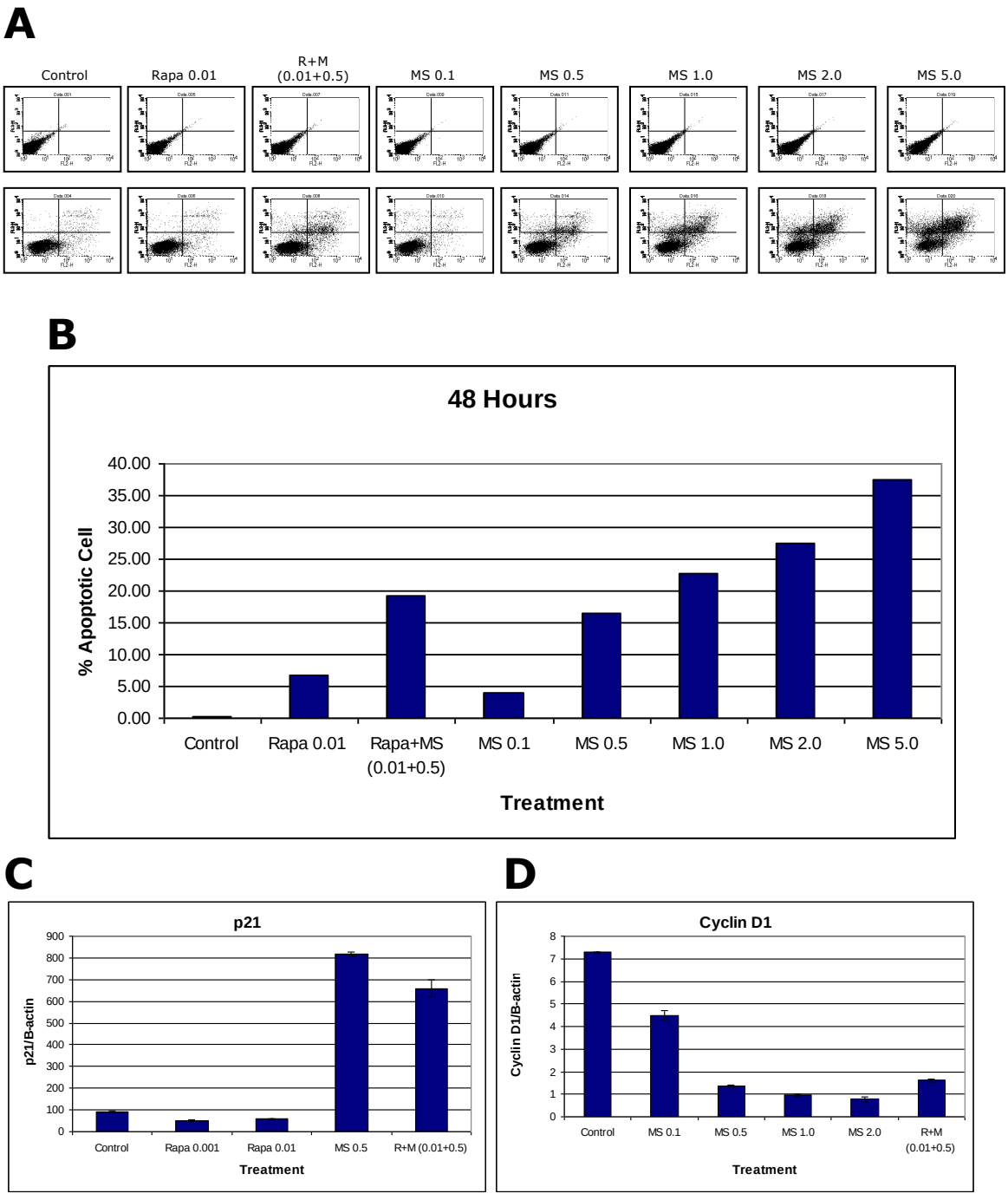

Figure 3. Treatment of U266 cells with rapamycin and MS-275 induced apoptosis and changes in gene expression. **(A)** After observing a large population of cells in the sub-G_0/G_1 phase, we tested for apoptosis using Annexin-VPE and 7-AAD staining (X axis, Annexin V-PE; Y axis, 7AAD). Each sample was measured unstained (top row of histograms) or stained (bottom row of histograms); cells in the lower right quadrant are in early-stage apoptosis and cells in the upper right quadrant are in late-stage apoptosis. **(B)** This graphical representation indicates the increasing levels of apoptosis after rapamycin (Rapa), MS-275 (MS), or combination (R+M) treatment. **(C)** We used quantitative real-time PCR to detect changes in p21 expression levels. p21 decreased after rapamycin treatments and increased after MS-275 treatments. **(D)** Treating U266 cells with increasing levels of MS-275 led to decreasing expression of cyclin D1. In a separate trial, rapamycin increased cyclin D1 expression (data not shown).

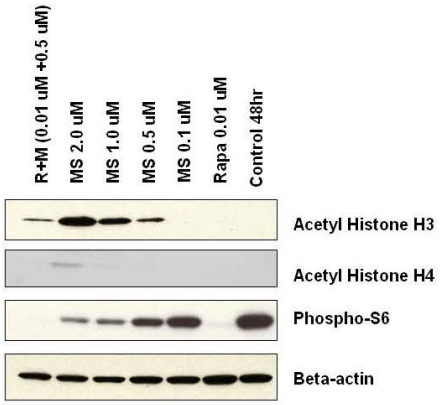

Figure 4. U266 cells were incubated with rapamycin (Rapa), MS-275 (MS), or combination (R+M) treatments for 48 hours. Increasing levels of acetyl-histone H3 and acetyl-histone H4 with increasing treatments of MS-275 indicate that the drug is successfully inhibiting the histone deacetylases, a process that leads to an increase in the transcription levels of specific genes. Decreased phosphorylation of S6 in cells treated with rapamycin (alone and in combination) indicates that the mTOR pathway has been blocked.

DISCUSSION

In this study, we examined the individual and combined effects of rapamycin and MS-275 on U266, a multiple myeloma cell line. Our study demonstrates that these drugs work synergistically together to inhibit cellular proliferation, primarily through G_0/G_1 cell cycle arrest and apoptosis.

Rapamycin demonstrated a cytostatic effect over a range of concentrations. This was associated with a high degree of cell cycle arrest and a decrease in relative cyclin-dependent kinase inhibitor p21 mRNA expression, as determined through quantitative real-time PCR. The decrease in p21 expression is consistent with data from other experiments[28,29,30] and several authors have suggested that this may be an important event after treating tumor cells with rapamycin. Interestingly, it has been postulated that this may be the result of the selective inhibition of p21 transcription as cells commit to apoptosis[31]; however, we only observed a minor increase in apoptosis levels after treatment with rapamycin.

Other authors suggest that a decrease in p21 expression levels may be advantageous in sensitizing tumor cells to treatment with other drugs[32,33,34]. Reasoning along these lines is consistent with the notion that an optimal basal level of p21 exists and that either a higher or lower level of p21 will inhibit cell proliferation[35]. Ultimately, due to the fact that p21 has numerous roles within a cell[36], it is difficult to accurately speculate about the exact causes and effects of a slight change in its expression without further investigation.

MS-275 demonstrated a dose-dependent effect over a range of concentrations. As quantitative real-time PCR indicated that increasing concentrations of MS-275 upregulated cyclin-dependent kinase inhibitor p21 while decreasing cyclin D1 expression, these data potentially provide a partial explanation for the increase in G_0/G_1 arrest. Additionally, the opposing effects of rapamycin and MS-275 on p21 and cyclin D1 expression is a reminder that the drugs are likely to work via distinct mechanisms.

We observed significant levels of apoptosis after treatment with MS-275, alone and in combination with rapamycin. Previous studies have shown that rapamycin does not typically induce apoptosis[37] while MS-275 typically does[38]. It is also important to note that we were unable to confirm the exact mechanisms by which apoptosis was induced. Previous studies indicate that MS-275 mediates caspase-dependent apoptosis[39], and while we did see an increase in poly(ADP-ribose) polymerase (PARP) cleavage, we did not check for the activation of any of the cleaved caspases that lie upstream of PARP[40].

Increased acetylation of histone H3 and histone H4 in our Western blot analysis indicates that MS-275 is effectively inhibiting the histone deacetylases. It is particularly comforting that acetylation increases with increasing concentrations of MS-275. Similarly, the lack of phospho-S6 in rapamycin-treated cells indicates that rapamycin is effectively inhibiting mTOR and blocking activation of its downstream targets.

While we have a great deal of evidence to suggest that both of these drugs have powerful anticancer properties, additional studies are needed to further elucidate the specific mechanisms by which they work. As other authors have suggested, thorough characterization of the various signaling pathways within a variety of tumor cell lines is a logical step, as this would allow for the potential clinical application of being able to select drugs that may be particularly effective in slowing the growth of a given tumor[41]. For example, tumors with a PTEN deletion, mutation, or hypermethylation that results in AKT phosphorylation (and, subsequently, mTOR activation, cell growth, and proliferation) can potentially be targeted with rapamycin and its derivatives[42].

Similarly, overexpression of bcl-2 is frequently associated with resistance to rapamycin, and thus, previous authors have suggested that bcl-2 may be used as a molecular marker to indicate that a given tumor type will not benefit from rapamycin treatment[43,44]. Interestingly, a recent study indicates that histone deacetylase inhibitors down-regulate bcl-2 expression and induce apoptosis in some lymphomas[45], which implies that histone deacetylases may be able to sensitize tumor cells to subsequent treatment with rapamycin (particularly in tumor cells with upregulated bcl-2). Thus, for our studies, it would be particularly prudent to examine the effect of MS-275 on bcl-2 expression. An upregulation of bcl-2 would indicate that the sequential treatment of MS-275 followed by rapamycin could yield increased inhibition against cell proliferation.

Previous experiments have also indicated that rapamycin and its derivatives induce autophagy in tumor cells[46]; in fact, downregulation of p21 has been linked to this process[47]. Thus, it might also be worthwhile to examine the effects of rapamycin and MS-275 on this process within this and other cell lines to gain a stronger understanding of the mechanisms by which these drugs work.

In summary, both of these drugs appear to be potent inhibitors of multiple myeloma, particularly in combination. Their synergistic effect is likely due to the fact that the drugs work via distinct mechanisms. And, while rapamycin and MS-275 have promising clinical applications, much more work should be done to further characterize their effects *in vitro* and *in vivo* to further elucidate how they can be used most effectively.

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