Drug Resistance to Inhibitors of the Human Double Minute-2 E3 Ligase is Mediated by Point Mutations of p53, but can be Overcome with the p53 Targeting Agent RITA

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Abstract

The human double minute (HDM)-2 E3 ubiquitin ligase plays a key role in p53 turnover, and has been validated pre-clinically as a target in multiple myeloma (MM) and mantle cell lymphoma (MCL). HDM-2 inhibitors are entering clinical trials, and we therefore sought to understand potential mechanisms of resistance in lymphoid models. Wild-type p53 H929 MM and Granta-519 MCL cells resistant to MI-63 or Nutlin were generated by exposing them to increasing drug concentrations. MI-63-resistant H929 and Granta-519 cells were resistant to Nutlin, while Nutlin-resistant cells displayed cross-resistance to MI-63. These cells also showed cross-resistance to bortezomib, doxorubicin, cisplatin, and melphalan, but remained sensitive to the small molecule inhibitor RITA. HDM-2 inhibitor-resistant cells harbored increased p53 levels, but neither genotoxic nor non-genotoxic approaches to activate p53 induced HDM-2 or p21. Resequencing revealed wild-type HDM-2, but mutations were found in the p53 DNA binding and dimerization domains. In resistant cells, RITA induced a G_2/M arrest, up-regulation of p53 targets HDM-2, PUMA, and NOXA, and PARP cleavage. Combination regimens with RITA and MI-63 resulted in enhanced cell death compared to RITA alone. These findings support the possibility that p53 mutation could be a primary mechanism of acquired resistance to HDM-2 inhibitors in MCL and MM. Furthermore, they suggest that simultaneous restoration of p53 function and HDM-2 inhibition is a rational strategy for clinical translation.

Keywords

HDM-2; p53; Multiple Myeloma; Mantle Cell Lymphoma; MI-63; Nutlin; RITA

Introduction

The ubiquitin-proteasome pathway is a key regulator of transcription factors and cellular proteins (1). Through its role in the poly-ubiquitination of target proteins and their subsequent proteasome-mediated degradation, this system influences angiogenesis, cell-cycle progression, DNA repair, and apoptosis (2). Tumors exploit key segments of this pathway to enhance carcinogenesis and circumvent cellular surveillance strategies aimed at eliminating malignant cells. One example is over-expression of the E3 ligase Human Double Minute (HDM)-2 in 10% of human malignancies (3), depleting the tumor suppressor p53. This leads to loss of cell cycle check points and uncontrolled proliferation. A single nucleotide polymorphism within the HDM-2 promoter has also been reported to enhance HDM-2 expression and tumor formation (4).

HDM-2 binds to the N-terminus of p53 to inhibit its transcriptional activity (5, 6), and ubiquitinates it for subsequent degradation (7, 8). This can be exploited therapeutically by agents such as Nutlin and MI-63 (Fig.1A), which bind the HDM-2 p53 binding site and prevent p53 ubiquitination (9, 10). Both have shown pre-clinical activity against mantle cell lymphoma (MCL) and multiple myeloma (MM), where they stabilize p53 and enhance its pro-apoptotic effects (11–16). RITA (reactivation of p53 and induction of tumor cell apoptosis)(17)(Fig.1B), disrupts binding of p53 and HDM-2, but inhibits the interaction by binding to the HDM-2 binding site in p53 (18).

Targeting the proteasome/E3 ligase system has recently become a focus in anti-cancer research with novel HDM-2 inhibitors (19). The advantage of inhibiting HDM-2 could be that specific inhibition of a single E3 ligase may reduce effects, such as neuropathy, which are seen in patients treated with bortezomib. Nutlin and MI-63 have been extensively studied in lymphoid malignancies (20, 21), and at physiologic concentrations their effects are due to disruption of the p53/HDM-2 interaction (22). One disadvantage of specific small molecule inhibitors is that resistance may be more likely to develop compared to genotoxic agents, since the latter may have a broader target range (23). Studies of mechanisms of innate or primary resistance to Nutlin and MI-63 have attributed this phenotype to p53 inactivation through HDM-X overexpression (24). Acquired, or secondary Nutlin resistance has been investigated in models of osteosarcoma, neuroblastoma, rhabdomyosarcoma and melanoma (25, 26). These studies identified drug-resistant isolates, in some of which wild-type p53 was retained, while others were characterized by p53 mutations that impaired its DNA binding and transactivation. However, no data have elucidated how lymphoid malignancies acquire HDM-2 inhibitor resistance. This is especially important since these tumors show a lower rate of p53 inactivation than solid tumors (27), suggesting they may be better targets for this drug class.

We therefore sought to examine the mechanisms responsible for MI-63 and Nutlin resistance by developing models that were insensitive to these pro-apoptotic drugs. Cell lines resistant to either agent demonstrated cross-resistance to the other, and were less sensitive to bortezomib, doxorubicin, cisplatin and melphalan, but not to RITA. Analysis of these cells showed enhanced p53 expression, while genomic sequencing revealed wild-type HDM-2 but mutated p53. Exposure of resistant cells to RITA induced cell cycle arrest and p53 transcriptional targets, supporting a restoration of p53 activity. Combination studies using RITA and MI-63 showed resensitization of resistant cells to MI-63. Taken together, our data support the hypothesis that translation of HDM-2 inhibitors to the clinic for chronic treatment of patients with MCL and MM could result in selection of resistant clones with p53 point mutations. Importantly, it may be possible to circumvent resistance by using molecules that inhibit the p53/HDM-2 interaction and refold mutant p53 into a wild-type conformation.

Materials and Methods

Reagents

HDM-2 inhibitors MI-63 and MI-219 were provided by Ascenta Therapeutics (Malvern, PA). Nutlin, doxorubicin and melphalan were from Sigma-Aldrich (St. Louis, MO), RITA was from Cayman Chemical Company (Ann Arbor, MI), bortezomib from Selleck Chemicals, LLC (Houston, TX), and cisplatin from the M.D. Anderson Cancer Center Pharmacy (Houston, TX).

Cell culture and generation of MI-63- and Nutlin-resistant cells

Wild-type p53 (*wtp53*) Granta-519 MCL and NCI-H929 MM cell lines were from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and American Type Culture Collection (ATCC)(Manassas, VA). Cell lines were validated in the M.D. Anderson Cell Line Validation Core Facility by short tandem repeat (STR) DNA fingerprinting (May 2012) using the AmpF*l*STR Identifiler kit (Applied Biosystems; Carlsbad, CA). The STR profiles were compared to known ATCC fingerprints, the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526) and the M.D. Anderson fingerprint database. To generate resistance to MI-63 and Nutlin, cells were exposed to a concentration of each drug that induced a 25% proliferation inhibition for one-week, followed by a drug holiday. The drug dose was then increased every two weeks until lines were resistant to 10μM of MI-63 or Nutlin. Cells were then maintained in drug-free media and the resistant phenotype was confirmed monthly. Experiments were performed in cells that had been drug-free for at least two weeks.

Semi-quantitative and real-time PCR

RNA was isolated using the RNeasy Plus kit (Qiagen; Valencia, CA), and cDNA was synthesized using Superscript II (Invitrogen; Carlsbad, CA). Real-time PCR was performed on a StepOnePlus PCR analyzer (Applied Biosystems; Carlsbad, CA) using inventoried real time Taqman-FAM and GAPDH-VIC probes. Relative transcript expression (RQ) was determined using vehicle treated cells as a calibrator with the $\Delta\Delta$CT method.

p53 and HDM-2 resequencing

Genomic DNA was extracted using the Genomic DNA extraction kit (Qiagen). Exons, introns, and regulatory regions of p53 and HDM-2 were sequenced by the M.D. Anderson DNA Analysis Core Facility. Exons were amplified using custom PCR primers, and Sanger sequencing was performed on a 3730xl DNA Analyzer using BigDye™ Terminator v3 (Applied Biosystems). Mutations analysis was performed using SeqScape® Software v2.5 (Applied Biosystems).

Immunoblotting

Protein expression was measured by immunoblot analysis as previously described (28). Antibodies against p53 were from Santa Cruz Biotechnologies (Santa Cruz, CA), anti-poly-(ADP-ribose) polymerase (PARP) was from Cell Signaling Technology (Danvers, MA), anti-p21, anti-PUMA, anti-NOXA, and anti-HDM-2 were from EMD Biosciences (Gibbstown, NJ), and anti-β-actin was from Sigma-Aldrich.

Cell cycle and cell death analysis

Cells were treated with drugs for 48-hours, fixed in 70% ethanol, and stained with propidium iodide (Sigma-Aldrich). Cell cycle data were obtained on a BD Facs Canto II flow cytometer (Becton-Dickson; Franklin Lakes, NJ) using FlowJo v.7.6.1 (Tree Star, Inc.;

Ashland, OR). Cell death was measured by staining with Annexin-V Pacific Blue and TO-PRO-3 (Invitrogen).

Cell proliferation assay

The WST-1 reagent (Roche Diagnostics; Indianapolis, IN) was used to determine the effects of chemotherapeutics (29). Median inhibitory concentration (IC_{50}) determinations were performed in 96-well plates in triplicate in 3 separate experiments performed on different days. Dose response curves were plotted using GraphPad Prism 5 (GraphPad; La Jolla, CA) showing standard error of the mean with a log scale and IC_{50} values calculated using the nonlinear regression competitive binding log IC_{50} algorithm.

Results

Generation of HDM-2 inhibitor resistant cells

To define the mechanisms through which MCL and MM could acquire HDM-2 inhibitor resistance, we exposed Granta-519 and H929 cells to increasing MI-63 and Nutlin concentrations. Median inhibitory concentrations (IC_{50}) for wild-type Granta-519 (Granta.WT) were 2.03μM for MI-63 and 0.96μM for Nutlin (Supplemental Table 1), and for NCI-H929 (H929.WT) the IC_{50} was 3.30μM and 3.54μM, respectively (Supplementary Table 1). Exposure of Granta-519 cells resistant to MI-63 (Granta.MI63R) or Nutlin (Granta.NutlinR) to MI-63 or Nutlin showed that both cell lines were insensitive to MI-63 and Nutlin (Fig.1C, 1E). Granta.MI63R showed IC_{50}'s of 60μM for MI-63 and 59.75μM for Nutlin, whereas Granta.NutlinR had an IC_{50} of 47.3μM for MI-63 and 44.75μM for Nutlin (Supplementary Table 1). A similar result was seen in the H929 resistant cells (Fig.1D, 1F) where H929.MI63R had an IC_{50} of 59.75μM to MI-63 and 56.65μM to Nutlin, whereas H929.NutlinR had an IC_{50} of 55.80μM for MI-63 and 47.60μM to Nutlin (Supplementary Table 1). Interestingly, enhancement of growth, particularly in H929.MI63R and H929.NutlinR, was noted in response to MI-63 or Nutlin (Fig.1D, 1F). Finally, we examined whether MI-63 and Nutlin resistance conveyed resistance to the small molecule RITA, which inhibits the p53/HDM-2 interaction by binding to p53 (18). Titration of RITA into Granta.MI63R and Granta.NutlinR cells revealed that they remained sensitive to RITA, though with a slightly increased IC_{50} of 1.72 and 0.84μM, respectively, compared to 0.272 μM in Granta.WT cells (Fig.1G; Supplementary Table 1). Lastly, H929.MI63R and H929.NutlinR showed some resistance to RITA, although this was not statistically significant (p>0.05), with IC_{50} values of 2.14μM and 1.99μM compared to 0.43μM for H929.WT cells (Fig.1H; Supplementary Table 1). These data indicate that our models were heavily cross-resistant to HDM-2 inhibitors that bind the p53 binding site on HDM-2, but not as resistant to HDM-2 inhibitors targeting the HDM-2 binding site on p53.

HDM-2 inhibitor-resistant cells demonstrate resistance against other therapeutics

We next considered whether MI-63 or Nutlin resistance could influence sensitivity to other anti-MCL (30) or MM (31) agents. Granta.MI63R and Granta.NutlinR cells displayed a slightly decreased sensitivity to doxorubicin, with an increase in the IC_{50} to 15.54 and 15.73nM, respectively, compared to 8.01nM in Granta.WT cells (Fig.2A; Supplementary Table 2). H929.MI63R and H929.NutlinR also showed a weak decrease in doxorubicin sensitivity (Fig.2B; Supplementary Table 2). Since the proteasome inhibitor bortezomib is effective against MCL and MM, Granta.MI63R and Granta.NutlinR cells were exposed to this agent, and showed a slight increase in resistance (Fig.2C; Supplementary Table 2). Similar results were found in H929.MI63R and H929.NutlinR cells (Fig.2D; Supplementary Table 2).

To further investigate whether this HDM-2 inhibitor resistance conveyed resistance to other DNA damaging agents, we examined the activity of melphalan and cisplatin. Granta.MI63R and Granta.NutlinR cells exposed to cisplatin displayed increased resistance, with IC_{50} values of 20 and 37.95μM, respectively, compared to 4.02μM for Granta.WT, representing a 4.9–9.4 fold increase in resistance (Fig.2E; Supplementary Table 2). H929.MI63R and H929.NutlinR cells treated with cisplatin had IC_{50}'s of 38.28 and 20.17μM compared to 2.55μM in the H929.WT cells (Fig.2F; Supplementary Table 2). Finally, treatment of Granta.MI63R and Granta.NutinR cells with melphalan resulted in IC_{50} values of 2.2 and 1.5μM, respectively, compared to 0.35μM in Granta.WT cells (Fig.2G; Supplementary Table 2). Similarly, H929.MI63R and H929.NutlinR cells were resistant to melphalan, with IC_{50} values of 4.77 and 2.59μM compared to 0.78μM in the H929.WT cells (Fig.2H; Supplementary Table 2), representing a 3.32- and 6.2-fold change.

Resistance to HDM-2 inhibitors is mediated by p53 point mutations

Since MI-63- and Nutlin-insensitive cells showed cross-resistance to DNA damaging agents which work in part in a p53-influenced manner, we considered the possibility that these cells had acquired p53 mutations. Granta.MI63R or H929.MI63R cells were therefore exposed to Nutlin, the *in vivo* MI-63 analogue MI-219, or doxorubicin, and p53, HDM-2, and p21 levels were evaluated. Both resistant cell lines showed elevated p53 levels in the vehicle controls compared to WT counterparts (Fig.3A, 3B). When Granta.WT and H929.WT cells were exposed to Nutlin or MI-219, a robust p53 increase was seen, resulting in strong HDM-2 and p21 induction. In contrast, Granta.MI63R and H929.MI63R cells showed little if any p53 increase in response to Nutlin, MI-63, or doxorubicin. Importantly, neither doxorubicin nor the HDM-2 inhibitors induced HDM-2 and p21, indicating the absence of transcriptionally active p53.

We probed the mutational status of p53 by sequencing, and analysis of Granta.MI63R cells identified two mutations, Q252Q and Y205Y. Q252Q is an exon 3 inactivating nonsense mutation, while Y205Y is an exon 5 inactivating missense mutation (Supplementary Table 3). H929.MI63R and H929.NutlinR cells both carried R175H and R248Q missense mutations within exons 4 and 6, respectively. Notably, HDM-2 sequencing revealed wild-type sequences throughout, indicating drug resistance was not mediated by mutation of the MI-63 or Nutlin binding site.

RITA induces cell cycle arrest and apoptosis in resistant cells

The unanticipated sensitivity of Granta.MI63R and H929.MI63R cells to RITA suggested that RITA may have a mechanism of action beyond inhibiting the p53/HDM-2 interaction. Treatment of Granta.WT cells with RITA resulted in G_2/M cell cycle arrest compared to vehicle controls (Fig.4A, upper panel), whereas MI-63 and Nutlin induced a G_1 arrest. Granta.MI63R cells showed no cell cycle changes in response to MI-63 or Nutlin but RITA induced a strong G_2/M arrest (Fig.4A, lower panel). When H929.WT cells were studied, they also showed a G_2/M cell cycle arrest with RITA, whereas MI-63 induced a strong G_1 arrest with a sub-G_1 apoptotic peak. Nutlin also induced a sub-G_1 apoptotic peak and increased the G_2/M fraction (Fig.4B, upper panel). Similar to the Granta model, RITA treatment of H929.MI63R cells increased the proportion of plasma cells in G_2/M, whereas MI-63 and Nutlin had no effect (Fig.4B, lower panel).

A novel insight into RITA's function has recently been reported by Zhao *et al* (32) and Messina *et al* (33), noting RITA could rescue the apoptosis-inducing function of mutant p53. We therefore treated MI-63-resistant cells with RITA, and evaluated its effect on p53 down-stream targets. Exposure of Granta.MI63R cells to RITA initially decreased HDM-2 expression at 3-hours, and though a small rebound was noted at 12-hours, recovery did not

occur to baseline levels, and almost full abrogation of expression was visible at 48–72-hours (Fig.4C). This HDM-2 decrease coincided with an increase in p53, and up-regulation of cleaved PARP. Two other p53-influenced apoptosis markers increased, including NOXA and p53 up-regulated modulator of apoptosis (PUMA). In contrast, in H929.MI63R cells, RITA up-regulated HDM-2 at 3–24-hours but, as in Granta cells, HDM-2 expression was virtually absent by 48–72-hours (Fig.4D). Similar to Granta.MI63R cells, p53 levels also increased in H929.MI63R cells as early as 6-hours, and RITA induced apoptosis in H929.63R cells in association with increased levels of NOXA and PUMA.

RITA resensitizes HDM-2 inhibitor resistant cells to MI-63

Since RITA induced the expression of PUMA and NOXA, we explored whether RITA could restore p53 function and resensitize MI-63 resistant cells to HDM-2 inhibitors. Thus, we performed combination experiments with MI-63 and RITA, and examined whether the drug exposure sequence affected the outcome. Initial studies determined the activity of RITA and MI-63 for 24- and 48-hours as single agents in the MI-63-resistant cells. RITA had no effect on cell viability at 24-hours in Granta.MI63R or H929.MI63R cells (Fig.5A, 5B). However, treatment with RITA for 48-hours reduced cell viability by 50% in Granta.MI63R and H929.MI63R at 1.25 and 2.5μM, respectively. Treatment with MI-63 had no effect at any of the concentrations used in the MI-63-resistant cells. To determine if the combination of MI-63 and RITA affected the resistant cells, RITA and MI-63 were titrated simultaneously, or cells were pre-treated with one for 24-hours, followed by addition of the other for 48-hours. When Granta.MI63R (Fig.5C, left panel) and H929.MI63R cells (Fig. 5D) were treated simultaneously with RITA and MI-63, significantly greater reductions in viability were seen than with either agent alone (p<0.05 to vehicle)(Fig.5A and 5B). Sequencing studies showed that pre-treatment of Granta.MI63R (Fig.5C) or H929.MI63R (Fig.5D) cells with RITA followed by MI-63 resulted in resensitization to MI-63 (p<0.05 to vehicle and simultaneous addition). Interestingly, pre-treatment of Granta.MI63R cells with MI-63, followed later by RITA, showed no resensitization (p<0.05 to simultaneous addition), and indeed the combination was ineffective at reducing viability (Fig.5C). Studies in H929.MI63R cells showed qualitatively similar findings, particularly at lower concentrations, in that MI-63 followed by RITA induced less cell death than RITA alone (p<0.05 to simultaneous addition), supporting the possibility that this treatment sequence was antagonistic.

Since simultaneous addition of clinically relevant analogues of RITA and MI-63 would be a convenient treatment approach for patients, we investigated the molecular effects of this regimen. RITA induced a modest amount of apoptosis in Granta.MI63R (Fig.6A) and H929.MI63R cells (Fig.6B), albeit to a lesser degree than in their wild-type counterparts. When MI-63 was used alone, as expected, it was ineffective against either, especially in comparison to the wild-type controls. However, when RITA and MI-63 were given simultaneously, the levels of apoptosis in the HDM-2 inhibitor-resistant models resembled those in the drug-naïve, p53 wild-type counterparts (p<0.01).

To evaluate if RITA was indeed restoring wild-type p53 function, Granta.MI63R and H929.MI63R cells were treated with RITA and MI-63, and expression of p21 and HDM-2 was analyzed by quantitative real-time PCR. RITA induced a 1.5-fold increase in p21, and a 1.25-fold increase in HDM-2 transcripts in Granta.MI63R cells (Fig.6C, 6E), while MI-63 alone failed to induce either. In contrast, the RITA/MI-63 combination increased p21 expression 3-fold, and HDM-2 nearly 2-fold. Similarly, in H929.MI63R cells, RITA induced a 2.5-fold increase in p21 and a 3-fold increase in HDM-2 (Fig.6D, 6F), whilst MI-63 failed at either. This failure was overcome by the addition of RITA and MI-63, resulting in a near 6-fold increase in p21, and a 5-fold increase in HDM-2. These data indicate that continuous exposure of MCL and MM models to the HDM-2 inhibitors MI-63

and Nutlin result in p53 point mutations as a mechanism of acquired drug resistance, and that RITA overcomes this resistance by restoring p53 function, thereby resensitizing cells to HDM-2 inhibitors.

Discussion

Inhibitors of HDM-2, including Nutlin and MI-63/MI-219, have been used as tool compounds to establish HDM-2 as a target for therapy in MCL and MM. In anticipation that HDM-2 inhibitors will be evaluated in the clinic, and since both MCL and MM are characterized by the emergence of drug resistance, it is important to develop models of such resistance to identify the responsible mechanisms. Here we report that acquired MI-63 and Nutlin resistance can be mediated by the acquisition of p53 point mutations that inactivate its activity as a transcriptional activator. DNA damaging therapeutics such as anthracyclines and radiation frequently induce p53 mutations (34, 35), but HDM-2 inhibitors are not felt to induce genotoxic stress or DNA damage (9), making this finding somewhat unexpected. Additional studies will be needed to determine if these mutations are due to a low level of genotoxic stress, outgrowth of a sub-population of mutant p53 clones present in the background of the original wild-type p53 cells, or another mechanism.

Cells with acquired resistance to one HDM-2 inhibitor were cross-resistant to another. Indeed, in cells resistant to MI-63 or Nutlin, further treatment with these inhibitors promoted cellular viability and growth (Fig.1). This may be because mutant p53 has been shown to enhance the development, maintenance, and metastatic spread of malignant cells (36–38). As HDM-2 can still ubiquitinate and degrade mutant p53 (7), HDM-2 inhibitors stabilize these p53 variants (25), and potentiate this gain of function, and potentially enhance proliferation. This suggests that the emergence of drug-resistant clones in patients could be associated with more rapid disease progression than is typically seen with other chemotherapeutics. Patients would therefore need to undergo regular measurements of their disease burden and its molecular characteristics to minimize this risk.

While HDM-2 inhibitor-resistant models showed cross-resistance to other chemotherapeutics, they remained sensitive to RITA. MI-63 and Nutlin differ in that while both bind residues Phe-19, Trp-23, and Leu-26 of the HDM-2 domain that interacts with p53, MI-63 also interacts with Leu-22 (10, 39, 40). Despite this, MI-63 was still inactive against Nutlin-resistant cells, likely demonstrating that cross-resistance to HDM-2 inhibitors is dependent upon the target binding the same p53 HDM-2 binding site. RITA, on the other hand, induced cell cycle arrest and apoptosis, and down-stream p53 targets, suggesting RITA was restoring wild-type p53 function. Confirming this possibility, simultaneous treatment with MI-63 and RITA, and pre-treatment with RITA resensitized cells to MI-63 (Fig.5, 6) with p21 and HDM-2 induction. Originally, the mechanism of action of RITA was thought to be limited to *wtp53* cells (18), but recent data indicate activity against *mutp53* tumors (32, 33). Indeed, RITA restored the transactivation and transrepression functions of p53 mutants, and demonstrated selectivity for mutant p53 cells (32, 33). These findings explain the ability of RITA to overcome HDM-2 inhibitor resistance, and restore MI-63 sensitivity. One interesting observation was the antagonistic effect of exposing cells to MI-63 prior to RITA, and the ability of Nutlin and MI-63 to enhance the growth of resistant cells, which may be related to the function of p53 as a tetramer. Mutant p53 molecules can disrupt the function of wild-type p53 tetrameric complexes by direct binding, resulting in p53 heterodimers that cannot form functional tetramers (41). Addition of MI-63 first may increase mutant p53 expression in the resistant cells, and when RITA is then added, the number of mutant p53 molecules may be in excess, precluding the ability of RITA to convert them to wild-type function. This may result in antagonism as the balance of mutant p53 molecules can compete off the restored wild-type p53 function. In contrast, addition of

RITA prior to MI-63 would allow conversion of mutant p53 to wild-type, and thereby change the balance of dimers, ultimately resulting in wild-type function.

Inactivation of p53 through mutation is linked to decreased sensitivity to many chemotherapeutics (34, 35). Resequencing of HDM-2 and p53 in our models indicated the presence of point mutations solely within p53 DNA binding and dimerization domains. This explains the increased expression of p53, since mutant p53 has a longer half-life. Also, this helps to explain the failure of bortezomib, doxorubicin, melphalan, cisplatin, Nutlin, or MI-63/MI-219 to induce a significant amount of p21 and HDM-2. A previous study examining Nutlin-induced resistance in SJSA osteosarcoma cells found one population had acquired mutations in the p53 DNA binding and dimerization domain (25). Another population retained *wtp53*, but when the cells were rechallenged with Nutlin, they still underwent growth arrest (25). A more recent study which examined several cell lines with induced Nutlin resistance, including neuroblastoma, melanoma and rhabdomyosarcoma cells in which p53 mutation is infrequent, found that 80% harbored p53 mutations (42). This resistance was generated using similar methods to ours, and of particular interest was the finding that among the cell lines with induced resistance to varying genotoxic agents, only 7.14% had mutated p53 (42). This suggests that Nutlin class HDM-2 inhibitors are prone to induce p53 mutations within chronically exposed cells. All of the p53 mutations found in our cells were heterozygous, and this was also the case in the neuroblastoma Nutlin-resistant cells previously reported (26). Of particular interest was the fact that MI-63- and Nutlin-resistant H929 cells both carried the R248Q and R175H hot spot mutations, which are the most common p53 mutation in B-cell malignancies (43). The finding that both cells acquired the same mutations suggests the presence of mutated cells within the wild-type H929 cell line. These cells may be infrequent and not readily detectable, but following treatment with HDM-2 inhibitors their abundance may be increased. In contrast, the Granta-519 MI-63 and Nutlin resistant cells both had different mutations, suggesting these cells developed unique mutations not present within the wild-type parental cells, which was also observed in Nutlin resistant neuroblastoma cells (26).

The HDM-2 inhibitor R05045337 is in phase I clinical trials targeting patients with solid tumors, and with acute and chronic leukemias. Outcomes from these and other trials, and especially analyses of primary samples obtained at baseline and at the emergence of drug resistance, will further delineate mechanisms of resistance *in vivo*. Until then, our data suggest that p53 mutations will play a prominent role and, importantly, provide a rationale for combining HDM-2 inhibitors with p53 reactivating agents such as RITA or PRIMA[MET] (44). These combinations may be of particular use in MCL, where ~15% of patients have mutated p53 with a median survival of only 1.3 years (45). Similarly, p53 mutations in MM fall in the 10–20% range (46), and increase with advanced disease (47). It is exciting to note the p53 reactivating agent APR-246 recently completed phase I clinical trials (48), and has shown activity *in vitro* in acute myeloid leukemia (49), and malignant melanoma (50). This indicates that MM and MCL patients with baseline or acquired p53 mutations could benefit from novel chemotherapies targeting HDM-2, and that APR-246 or RITA could help to restore wild-type p53 function, leading to enhanced cellular responses to therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

DOX	doxorubicin
Granta.WT	Granta-519 wild-type
Granta.MI63R	Granta-519 MI-63 resistant cells
Granta.NutlinR	Granta-519 Nutlin resistant cells
HDM-2	Human Double Minute protein -2
H929.WT	H929 wild-type
H929.MI63R	H929 MI-63 resistant cells
H929NutlinR	H929 Nutlin resistant cells
IC$_{50}$	Median inhibitory concentration
MCL	mantle cell lymphoma
MM	multiple myeloma
mutp53	mutant p53
PARP	poly-(ADP-ribose) polymerase
PUMA	p53 up regulated modulator of apoptosis
RITA	reactivation of p53 and induction of tumor cell apoptosis
RQ	relative transcript expression
wtp53	wild-type p53

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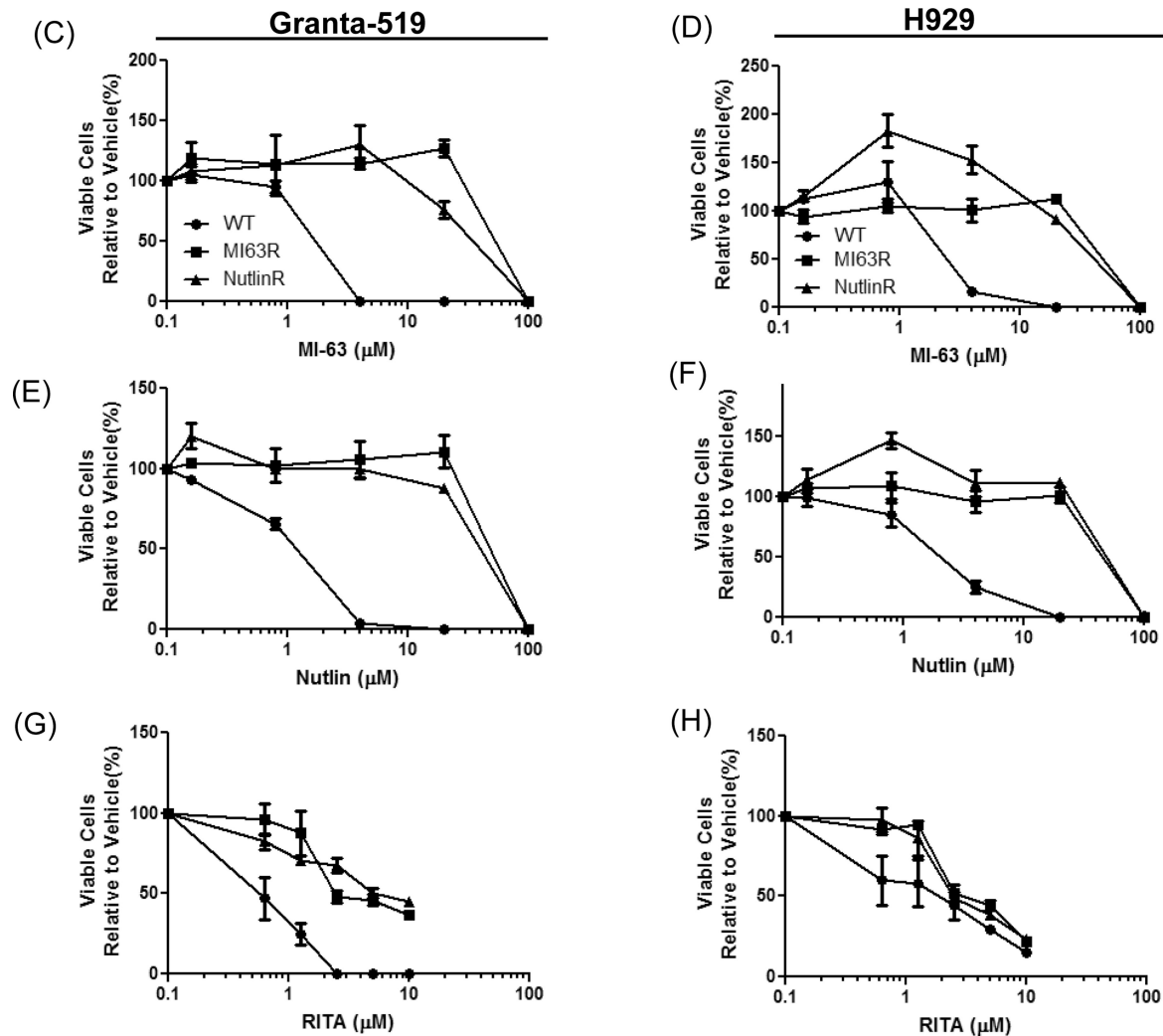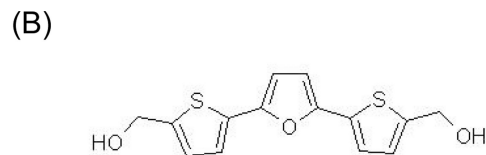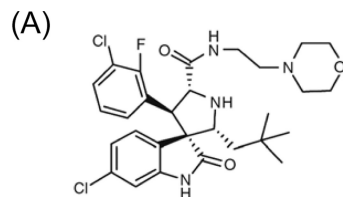
Figure 1. MI-63- and Nutlin-resistant cells show cross-resistance to HDM-2 inhibitors
(A) Chemical structure MI-63. **(B)** Chemical structure RITA. Granta-519 **(C, E, G)** and H929 **(D, F, H)** wild type (WT) cells, along with their MI-63-resistant (MI63R) and Nutlin-resistant (NutlinR) counterparts, were treated for 72-hours with MI-63, Nutlin or RITA. Cell viability was determined using the WST-1 reagent, and results were expressed as the percentage viability relative to vehicle controls, which were arbitrarily set at 100%. IC_{50} values for MI-63, Nutlin and RITA-exposed conditions were determined, and fold changes in the IC_{50} were calculated relative to WT cells (Supplementary Table 1).
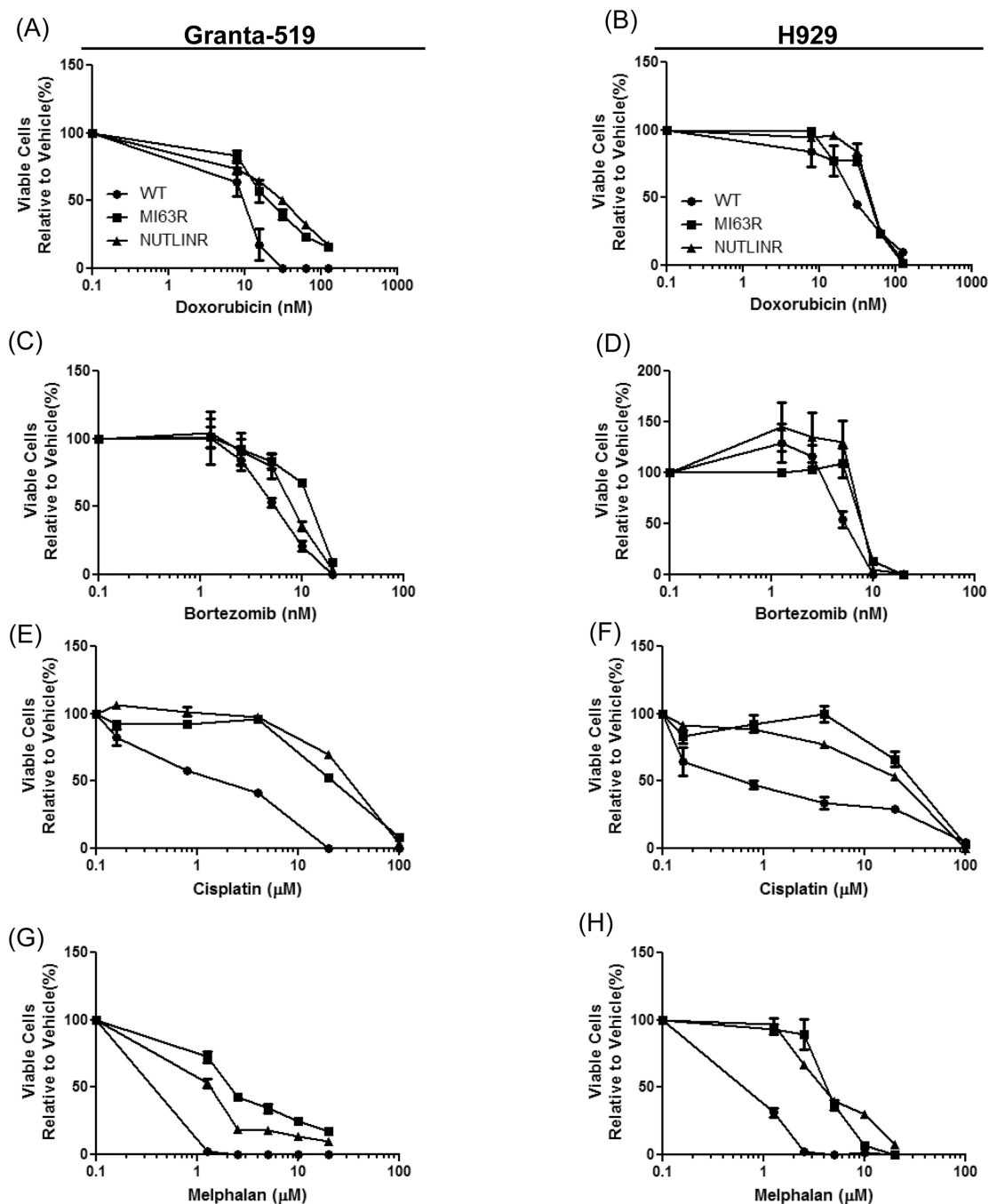
Figure 2. HDM-2 inhibitor-resistant cells show cross-resistance to some chemotherapeutics
Granta-519 **(A, C, E, G)** and H929 **(B, D, F, H)** WT, MI63R, and NutlinR cells were treated
with doxorubicin, bortezomib, cisplatin or melphalan for 72-hours. Cell viability was
determined using the WST-1 reagent. IC$_{50}$ values were determined for each cell line, and
fold changes were calculated relative to the WT cells (Supplementary Table 2).
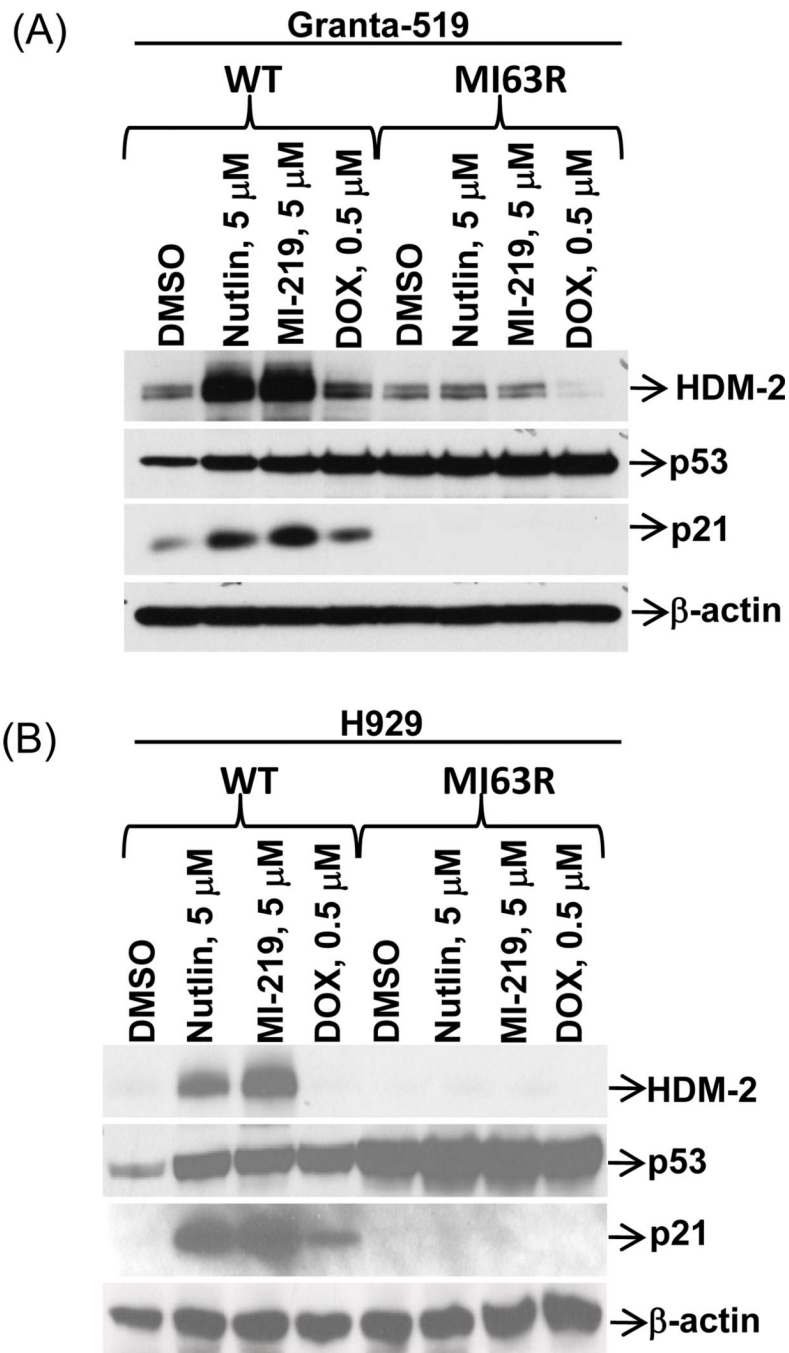
Figure 3. MI-63-resistant cells over-express p53, but cannot induce p21 in response to HDM-2 inhibition

Granta-519 **(A)** and H929 **(B)** WT and MI63R cells were treated for 24-hours with Nutlin, MI-219, or doxorubicin (DOX), and protein lysates were subjected to Western blotting. Representative images are shown in both panels from one of three independent experiments.
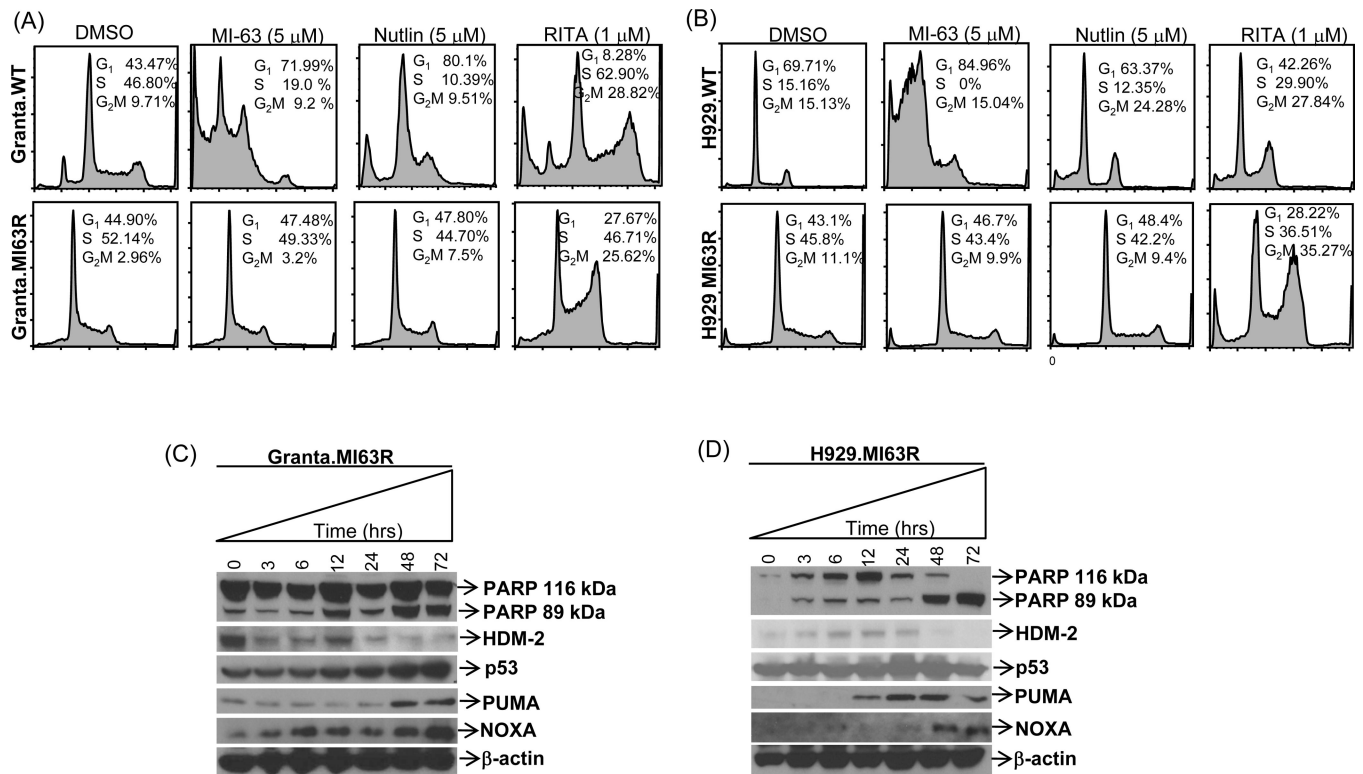
Figure 4. RITA induces G₂/M cell cycle arrest, and up-regulates p53 pro-apoptotic targets
Granta-519 **(A)** and H929 **(B)** WT and MI63R resistant counterparts were treated with vehicle, MI-63, Nutlin or RITA for 48 hours, and cell cycle analysis was performed. Granta.MI63R **(C)** and H929.MI63R **(D)** cells were treated with 5μM RITA, samples were harvested at the indicated time points, and protein lysates were subjected to Western blotting.
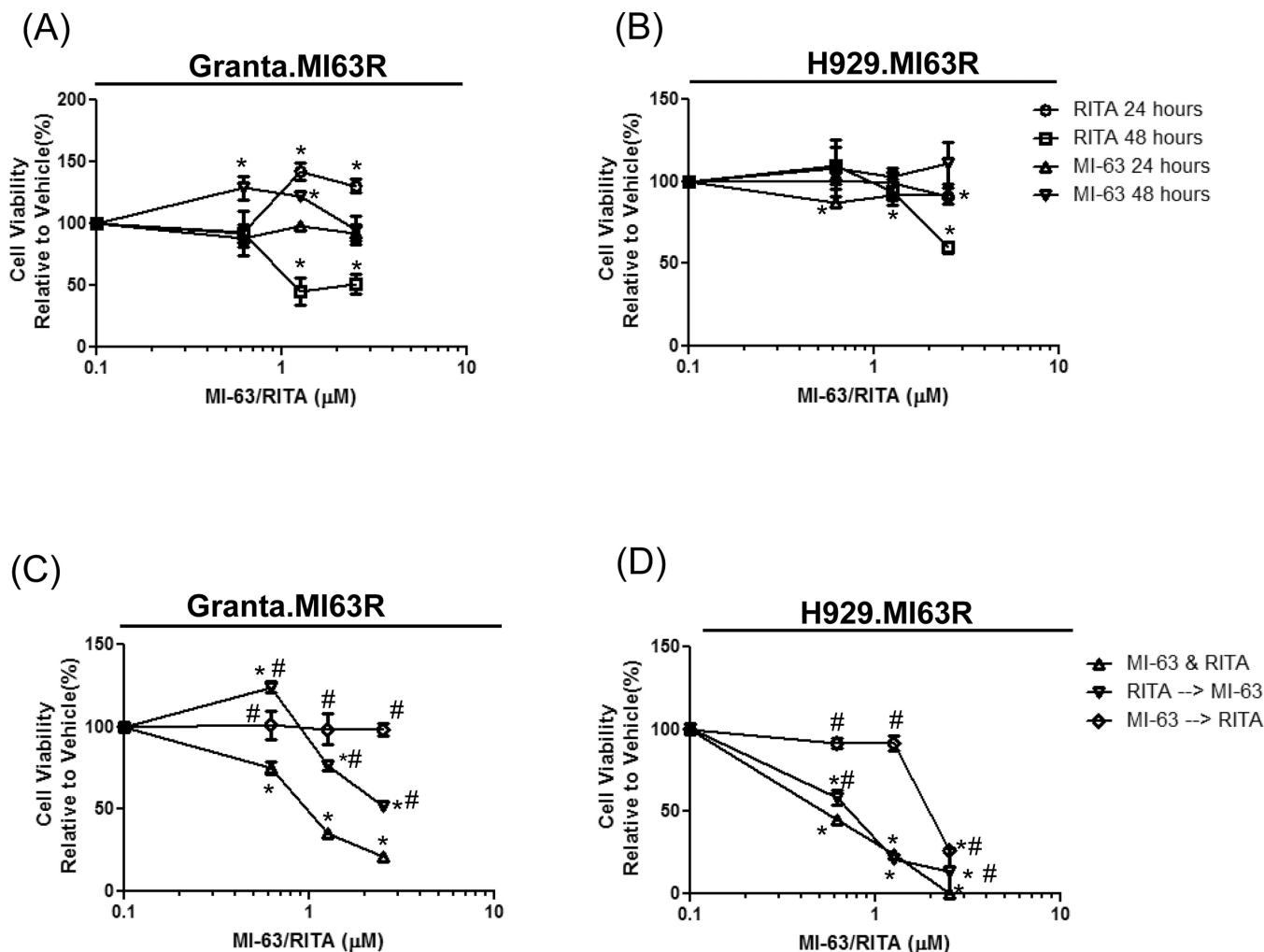
(A)

Granta.MI63R

(B)

H929.MI63R

- RITA 24 hours
- RITA 48 hours
- MI-63 24 hours
- MI-63 48 hours

(C)

Granta.MI63R

(D)

H929.MI63R

- MI-63 & RITA
- RITA --> MI-63
- MI-63 --> RITA

Figure 5. Simultaneous addition of RITA with MI-63 is synergistic in MI-63-resistant cell lines
Granta.MI63R **(A)** and H929.63R **(B)** cells were incubated with MI-63 or RITA for 24- and 48-hours. In parallel, Granta.MI63R **(C)** and H929.63R **(D)** cells were exposed to MI-63 and RITA simultaneously, or first to MI-63 for 24-hours followed by RITA and MI-63 for 48-hours, or to RITA first for 24-hours followed later by MI-63 and RITA for 48-hours. Cell viability was determined using the WST-1 reagent, and results were expressed relative to the vehicle control. An unpaired T test was performed on conditions in panels C and D, where * designates $p < 0.05$ relative to the vehicle control, and # designates $p < 0.05$ relative to the simultaneous addition of RITA and MI-63.
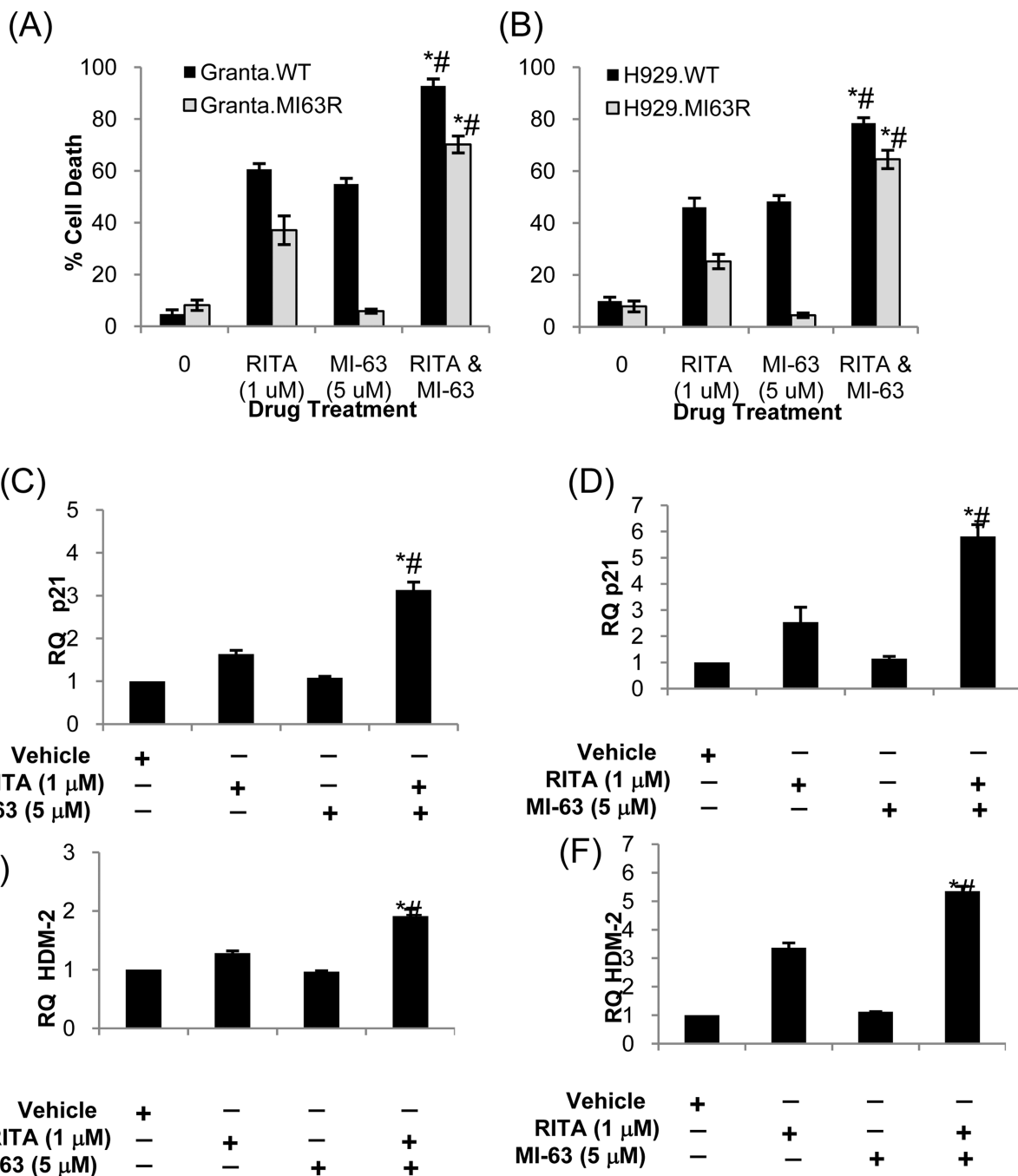
Figure 6. RITA restores sensitivity to MI-63 resulting in p21 and HDM-2 induction
Granta-519 (**A**) and H929 (**B**) WT, and MI-63R resistant counterparts were treated with
RITA, MI-63, or both simultaneously for 48-hours. FACS analysis was performed with
Annexin-V and TO-PRO-3, and the percentage apoptosis calculated. Granta.MI63R (**C, E**)
and H929.MI63R (**D, F**) were treated with RITA, MI-63, or both simultaneously for 24-
hours. RNA was extracted and cDNA was synthesized, and p21 and HDM-2 were measured
by quantitative real-time PCR using the $\Delta\Delta CT$ method, with the vehicle treated cells used
as a relative calibrator. An unpaired t-test was performed to evaluate for significance, and

"*" denotes p<0.01 relative to RITA alone, while "#" denotes p<0.01 relative to MI-63 alone.