

Restoration of p53 Pathway by Nutlin-3 Induces Cell Cycle Arrest and Apoptosis in Human Rhabdomyosarcoma Cells

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Abstract Purpose: Seventy to eighty percent of rhabdomyosarcoma (RMS) tumors retain wild-type p53. The tumor suppressor p53 plays a central role in inducing cell cycle arrest or apoptosis in response to various stresses. p53 protein levels are regulated by MDM2 through ubiquitin-dependent degradation. In this study, we evaluated whether nutlin-3, a recently developed small-molecule antagonist of MDM2, has an effect on p53-dependent cell cycle arrest and apoptosis in cultured human RMS cell lines.

Experimental Design: Five RMS cell lines with different p53 statuses and MDM2 expression levels were treated with nutlin-3. Gene expression patterns, cell viability, cell cycle, and apoptosis after nutlin-3 treatment, and antitumor activity of combination treatment with vincristine or actinomycin D were assessed.

Results: Significant p53 activation was observed in wild-type p53 cell lines after nutlin-3 treatment. p53 activation led to cell cycle arrest in parallel with increased p21 expression. Furthermore, these cell lines underwent p53-dependent apoptosis, concomitant with elevation of proapoptotic genes and activation of caspase-3. The effect of nutlin-3 was almost the same in terms of half maximal inhibitory concentration and apoptosis whether or not MDM2 was overexpressed. Nutlin-3 did not induce either cell cycle arrest or apoptosis in p53 mutant cell lines. A combination of vincristine or actinomycin D with nutlin-3 enhanced the antitumor activity in RMS cell lines with wild-type p53.

Conclusions: Nutlin-3 effectively restored p53 function in both normal MDM2 expression and MDM2 overexpression RMS cell lines with wild-type p53. p53 restoration therapy is a potential therapeutic strategy for refractory RMS with wild-type p53.

The tumor suppressor p53 plays an essential role in protecting cells from malignant transformation by inducing cell cycle arrest or apoptosis in response to various stresses (1). p53 mutations are found in half of human malignancies (2, 3). However, they are found less frequently in pediatric malignancies and occur in only 20% to 30% of rhabdomyosarcoma (RMS) cases (4).

MDM2 is an E3 ubiquitin ligase that regulates the protein level of p53 through ubiquitin-dependent degradation (5). Overexpression of MDM2 is found in several human malignancies (6), in which it makes p53 functionally inactive and contributes to tumor development (7). MDM2 is overexpressed in 10% to 20% of RMS cases (4).

Because p53 and MDM2 play important roles in tumor development and growth, inhibition of the MDM2-p53 pathway has been considered a promising therapeutic target for malignancies with wild-type p53. Inhibition of MDM2 expression by antisense oligonucleotides was shown to activate p53 and to suppress tumor growth in mouse xenograft models (8, 9). However, it is still difficult to apply antisense treatment in clinical settings. Therefore, development of a small-molecule inhibitor is needed.

Nutlin-3, a recently developed small-molecule antagonist of MDM2, inhibits the MDM2-p53 interaction and restores the p53 pathway *in vitro* and *in vivo* (10). Nutlin-3 has recently been shown to be effective against a variety of cancer cells with wild-type p53, including neuroblastoma (11), retinoblastoma (12), osteosarcoma (10), and leukemia (13–15).

In this study, we evaluated whether nutlin-3 has an effect on p53-dependent cell cycle arrest and apoptosis in cultured human RMS cell lines and whether nutlin-3 could enhance the antitumor activity of currently used chemotherapeutic agents against RMS. We show that inhibiting the MDM2-p53 pathway

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Translational Relevance

Seventy to eighty percent of rhabdomyosarcoma (RMS) tumors retain wild-type p53. Nutlin-3, a recently developed small-molecule antagonist of MDM2, effectively restored p53 function in both normal MDM2 expression and MDM2 overexpression RMS cell lines with wild-type p53, leading to cell cycle arrest and apoptosis. Furthermore, a combination of vincristine or actinomycin D (currently used in RMS treatment regimens) with nutlin-3 enhanced the antitumor activity in the RMS cell lines with wild-type p53. Because p53 activation by nutlin-3 is nongenotoxic, treatment regimens with less genotoxic side effects might be possible. p53 restoration therapy is a potential therapeutic strategy for refractory RMS with wild-type p53.

with nutlin-3 suppresses tumor growth as well as induces the death of RMS cells, and thus can be a potential therapy for patients with RMS.

Materials and Methods

Cell lines and reagents. Human alveolar RMS cell lines SJ-Rh18 (Rh18), SJ-Rh30 (Rh30), and SCMC-RM2 (RM2; ref. 16), and embryonal RMS cell lines RMS-YM (17) and RD were used. Cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ incubator. Nutlin-3 (Cayman Chemical) was dissolved in DMSO and stored as a 16-mmol/L stock solution in small aliquots at -20°C. Vincristine (Sigma) was stored as a 1-mmol/L stock solution in distilled water at -20°C, and actinomycin D (Sigma) was stored as a 1-mmol/L stock solution in DMSO.

Reverse transcription-PCR and direct sequencing of p53 cDNA. Total RNA was extracted from untreated cells with the use of an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized with the use of the SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen) according to the manufacturer's instructions. The entire coding region of p53 was PCR amplified in two overlapping fragments with the use of the following primer pairs: 5'-GTGACACGC TTCCCTGGAT T-3' (forward) and 5'-GCACCACCACACTATGTCGAA-3' (reverse), and 5'-TGGCCATCTACAAGCAGTCA-3' (forward) and 5'-GCAAGCAAGGGTTC AAGACC-3' (reverse). PCR products were sequenced with the use of the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and the ABI PRISM 377 Sequence Detection System (Applied Biosystems).

Real-time quantitative reverse transcription-PCR-based quantification of mRNA expression. The cells were treated with 4 or 10 μmol/L nutlin-

3 or an equivalent amount of DMSO. Total RNA was extracted from cells, and cDNA was synthesized as described above. Real time reverse transcription-PCR was carried out in a 7300 Real-Time PCR System (Applied Biosystems) with SYBR Green I (Takara Bio). Relative target mRNA expression was determined with the use of the ΔCt method (value obtained by subtracting the Ct value of glyceraldehyde-3-phosphate dehydrogenase mRNA from the Ct value of the target mRNA). Data were expressed as the ratio (calculated with the use of 2^{-(ΔCt)}) of target mRNA to glyceraldehyde-3-phosphate dehydrogenase mRNA (18). Relevant primers are shown in Supplementary Table S1.

Western blot analysis. Cells were lysed with Laemmli sample buffer, which contained no β-mercaptoethanol or bromophenol blue. Protein concentrations of the cell lysates were measured by BioRad DC Protein Assay (BioRad). Before loading the samples onto polyacrylamide gels, β-mercaptoethanol and bromophenol blue were added to the lysates. Samples were boiled for 5 min, separated by SDS-PAGE with the use of equal amounts of protein in each lane, transferred to an Immobilon-P membrane, and immunoblotted with the use of p53 antibody (1:1,000; Cell Signaling), p21^{WAF1/CIP1} antibody (1:2,000; Cell Signaling), MDM2 antibody (1:200; Santa Cruz Biotechnology), whole caspase-3 antibody (1:2,000; BD Biosciences), cleaved caspase-3 antibody (1:1,000; Cell Signaling), and β-actin antibody (1:2,000; Sigma). Antibody binding was detected with an enhanced chemiluminescence detection system (Amersham).

WST-8 cell viability assay. WST-8 colorimetric assays were done with the use of the Cell Counting Kit-8 (Nakalai Tesque) according to the manufacturer's guide. Cells were seeded into 96-well plates in 100 μL of culture medium for 24 h, and then various reagents were added. Cell viability was determined colorimetrically by the optical density at a wavelength of 450 nm, with a microplate reader (Multiscan IX; Dainippon Sumitomo Pharmaceutical) as described previously (19).

Cell growth assay. Cells were plated in normal growth medium into 24-well cell plates. After 24 h, cells were treated with nutlin or DMSO. The cells were lysed under hypotonic conditions, and nuclei were counted every 24 h for 3 d with a Coulter counter.

Cell cycle analysis. For analysis of cell cycle distribution, cells were cultured in the presence of 4 or 10 μmol/L nutlin-3 or an equivalent amount of DMSO for 24 h, scraped, washed with PBS, and incubated for 30 min with propidium iodide to stain DNA. DNA content was determined with the use of a FACS Calibur flow cytometer (BD Biosciences), and the cell cycle was analyzed with the use of ModFit LT software (Verity Software House) as described previously (20).

Analysis of apoptosis by flow cytometry. Cell death was determined through Annexin V-FITC/propidium iodide staining with the use of a TACS Annexin V-FITC apoptosis detection kit (R&D Systems) according to the manufacturer's instructions. Data were analyzed with the use of Cell Quest software (BD Biosciences).

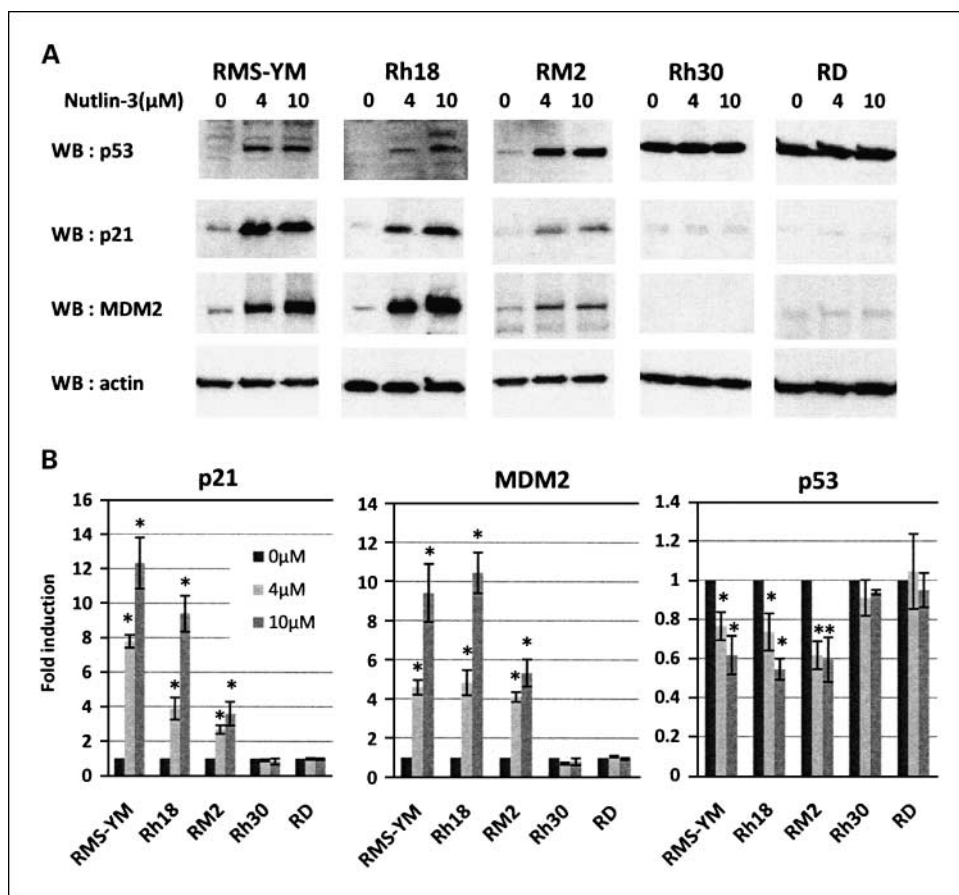
Statistical analysis. Average values are expressed as mean ± SD. The two-tailed Student's *t* test was used for comparison of the means between groups, with the use of a significance level of *P* < 0.05. The combination index was calculated with the use of the Chou-Talalay method (21), and the combination index was determined in relation to the fraction of cells affected. A combination index of 1 indicates an

Table 1. p53 mutational status and MDM2 expression status of RMS cell lines studied

Cell line	ARMS/ERMS	MDM2 overexpression	p53
RMS-YM	ERMS	+	wt
Rh18	ARMS	+	wt
RM2	ARMS	-	wt
Rh30	ARMS	-	R273C heterozygous mutation
RD	ERMS	-	R248W homozygous mutation

Abbreviations: ARMS, alveolar RMS; ERMS, embryonal RMS; wt, wild-type.

Fig. 1. Restoration of p53 pathway by nutlin-3 in RMS cell lines with wild-type p53. **A**, western blot analysis of p53, MDM2, and p21 expression in RMS cell lines with wild-type p53 (RMS-YM, Rh18, RM2) and mutant p53 (Rh30, RD) after treatment with 4 or 10 $\mu\text{mol/L}$ or an equivalent amount of solvent for 24 h. β -actin is used as a loading control. **B**, expression of p53, p21, and MDM2 transcripts. The results are shown as fold induction relative to cells treated with DMSO. *, $P < 0.05$ compared with untreated.



additive effect; a combination index >1 , an antagonistic effect; and a CI <1 , a synergistic effect.

Results

Mutation status of p53 and expression status of MDM2 in RMS cell lines. Sequencing of full-length cDNAs revealed that three of the five RMS cell lines (Rh18, RM2, and RMS-YM) had wild-type p53 (Table 1). In Rh30 cells, p53 had a heterozygous 817C>T (R273C) missense mutation and, in RD cells, it had a homozygous 742C>T (R248W) missense mutation, as previously reported (4). Western blot analysis confirmed that two RMS cell lines, Rh18 and RMS-YM, overexpressed the MDM2 protein compared with the other three cell lines (Table 1; Supplementary Fig. S1).

Nutlin-3 induces p53 protein and restores the p53 pathway in RMS cell lines with wild-type p53. We next asked if treatment with nutlin-3 would increase p53 protein and induce expression of p53-dependent genes in RMS cell lines. Five RMS cell lines, three with wild-type p53 and two with mutant p53, were incubated with nutlin-3 for 24 h. A western blot analysis showed an increase of p53 protein in RMS cell lines with wild-type p53 after nutlin-3 treatment (Fig. 1A). Furthermore, nutlin-3 treatment induced dose-dependent increases (3-fold to 12-fold increases) in the mRNA levels of p21 and MDM2, which are major transcription targets of p53 (Fig. 1B). These increases were also observed at the protein level by western blotting (Fig. 1A). Because the observed increase in p53 protein

was not accompanied by an increase in the p53 mRNA level (Fig. 1B), the p53 protein level seems to have increased through a post-transcriptional mechanism. Nutlin-3 had little effect on the mRNA and protein levels of p53, p21, and MDM2 in the RMS cell lines with mutated p53 (Fig. 1A and B).

Nutlin-3 inhibits cell growth in RMS cell lines. We examined the effect of the MDM2 inhibitor on the growth of RMS cell lines. Nutlin-3 dose-dependently inhibited the growth of RMS-YM, RM2, and Rh18 but not of RD and Rh30 cells with mutant p53 (Fig. 2A; Supplementary Fig. S2A). Nutlin-3 slowed cell proliferation in both normal MDM2 expression and MDM2 overexpression RMS cell lines with wild-type p53. The half maximal inhibitory concentration values in the cells with wild-type p53 were 2.5 $\mu\text{mol/L}$ (RMS-YM), 2.5 $\mu\text{mol/L}$ (RM2), and 3.5 $\mu\text{mol/L}$ (Rh18; Fig. 2B; Supplementary Fig. S2B). The half maximal inhibitory concentration values were not dependent on MDM2 expression status.

p53 activation leads to cell cycle arrest in RMS cell lines with wild-type p53. Nutlin-3 strongly arrested the cell cycle of RMS cells with wild-type p53 (Fig. 3). At 24 hours after treatment with nutlin-3, the S-phase fraction decreased from 30% to 40%, to 5% to 20%. Nutlin-3 caused G₁ arrest in the Rh18 and RM2 cell lines, and G₂ arrest in the RMS-YM cell line. In contrast, nutlin-3 did not have any effect on the cell cycle progression of Rh30 and RD cell lines with mutant p53.

p53 activation leads to apoptosis in RMS cell lines with wild-type p53. At 48 hours after treatment with nutlin-3, Annexin V-positive cells increased by 30% to 50% in RMS cell lines with

wild-type p53 (Fig. 4A and B) but changed little in Rh30 and RD cells. In a concentration-dependent manner, nutlin-3 increased the mRNA levels of *PUMA*, a known transcriptional target of p53, by 2-fold to 10-fold and increased the mRNA levels of *BAX* and *NOXA* by 1.3-fold to 2.5-fold (Fig. 4C) at 8 hours after treatment with nutlin-3. In contrast, only slight differences in the mRNA levels of *PUMA*, *BAX*, and *NOXA* were found in Rh30 and RD cell lines with mutant p53. Caspase-3 activation was observed after activation of proapoptotic genes (Fig. 4D).

Nutlin-3 enhances the cytotoxicity with chemotherapeutic agents against RMS cell lines with wild-type p53. Incubation with 0.5 $\mu\text{mol/L}$ nutlin-3 alone had only a slight effect on the viability of the three RMS cell lines with wild-type p53, whereas in combination with vincristine or actinomycin D, it enhanced drug-induced cytotoxicity in each of these lines (Fig. 5A; Supplementary Figs. S3A and S4A). The combination effect was synergistic for both vincristine and actinomycin D in RM2 and Rh18 cells because the combination index values were <1.0 (Fig. 5B; Supplementary Fig. S4B). The combination effect was synergistic for actinomycin D and additive for vincristine in RMS-YM cells (Supplementary Fig. S3B). Annexin V-positive

cells were about twice as abundant in the group treated with two agents than in the single-agent treated groups in RM2 cells (Fig. 5C). The increase in apoptosis was less pronounced in RMS-YM cells (Supplementary Fig. S3C) and Rh18 cells (Supplementary Fig. S4C). The p53 protein levels in the RMS cell lines treated with nutlin-3 and vincristine or actinomycin D were higher than in cells treated with either drug alone (Fig. 5D; Supplementary Figs. S3D and S4D).

Discussion

In our study, nutlin-3 effectively restored the p53 pathway in RMS cell lines with wild-type p53. Our data confirmed that nutlin-3 activated the p53 protein via a post-transcriptional mechanism because nutlin-3 treatment did not induce p53 at the mRNA level but it significantly increased p53 at the protein level. This result is consistent with the previous finding that nutlin-3 interferes with the MDM2-p53 interaction by binding the p53 binding pocket of MDM2 protein and prevents the degradation of p53 by MDM2 (10).

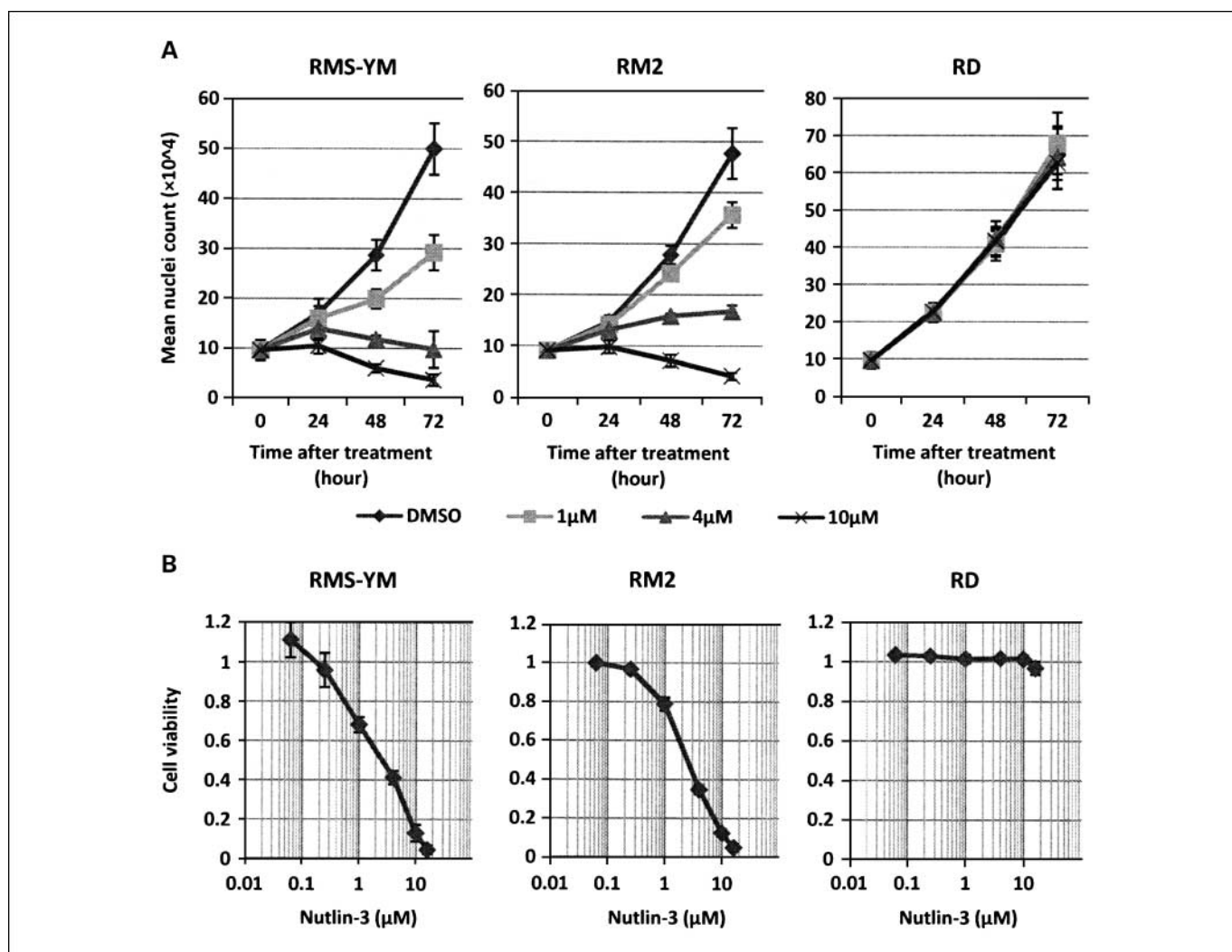


Fig. 2. Growth inhibitory effect of nutlin-3. *A*, exponentially growing cancer cells with wild-type p53 (RMS-YM and RM2) or mutant p53 (RD) were incubated with the indicated nutlin-3 concentrations, and growth was assessed by counting nuclei every 24 h. *B*, exponentially growing cancer cells with wild-type p53 (RMS-YM and RM2) or mutant p53 (RD) were incubated with the indicated nutlin-3 concentrations for 48 h, and the viability was measured with the WST-8 assay.

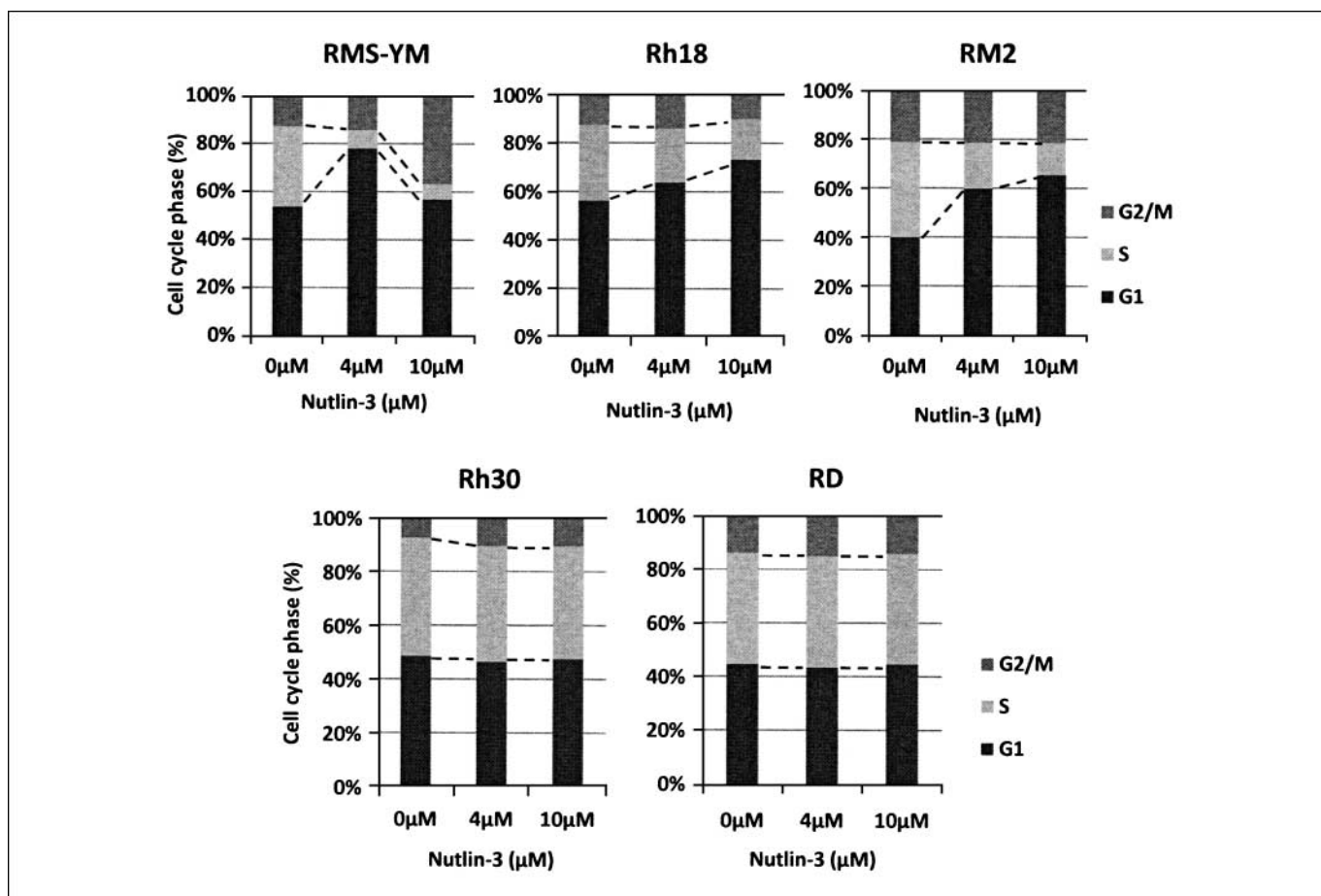


Fig. 3. Induction of cell cycle arrest by nutlin-3. Cell cycle distribution was monitored by flow cytometry after treatment with 4 or 10 $\mu\text{mol/L}$ nutlin-3 or an equivalent amount of DMSO for 24 h.

We also showed that the p53 protein induced by nutlin-3 was transcriptionally active and effectively induced both cell cycle arrest and apoptosis. The induced p53 protein increased expression of the cyclin-dependent kinase inhibitor p21 and proapoptotic genes in RMS cell lines with wild-type p53. The induction of p21 and proapoptotic genes showed that the downstream p53 pathway of the RMS cell lines with wild-type p53 used in this study was active. On the other hand, nutlin-3 did not have any effect on cell cycle arrest or apoptosis in RMS cell lines with mutated p53, indicating the effect of nutlin-3 as a single agent was dependent on the p53 status.

In our study, nutlin-3 treatment led to accumulations of cells in the G_1 phase in the Rh18 and RM2 cell lines, and in the G_2 phase in the RMS-YM cell line. Recently, Kan et al. showed that nutlin-3 can induce G_1 arrest through suppression of retinoblastoma protein phosphorylation and can also induce G_2 arrest through suppression of CDC2 and cyclin B (22). Which pathway is suppressed more strongly apparently depends on the cell type. Thus, a possible explanation for the difference in arrests is that retinoblastoma protein phosphorylation is more strongly suppressed in the Rh18 and RM2 cells, whereas the CDC2/cyclin B pathway is more suppressed in the RMS-YM cells.

Four studies have looked into the correlation between p53 status and prognosis with differing results (4, 23–25). Two studies showed that p53 overexpression is correlated with poor

prognosis (23, 24). On the other hand, another study showed that p53 mutation is not associated with poor prognosis (4), and a recent study of 150 RMS cases did not confirm an association between elevated p53 expression and poor prognosis (25). The relationship between p53 status and prognosis remains to be elucidated. Therefore, RMS with poor prognosis may retain wild-type p53 and patients with refractory RMS could be rescued by nutlin-3. If nutlin is used in a clinical trial, the p53 status of the tumor should be checked before treatment. It is important to learn how p53 status is related to prognosis.

There is some controversy over whether the effect of nutlin-3 is correlated with MDM2 expression. Some reports have shown that nutlin-3 was more effective against cancer cells with high MDM2 expression than against cancer cells with normal MDM2 expression (13, 14), and other studies showed that the effect of nutlin-3 did not correlate with MDM2 expression level (11, 26). In our study, the effect of nutlin-3 was almost the same in terms of half maximal inhibitory concentration and apoptosis whether or not MDM2 was overexpressed. It is likely that other factors besides MDM2 expression status affect the effectiveness of nutlin-3. One factor may be the potency of the p53 downstream apoptotic pathway. With the use of nutlin-3 to probe downstream p53 signaling, Tovar et al. found that the cell cycle arrest function of the p53 pathway was preserved in every tested tumor-derived cell line expressing wild-type p53,

but the ability to undergo p53-dependent apoptosis was lost in some of the cell lines tested (27). They also showed that cancer cells with an aberrant apoptotic pathway were less sensitive to nutlin-3 (27). Our results showed that the three RMS cell lines with wild-type p53 retained the ability to undergo p53-dependent apoptosis (Fig. 4). We speculate that this potency of the apoptotic pathway is the reason why nutlin-3 was almost equally effective against the three RMS cell lines with wild-type p53 in our study. Nutlin-3 seems to be effective irrespective of MDM2 status as long as the apoptotic pathway of the cancer cells is potent.

Vincristine and actinomycin D are used in current standard treatments of RMS. A low concentration of nutlin-3 in combination with vincristine or actinomycin D had an enhanced antitumor effect. Combination treatment with nutlin-3 and vincristine or actinomycin D led to activation of the p53 protein and was more effective at inducing apoptosis than treatment with either agent alone. These observations suggest that a

combination of an MDM2 antagonist and a currently used chemotherapeutic agent might be a useful approach to enhance antitumor efficacy in RMS cells.

After the introduction of intense chemotherapy, the prognosis of childhood cancers has improved. However, the side effects of genotoxic treatments are becoming major problems for childhood cancer survivors. Treatment regimens with a less genotoxic agent are desirable. When p53 is activated by nutlin-3, Ser15 of p53 is not phosphorylated, and this means p53 activation is nongenotoxic (10). Because nutlin-3 is effective with genotoxic agents in various cancers (15, 28–31), it may be possible to decrease the dose of genotoxic agents.

Recent studies suggest that nutlin-3 is less toxic to normal cells than to neoplastic cells. Although transient p53 activation by blockade of the MDM2-p53 interaction led to cell cycle arrest in both normal cells and neoplastic cells, normal cells were more resistant to apoptosis than neoplastic cells (31–33). In the xenograft mouse model, neither weight loss nor

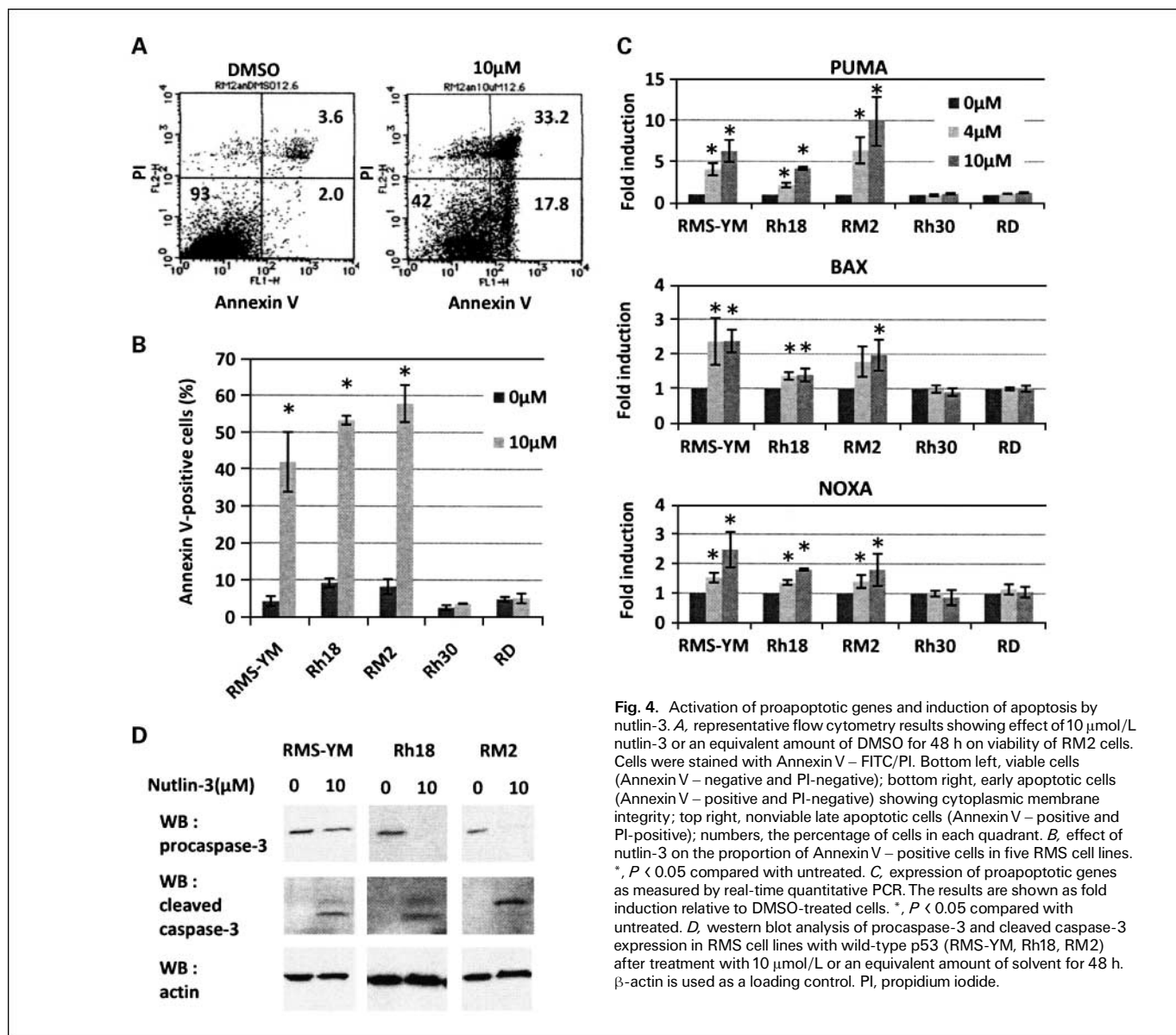


Fig. 4. Activation of proapoptotic genes and induction of apoptosis by nutlin-3. *A*, representative flow cytometry results showing effect of 10 µmol/L nutlin-3 or an equivalent amount of DMSO for 48 h on viability of RM2 cells. Cells were stained with Annexin V – FITC/PI. Bottom left, viable cells (Annexin V – negative and PI-negative); bottom right, early apoptotic cells (Annexin V – positive and PI-negative) showing cytoplasmic membrane integrity; top right, nonviable late apoptotic cells (Annexin V – positive and PI-positive); numbers, the percentage of cells in each quadrant. *B*, effect of nutlin-3 on the proportion of Annexin V – positive cells in five RMS cell lines. *, *P* < 0.05 compared with untreated. *C*, expression of proapoptotic genes as measured by real-time quantitative PCR. The results are shown as fold induction relative to DMSO-treated cells. *, *P* < 0.05 compared with untreated. *D*, western blot analysis of procaspase-3 and cleaved caspase-3 expression in RMS cell lines with wild-type p53 (RMS-YM, Rh18, RM2) after treatment with 10 µmol/L or an equivalent amount of solvent for 48 h. β-actin is used as a loading control. PI, propidium iodide.

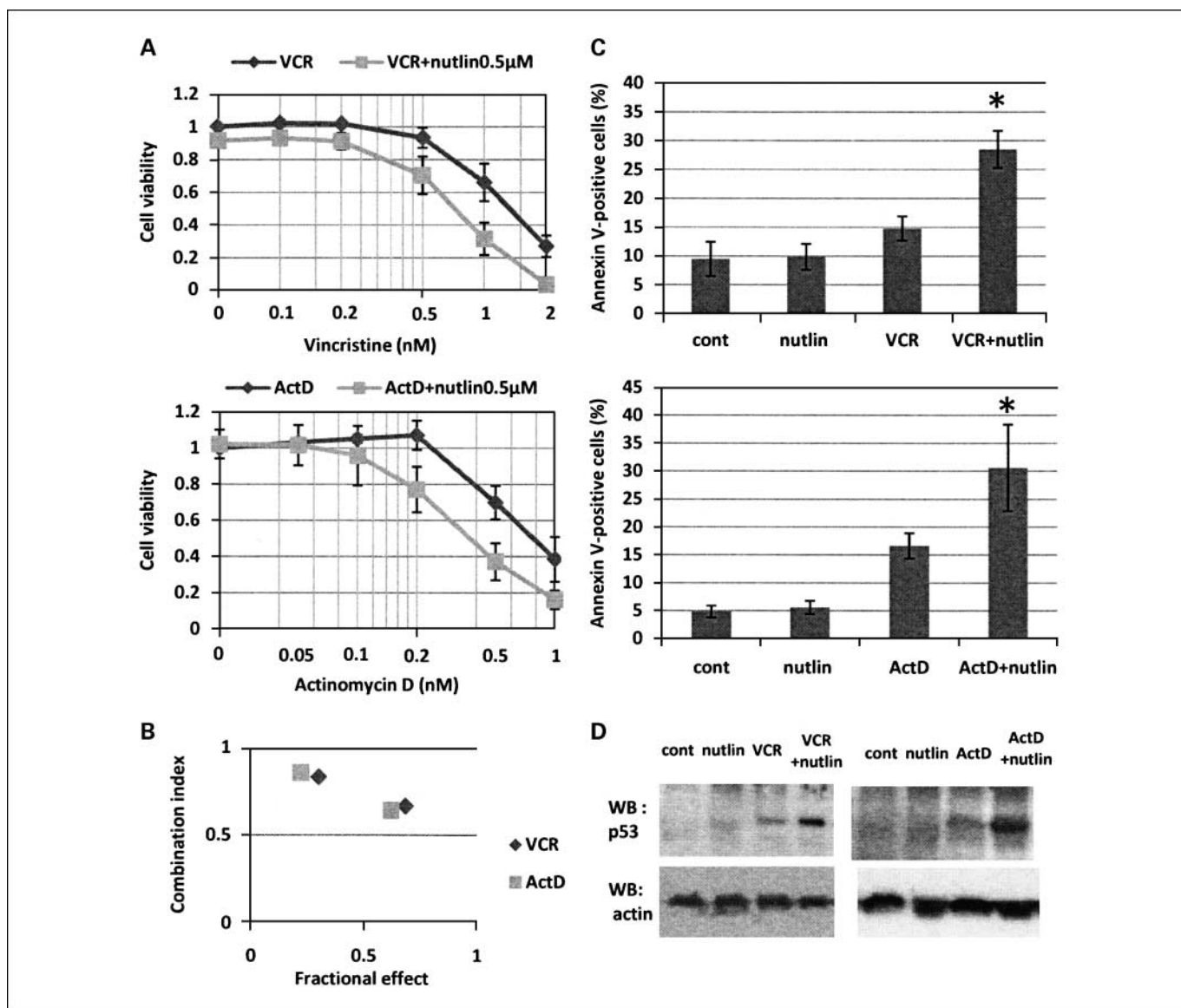


Fig. 5. Nutlin-3 enhances the cytotoxicity of currently used chemotherapeutic agents against RM2 cell line. *A*, RM2 cell line with wild-type p53 was incubated for 96 h with increasing dose of vincristine or actinomycin D alone or in combination with 0.5 μmol/L nutlin-3. Cell viability was assessed with the use of WST-8 assay. *B*, combination index was determined in relation to the fraction of cells affected with the use of the Chou-Talalay method to characterize interactions of nutlin-3 with vincristine or actinomycin D. *C*, effect of nutlin-3 in combination with vincristine and actinomycin D. Viability was assessed by Annexin V – FITC/PI staining and measured by flow cytometry. Cells were treated with 0.5 μmol/L nutlin-3, 0.5 nmol/L vincristine, 0.5 nmol/L actinomycin D, or a combination of nutlin-3 and vincristine or actinomycin D for 96 h. *, $P < 0.05$ compared with untreated and single-agent treated. *D*, western blot analysis of p53 expression in RM2 cell line after exposure to 0.5 μmol/L nutlin-3, 0.5 nmol/L vincristine, 0.5 nmol/L actinomycin D, or a combination of nutlin-3 and vincristine or actinomycin D. β-actin is used as a loading control.

significant pathologic changes of normal tissues were observed in mice given therapeutically effective doses of nutlin-3 (27). So far, none of the results of these experiments indicate any safety problems with nutlin-3. Further animal studies and clinical trials are needed to evaluate the safety of MDM2 antagonists.

Taken together, these results show that nutlin-3 restores p53 downstream signaling in RMS. Our data suggest that specific pharmacologic activation of p53 by an MDM2 antagonist can be achieved in RMS cells. Selective p53 activation with the nongenotoxic MDM2 antagonist nutlin-3 induces cell cycle arrest and apoptosis in both normal MDM2 expression and MDM2 overexpression RMS cell lines with wild-type p53. Nutlin-3 was effective against both the embryonal and alveolar

subtypes of RMS. Nutlin-3 enhanced cytotoxicity with chemotherapeutic agents against RMS cell lines with wild-type p53. Thus, nongenotoxic activation of the p53 pathway could be a promising future treatment option for patients with RMS.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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