Inhibition of p53-Murine Double Minute 2 Interaction by Nutlin-3A Stabilizes p53 and Induces Cell Cycle Arrest and Apoptosis in Hodgkin Lymphoma

Elias Drakos,[1] Athanasios Thomaides,[1] L. Jeffrey Medeiros,[1] Jiang Li,[1] Vasiliki Leventaki,[1] Marina Konopleva,[2] Michael Andreeff,[2] and George Z. Rassidakis[1]

Abstract

Purpose: p53 is frequently expressed but rarely mutated in Hodgkin and Reed-Sternberg (HRS) cells of Hodgkin's lymphoma (HL). p53 protein levels are regulated by murine double minute 2 (MDM2) through a well-established autoregulatory feedback loop. In this study, we investigated the effects of nutlin-3A, a recently developed small molecule that antagonizes MDM2 and disrupts the p53-MDM2 interaction, on p53-dependent cell cycle arrest and apoptosis in cultured HRS cells.

Experimental Design: HL cell lines carrying wild-type (wt) or mutated *p53* gene were treated with the potent MDM2 inhibitor nutlin-3A or a 150-fold less active enantiomer, nutlin-3B.

Results: We show that nutlin-3A, but not nutlin-3B, stabilizes p53 in cultured HRS cells carrying wt *p53* gene resulting in p53-dependent cell cycle arrest and apoptosis. Cell cycle arrest was associated with up-regulation of the cyclin-dependent kinase inhibitor p21. Nutlin-3A–induced apoptotic cell death was accompanied by Bax and Puma up-regulation and caspase-3 cleavage and was abrogated, in part, by inhibition of caspase-9 and caspase-3 activity. By contrast, no effects on cell cycle or apoptosis were found in HL cell lines harboring mutated *p53* gene. Furthermore, combined treatment with nutlin-3A and doxorubicin revealed enhanced cytotoxicity in HRS cells with wt *p53* gene. Blocking of nuclear export by leptomycin B, or inhibition of proteasome by MG132, stabilized p53 at a level comparable with that of nutlin-3A treatment in HRS cells with wt *p53*.

Conclusions: These data suggest that nutlin-3A stabilized p53 by preventing MDM2-mediated p53 degradation in HRS cells. wt *p53* stabilization and activation by nutlin-3A may be a novel therapeutic approach for patients with HL.

Functional integrity of the *p53* gene is an essential cellular defense against neoplastic transformation (1). This is highlighted by the fact that ~50% of human cancers harbor mutated *p53* (mt *p53*; ref. 2). On genotoxic or nongenotoxic cellular stress, p53 is activated orchestrating several cell cycle and apoptotic regulatory pathways (1). Prominent among these is the transcriptional up-regulation of the cyclin-dependent kinase inhibitor p21, which leads to cell cycle arrest (1). In parallel, p53 may lead to apoptosis through transcriptional up-regulation of proapoptotic proteins, such as Bax, or BH3 family members, such as Puma, in concert with direct targeting of mitochondria and neutralization of antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xL (3).

Murine double minute 2 (*MDM2*; *HDM2* in humans) is a master regulator of p53 (1, 4, 5). MDM2 inhibits p53 through three mechanisms: first, binding of MDM2 to the transactivation domain of p53 inhibits p53 transcriptional activity; second, binding of MDM2 to p53 facilitates its export from the nucleus toward proteasomal degradation; and third, MDM2 acts as p53-ubiquitin ligase augmenting degradation by the proteasome and thus down-regulating p53 protein levels (4, 5). In turn, MDM2 is a transcriptional target of *p53* establishing an autoregulatory loop: p53 induces *MDM2* gene expression, and MDM2, in turn, leads to p53 inactivation and degradation (1). Thus, amplification of *MDM2* gene has been proposed as an alternative mechanism of inactivation of the p53 pathway in human cancers (6).

Because almost half of human cancers harbor unmutated or wild-type (wt) *p53* gene, many efforts have been made in developing therapeutic agents that specifically activate p53, with MDM2 antisense oligonucleotides being a paradigm (7–9). Recently, nutlin-3A, a small molecule that binds the p53-binding site of MDM2, was developed (10). By disrupting

Authors' Affiliations: Departments of [1]Hematopathology and [2]Blood and Marrow Transplantation, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Received 10/25/06; revised 2/28/07; accepted 3/20/07.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

E. Drakos did experiments and contributed to the writing of the manuscript; A. Thomaides, J. Li, and V. Leventaki did experiments; M. Andreeff, M. Konopleva, and L.J. Medeiros contributed vital reagents and contributed to the writing of the manuscript; and G.Z. Rassidakis designed experiments and contributed to the writing of the manuscript.

Requests for reprints: George Z. Rassidakis, Department of Hematopathology, The University of Texas M. D. Anderson Cancer Center, Unit 54, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-745-2535; Fax: 713-792-7273; E-mail: gzrassidakis@mdanderson.org.

doi:10.1158/1078-0432.CCR-06-2581

the interaction between MDM2 and p53, nutlin-3A has been shown to stabilize p53 protein, specifically activate the p53 pathway, and have *in vitro* and *in vivo* antitumor activity against a variety of solid tumors and some hematologic malignancies harboring wt *p53*. The later include plasma cell myeloma, acute myeloid leukemias, and chronic lymphocytic leukemia (10–16).

Classic Hodgkin's lymphoma (HL) accounts for ~30% of all lymphomas (17) and approximately two thirds of HL patients can be cured with standard chemotherapeutic agents (18). However, despite increased biological understanding and refinement of traditional therapeutic schemes, approximately one third of patients fail therapy and eventually die of disease (18). In addition, the high frequency of late complications, including secondary malignancies, in long-term survivors, points to the necessity for "targeted" and more relevant biological therapies (19–22).

Many studies have shown that Hodgkin and Reed-Sternberg (HRS) cells of most HL tumors have wt *p53* gene and express p53 protein, which is functional and capable of inducing expression of target genes, such as *MDM2* or *p21* (23–29). In this study, we hypothesized that wt *p53* can be stabilized by inhibiting MDM2-mediated degradation of the protein in HL. Using an *in vitro* system with cultured HRS cells carrying wt or mt *p53* genes, we show here that nutlin-3A can stabilize wt *p53* by inhibiting its degradation and induce nongenotoxic activation of the p53 pathway resulting in G_1-S cell cycle arrest and apoptosis. In addition, we show that nutlin-3A synergizes with classic chemotherapeutic agents and enhances their activity against HRS cells. This is the first study to provide evidence for the antitumor activity of MDM2 antagonists in HRS cells and the potential of wt *p53* stabilization and activation as a novel therapeutic strategy for patients with HL.

Materials and Methods

Cell lines and reagents. Six HL cell lines were used in this study, including MDA-V and KM-H2 (wt *p53* gene) and L-428, L1236, HDML2, and HD-MyZ (mt *p53* gene; ref. 30). MDA-V is a novel cell line (gift from Dr. R. Ford, The University of Texas M. D. Anderson Cancer Center, Houston, TX) that has been established recently and characterized in our Institution.[3] MDA-V was established from lymph nodes of a previously untreated patient with stage I classic HL of nodular sclerosis type. Karpas 299, an anaplastic large cell lymphoma cell line known to express high levels of p53 protein due to a point mutation at codon 273 of exon 8, was used as a positive control (31). All cells were maintained in RPMI 1640 supplemented with 15% FCS (Invitrogen Corp.), at 37°C, in a humidified atmosphere containing 5% CO_2.

The selective small-molecule antagonists of MDM2, nutlin-3A and its 150 times less active enantiomer nutlin-3B (diluted in DMSO, as 10 mmol/L stock solution), were kindly provided by Dr. Lyubomir T. Vassilev (Hoffmann-La Roche, Nutley, NJ). Doxorubicin, leptomycin B, the proteasome inhibitor MG132, the caspase-3 inhibitor DEVD-CHD, and the caspase-9 inhibitor Z-LEHD-FMK were purchased from Calbiochem. All reagents were used in different concentrations as indicated.

Reverse-transcription PCR and direct sequencing of p53 cDNA. Total RNA was extracted from the cells using the RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using the SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen Life Technologies) according to the manufacturer's instructions. The quality of cDNA was tested by PCR, using primers specific for β-actin cDNA. To amplify the entire open reading frame of the *p53* gene, the following primers were used: 5′-AAGTCTAGAGCCACCGTCCA-3′ (forward) and 5′-TGCAAG-CAAGGGTTCAAAGAC-3′ (reverse). The PCR program included cDNA denaturation at 95°C (5 min) followed by 35 cycles of 95°C (30 s), 56°C to 58°C (30 s), and 72°C (1 min) and lastly extension at 72°C (5 min). The PCR products were subcloned into pCR2.1-TOPO vector (Invitrogen) and sequenced. Sequencing was done using the GeneAmp PCR System 9600 (Perkin-Elmer), fluorescently labeled M13 forward and reverse primers, and AmpliTaq-FS DNA polymerase (Perkin-Elmer) according to the manufacturer's directions.

Tumor samples and immunohistochemistry. Mouse monoclonal antibodies to p53 and MDM2 (both from DakoCytomation) were used. Immunohistochemical methods were used to assess protein expression in formalin-fixed, paraffin-embedded tumor samples and cell blocks from MDA-V, KM-H2, and L-428 using methods described previously (32). In addition, 27 classic HL tumors obtained from previously untreated patients under an Institutional Review Board–approved protocol were assessed for p53 expression using immunohistochemistry and the same anti-p53 antibody. In each tumor sample, at least 100 HRS cells in representative fields were manually counted to determine the percentage of p53-positive HRS cells.

3-(4-5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay. MDA-V, KM-H2, and L-428 cells were treated with nutlin-3A, nutlin-3B, chemotherapeutic agents, or combinations in six-well plates using different concentrations as indicated. At 48 h, a tetrazolium compound [3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] was added to each well and 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt–positive cells were counted using the CellTiter 96 Aqueous cell proliferation assay (Promega) and μQuant spectrophotometer (Bio-Tek Instruments, Inc.) according to the manufacturer's instructions.

Cell cycle analysis. Cells were fixed overnight in ice-cold ethanol (70% v/v) and stained for 30 min with propidium iodide solution [50 μg/mL propidium iodide and 200 units/mL DNase-free RNase, in phosphate buffer solution (pH 7.4); Roche Applied Science] at 37°C. DNA content was determined using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems) and the cell cycle was analyzed using ModFit LT software (Verity Software House).

Cell viability and apoptosis studies. Cell viability was evaluated using trypan blue exclusion cell counts in triplicate. Annexin V staining (BD Biosciences PharMingen) detected by flow cytometry was used to assess apoptosis according to the manufacturer's instructions. Briefly, the cells were washed in ice-cold PBS and resuspended in binding buffer at a concentration of 1×10^6 cells/mL. Aliquots of 100 μL (1×10^5 cells/mL) were incubated with 5 μL Annexin V-FITC for 15 min followed by 5 μL propidium iodide for 1 min in the dark at room temperature. Ungated cells (1×10^4) were then counted using a flow cytometer (FACSCalibur). Cytospin preparations of nutlin-3A– or nutli-3B–treated cells were stained with 4′,6-diamidino-2-phenylindole and examined by fluorescence microscopy for morphologic evidence of apoptosis. Control cells were included in each set of experiments. All experiments were done at least twice.

Western blot analysis. Cells in log-phase growth were collected, washed twice in cold PBS, and lysed at 4°C in lysis buffer using protease and phosphatase inhibitors as described previously (32). Cell lysates containing 50 mg total protein were resolved in 10% SDS-PAGE, transferred to nitrocellulose polyvinylidene difluoride membranes (Amersham Pharmacia), and probed with primary antibodies. The antibodies used included p53, p21, Bax, Bcl-2 (DakoCytomation), ^{Ser15}p-p53, Puma (Cell Signaling Technology), total and activated caspase-3 (BD Biosciences PharMingen), and β-actin (control for protein load; Sigma). Detection and signal visualization were done using appropriate secondary antibodies conjugated with horseradish peroxidase (Bio-Rad) and enhanced chemiluminescence reagents (Amersham Pharmacia).

[3] In preparation.

Immunofluorescence and confocal microscopy. The primary antibodies used for immunofluorescence were the mouse monoclonal antibodies anti-p53 and anti-MDM2 (both from DakoCytomation). Briefly, cytospin cell preparations were washed twice with PBS, fixed, and permeabilized with ice-cold acetone and ethanol solution (70-30% v/v). Nonspecific binding of primary antibodies was blocked with incubation with 1% bovine serum for 30 min. After washing, cell preparations were incubated with primary antibody overnight at 4°C and then with Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes) diluted in 1% bovine serum for 30 min. ToPro3 was used as counterstain. Immunofluorescence was detected using a 60×/1.40 PlanApo objective lens on an Olympus FV500 confocal microscope with Fluoview version 4.3 software (Olympus). Staining of cells omitting the primary antibodies step served as negative controls in these experiments.

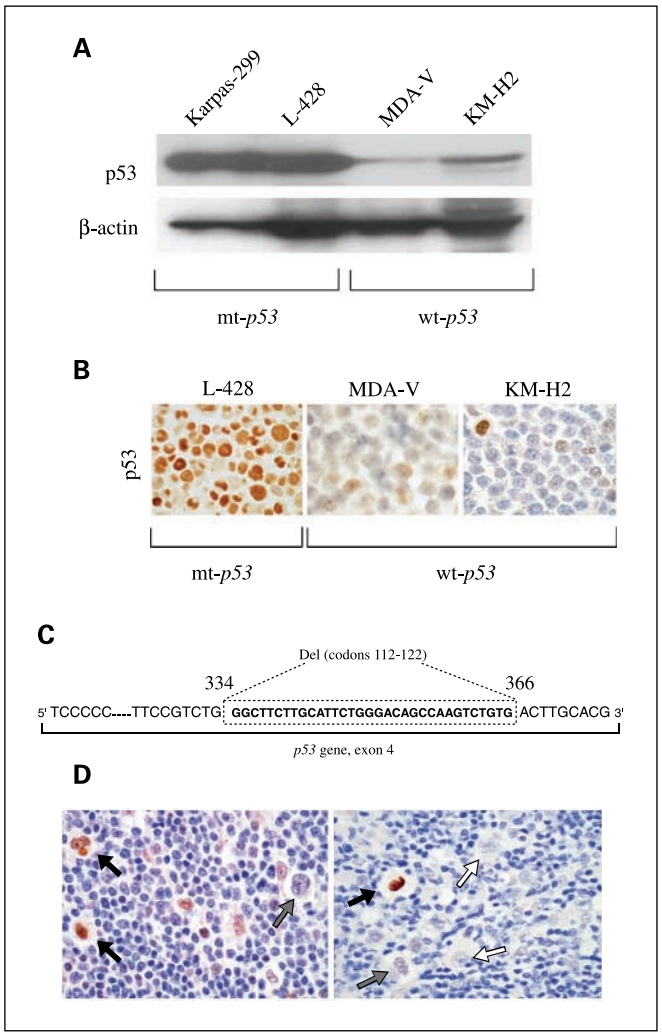


Fig. 1. Expression of p53 in HRS cells. *A* and *B,* expression levels of p53 was assessed in three HL cell lines using Western blot analysis and immunohistochemical methods. Karpas 299, an anaplastic large cell lymphoma cell line known to express high levels of mt *p53* (31), was used as a positive control. Immunoblots showed high levels of p53 in L-428, whereas KM-H2 and MDA-V expressed very low levels of p53. Immunohistochemistry of cell blocks showed that MDA-V and KM-H2 express p53 at a very low level compared with that of L-428 that carry a mutant *p53* gene. *C,* amplification of p53 cDNA and direct sequencing of the entire open reading frame of the *p53* gene showed that MDA-V and KM-H2 cells carry wt *p53* gene (not shown). By contrast, a 12-codon deletion in exon 4 corresponding to the DNA-binding domain was detected in L-428 cells. *D,* immunohistochemical analysis in HL tumors showed that p53 is expressed by a highly variable percentage of primary neoplastic HRS cells ranging from 11% to 99% with variable staining intensity indicating the potential of further p53 up-regulation in these cells. p53 immunoreactivity was predominantly nuclear. Black arrows, strongly p53-positive HRS cells; gray arrows, weakly p53-positive HRS cells; white arrows, p53-negative HRS cells.

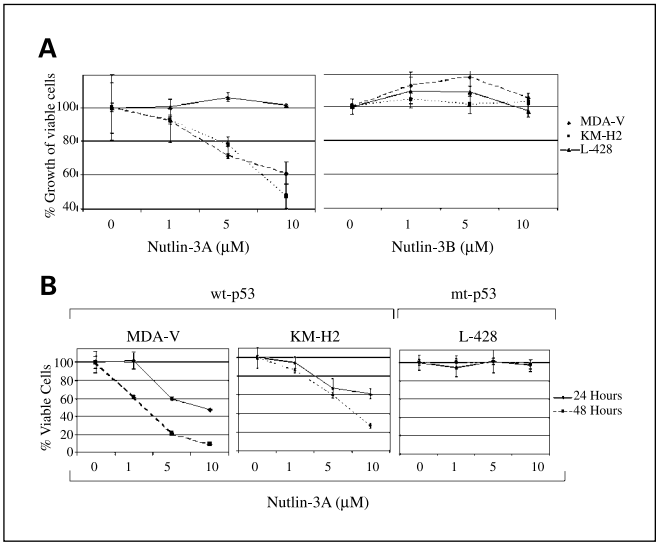


Fig. 2. Nutlin-3A inhibits the growth of HL cells that harbor wt *p53 gene. A,* 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay showed a dose-dependent inhibition of cell growth in MDA-V and KM-H2 but not L-428 treated with nutlin-3A. No effect was observed in cells treated with nutlin-3B. At 48 h after treatment with 10 μmol/L nutlin-3A, the growth of MDA-V and KM-H2 cells was inhibited by 40% and 52%, respectively. *B,* the number of viable cells, as measured by trypan blue exclusion assay, was reduced dramatically after nutlin-3A in MDA-V and KM-H2, by 90% and 73%, respectively. By contrast, no change in the number of viable cells was observed in L-428 after nutlin-3A nor in all three cell lines treated with the same concentrations of nutlin-3B. All experiments were repeated at least trice with similar results.

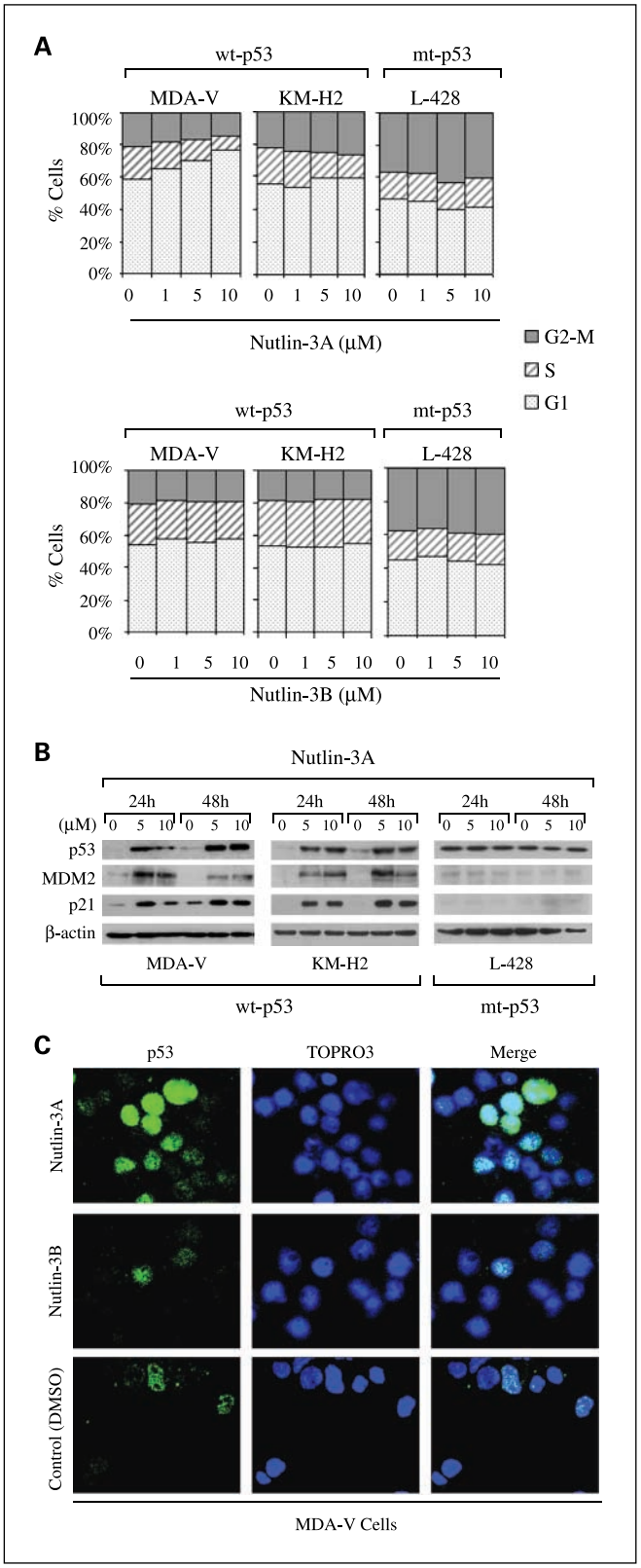
Results

Expression and mutation status of p53 in HRS cells. To investigate the expression levels and mutation status of the *p53* gene in HL cell lines, Western blot and immunohistochemical analysis were done in cultured HRS cells. As shown in Fig. 1A, KM-H2 and MDA-V cells showed low levels of p53 expression compared with those of L-428 that carry a mutant *p53* gene (Fig. 1A). Immunohistochemical analysis of p53 expression in MDA-V, KM-H2, and L-428 cell blocks confirmed the Western blot data (Fig. 1B). Direct sequencing of the entire open reading frame of the *p53* gene following cDNA amplification confirmed the *p53* gene status in all HL cell lines used in the study (Fig. 1C; Supplementary Fig. S1).

Immunohistochemical analysis of p53 expression in 27 classic HL tumors showed that the neoplastic HRS cells express p53 in all cases (Fig. 1D). However, the percentage of p53-positive HRS cells was highly variable among the classic HL tumors ranging from 11% to 99% (median, 48%). The intensity of p53 staining reflecting the p53 protein expression level also varied significantly from weak to strong in HRS cells (Fig. 1D). These data are in accordance with previously published results (26, 33) and indicate the potential of further up-regulation of wt *p53* by modulation of p53 stabilization mechanisms in primary HRS cells.

Antitumor effect of nutlin-3A in HRS cells is dependent on the p53 mutation status. To show that the antitumor effects of

nutlin-3A are dependent on *p53* gene status in HRS cells, p53-mutated and p53 unmutated HL cell lines were treated with increasing concentrations of the MDM2 antagonist nutlin-3A or nutlin-3B. As shown in Fig. 2A, treatment with nutlin-3A showed a dose-dependent antitumor activity against MDA-V and KM-H2 cells but not in L-428 cells. 3-(4-5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay showed that, at 48 h after incubation with 10 μmol/L nutlin-3A, the growth of viable MDA-V and KM-H2 cells was inhibited by 40% and 26%, respectively. More importantly, the total number of viable cells, as measured by trypan blue exclusion assay, was dramatically decreased by 90% and 73%, in MDA-V and KM-H2, respectively (Fig. 2B). By contrast, treatment with the 150 times less active nutlin-3B had no effect on HRS cell viability or growth irrespective of *p53* mutation status (Fig. 2A and B).



Nutlin-3Q induces cell cycle arrest in HRS cells through stabilization of p53 and up-regulation of p21. To investigate the effects of p53 stabilization on cell cycle progression, we did cell cycle analysis. Nutlin-3A treatment of HRS cells harboring wt *p53* showed dose-dependent reduction of S phase fraction (Fig. 3A). At 24 h after treatment with 10 μmol/L nutlin-3A, the fraction of MDA-V and KM-H2 cells in S phase decreased approximately by 60% and 35%, respectively, associated with a concentration-dependent increase of the G_1 phase fraction (MDA-V) or G_1 and G_2-M (KM-H2), indicating cell cycle arrest at G_1-S checkpoint or both G_1-S and G_2-M checkpoints. In contrast, no effect on cell cycle progression was observed in L-428 cells with mt *p53*. Treatment with nutlin-3B had no effect in all cell lines tested, suggesting that the effects on cell cycle are largely attributed to inhibition of p53-MDM2 interaction by the active nutlin-3A (Fig. 3A). This was further supported by Western blot analysis, which showed stabilization of p53 protein levels associated with a parallel increase in MDM2 levels in HRS cells with wt *p53* gene. Cell cycle changes were associated with a dose-dependent increase in the expression levels of the cyclin-dependent kinase inhibitor p21, a known transcriptional target of p53, indicating that stabilized p53 is functional and capable of inducing p21 expression in our *in vitro* system (Fig. 3B). Increased p53 protein levels after treatment with nutlin-3A in HRS cells with wt *p53* were also shown using immunofluorescence and confocal microscopy (Fig. 3C).

Nutlin-3A induces apoptotic cell death in HRS cells through activation of the p53 apoptotic pathway. Treatment with nutlin-3A resulted in a dose-dependent decrease in cell viability of HRS harboring wt *p53* but not in cells with mt *p53* gene. Nutlin-3A–induced cell death was substantially higher the 2nd day of treatment. Forty-eight hours after incubation with

Fig. 3. Nutlin-3A induces cell cycle arrest in HRS cells through activation of the p53 pathway. *A,* at 24 h after treatment with 10 μmol/L nutlin-3A, the fraction of cells in S phase in MDA-V and KM-H2 decreased dramatically by 60% and 35%, respectively, with a concomitant concentration-dependent increase in G_1 phase (MDA-V) or G_1 and G_2-M phase (KM-H2), indicating G_1-S or G_1-S and G_2-M phase cell cycle arrest. No effect on the cell cycle was observed in the p53-mutated HL cell line L-428 or after control treatment with nutlin-3B. *B,* Western blot analysis showed a dose-dependent increase in the level of the cell cycle inhibitor p21, parallel with the increase in p53 and MDM2 levels, in KM-H2 and MDA-V at 24 and 48 h after treatment with nutlin-3A. No change in p53, MDM2, or p21 protein level was seen in L-428 cells treated with nutlin-3A or in any cell line used after treatment with nutlin-3B. *C,* increased levels of p53 protein at 24 h following treatment with nutlin-3A in MDA-V and KM-H2 cells also was found using immunofluorescence and confocal microscopy. Results with MDA-V cells are shown. p53 localization was predominantly nuclear (*top*). No change in the expression level of p53 was seen in HRS cells treated with nutlin-3B (*bottom*). ToPro3 (*blue*) was used for counterstain of the nuclei.

10 μmol/L nutlin-3A, the viability of MDA-V and KM-H2 cells decreased from ~95% (untreated) to 36% and 48%, respectively (Fig. 4A). After 72 h, the viability of KM-H2 cells was further decreased to 30%, whereas no additional cell death was observed in MDA-V cells (data not shown). By contrast, nutlin-3A–treated L-428 cells showed no change in cell viability. In addition, treatment with nutlin-3B had no effect in cell viability.

To investigate whether apoptotic mechanisms were involved in nutlin-3A–induced cell death, Annexin V staining and flow cytometry studies were done. As shown in Fig. 4B, there was a concentration-dependent increase in Annexin V binding in MDA-V and KM-H2 but not in L-428 cells. At 48 h following treatment with 10 μmol/L nutlin-3A, Annexin V binding was increased from ~6% (untreated) to 39% in both MDA-V and KM-H2 cells (Fig. 4B). Apoptotic cells were morphologically evident with 4′,6-diamidino-2-phenylindole staining and immunofluorescence, showing the presence of nuclear condensation and fragmentation in nutlin-3A–treated cells (Fig. 4C). Furthermore, selective inhibition of caspase-3 or caspase-9 using specific inhibitors rescued a significant proportion of nutlin-3A–treated MDA-V and KM-H2 cells, suggesting that apoptosis is mediated, in part, through activation of the intrinsic (mitochondrial) pathway (Fig. 4D) Again, no increase in Annexin V binding was observed in nutlin-3A–treated L-428 cells or after treatment with equivalent concentrations of nutlin-3B, indicating that induction of p53-dependent apoptosis is due to disruption of p53-MDM2 interaction and subsequent p53 stabilization.

To examine the possible mechanisms underlying p53-mediated cell death, Western blot analysis was performed. As shown in Fig. 4E, a slight increase of the proapoptotic Bax and a concentration-dependent increase of Puma, a known transcriptional target of p53, was observed 48 h after nutlin-3A treatment, at the time point when significant cell death was seen in MDA-V, whereas the levels of the antiapoptotic protein Bcl-2 remained constant (Fig. 4E). In contrast, in KM-H2 cells, only slight differences in the levels of Bax and Puma were found. However, a significant down-regulation of Bcl-2 was observed. Therefore, more than one mechanism may be involved in nutlin-3A–induced apoptosis in HRS cells.

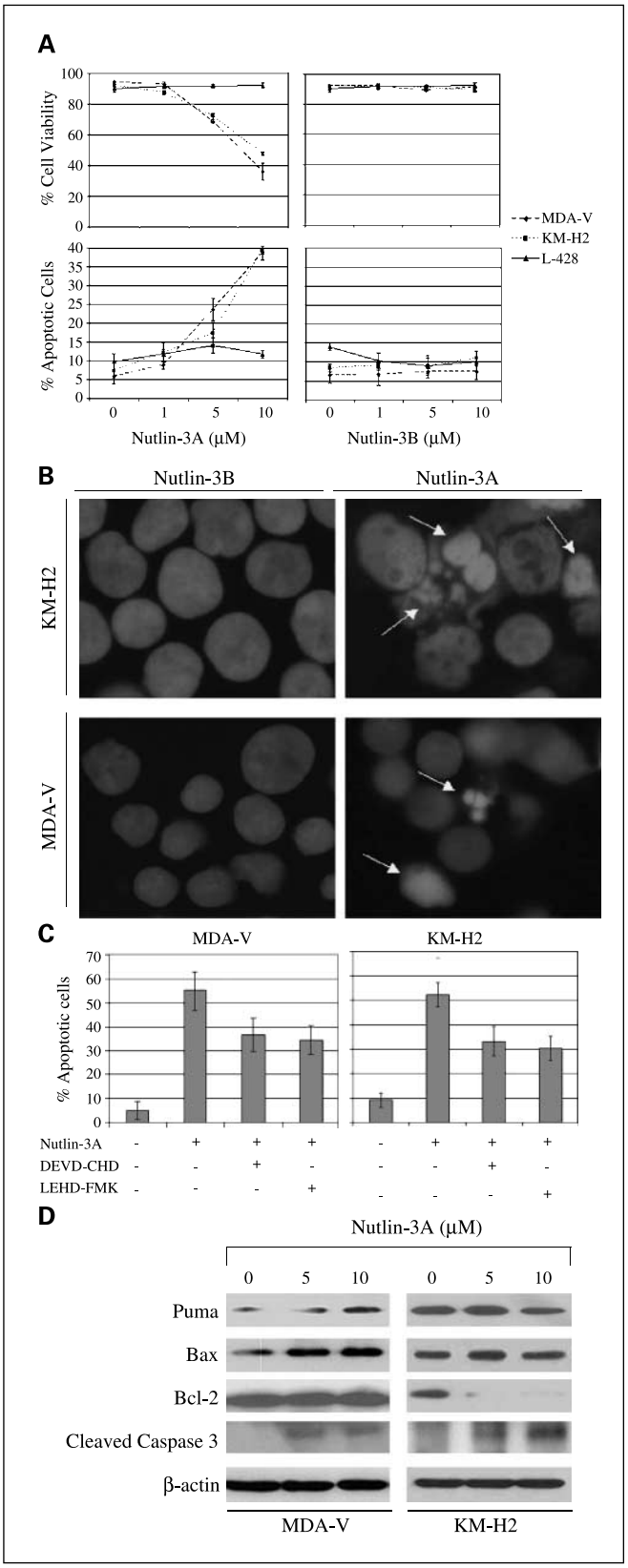


Fig. 4. Nutlin-3A induces apoptotic cell death in HL cells through activation of the p53 pathway. *A,* 48 h after incubation with 10 μmol/L nutlin-3A, the viability of MDA-V and KM-H2 cells decreased from above 95% of untreated cells to 36% and 48%, respectively (*top*). No change in cell viability was observed in L-428 cells or in any HL cell line tested after treatment with nutlin-3B. Annexin V binding in MDA-V and KM-H2 cells increased up to 39%, in a concentration-dependent manner at 48 h after treatment with different concentrations of nutlin-3A (*bottom*). Again, no change in Annexin V binding was observed in L-428 cells or after treatment with nutlin-3B. *B,* microscopic examination of the 4′,6-diamidino-2-phenylindole–stained cell preparations at 48 h after treatment with 10 μmol/L nutlin-3A showed morphologic evidence of apoptosis, including nuclear condensation and fragmentation in cultured HRS cells harboring wt *p53*. By contrast, control treatment with nutlin-3B using the same concentration showed no morphologic evidence of apoptosis. *C,* incubation of HRS cells with caspase-9 or caspase-3 inhibitors rescued, in part, nutlin-3A–induced apoptotic cell death in HRS cells. The percentage of Annexin V–positive cells is substantially decreased in MDA-V or KM-H2 cells treated with nutlin-3A in the presence of DEVD-CHD (caspase-3 inhibitor) or Z-LEHD-FMK (specific caspase-9 inhibitor), suggesting that apoptosis is partly mediated through activation of caspse-9 (intrinsic apoptotic pathway). *D,* Western blot analysis showed a slight increase of the proapoptotic protein Bax and Puma, after treatment of MDA-V or KM-H2 with nutlin-3A. Both Bax and Puma are known transcriptional targets of p53. A significant decrease of Bcl-2 levels was found observed after nutlin-3A treatment in KM-H2 but not in MDA-V cells, indicating that more than one mechanism might be involved in nutlin-3A–induced apoptosis of HRS cells. Lysates were prepared at 48 h following nutlin-3A treatment, a time point when significant nutlin-3A–induced cell death was found in MDA-V and KM-H2 cells. Western blot analysis also showed cleavage of caspases, predominantly caspase-9 and caspase-3.

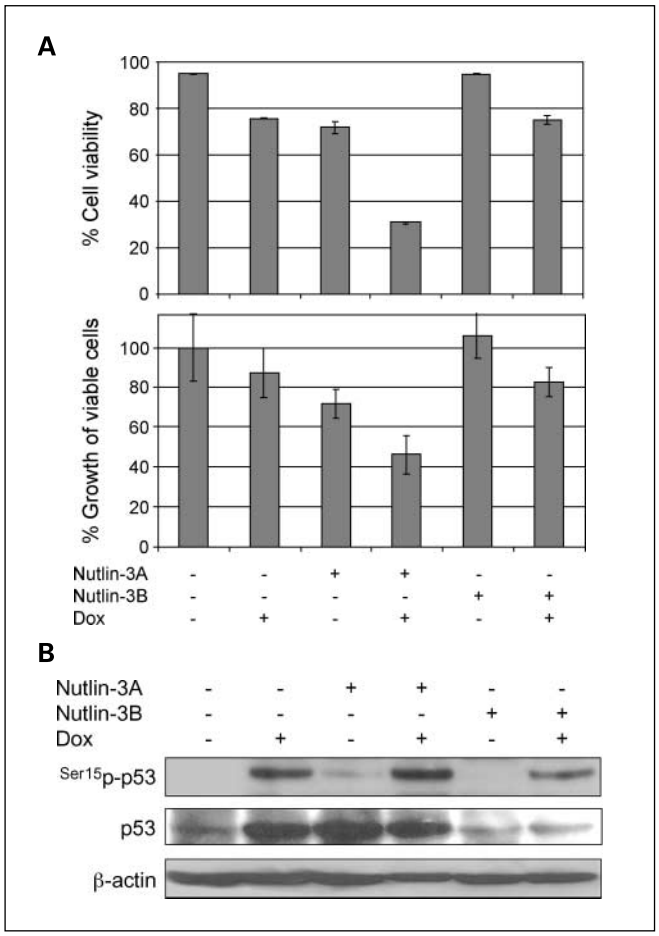


Fig. 5. Nutlin-3A enhances the antitumor activity of chemotherapeutic agents against HRS cells. *A,* combined treatment of MDA-V cells with a relatively low concentration (2 μmol/L) of nutlin-3A, compared with the high dose of 10 μmol/L, and 0.1 μmol/L doxorubicin (*Dox*) showed a substantial decrease of cell growth by 55% and an even higher decrease in cell viability by 70%, 48 h after treatment. By contrast, treatment with 0.1 μmol/L doxorubicin alone resulted in <13% decrease of cell growth and <24% decrease in cell viability. Similarly, treatment with 2 μmol/L nutlin-3A alone resulted in 29% and 28% decrease in cell growth and viability, respectively. Therefore, the effects of combined treatment (45% < 71% × 87% = 61.7% and 30% < 72% × 76% = 54.7%, respectively) indicated an at least additive effect of nutlin-3A with chemotherapeutic agents. *B,* to investigate the effects of nutlin-3A on p53 phosphorylation, the levels of ^{Ser15}p-p53 were assessed by Western blot analysis. Immunoblots showed that nutlin-3A induced p53 phosphorylation (^{Ser15}p-p53) at a very low level. By contrast, treatment with doxorubicin was associated with high expression levels of ^{Ser15}p-p53, indicating the nongenotoxic mode of p53 activation by nutlin-3A.

Nutlin-3Q enhances the activity of chemotherapeutic agents against HRS cells. To investigate whether stabilization and activation of p53 by nutlin-3a can enhance the antineoplastic activity of chemotherapeutic agents commonly used against HRS cells, combined treatment of MDA-V with a relatively low concentration of nutlin-3A and doxorubicin was used. After 48-h exposure to a concentration of 2 μmol/L nutlin-3A and 0.1 μmol/L doxorubicin, MDA-V cells showed decreased cell growth by 55% and cell viability by 70%. This decrease in cell growth and viability was more than the additive effect of each reagent separately (cell growth 45% < 71% × 87% = 61.7% and cell viability 30% < 72% × 76% = 54.7%, respectively), indicating an at least additive effect of nutlin-3A with chemotherapeutic agents (Fig. 5A and B).

Western blot analysis showed that the total level of p53 of the combined treatment was almost equal to that observed in the two separate treatments. However, analysis of p53 phosphorylation status showed that nutlin-3A induced p53 phosphorylation at Ser^{15} (^{Ser15}p-p53) at a low level compared with high levels of ^{Ser15}p-p53 after treatment with doxorubicin alone, further supporting the nongenotoxic mode of p53 activation by nutlin-3A (Fig. 5C).

Nutlin-3A–induced stabilization of p53 is posttranslational due to decreased degradation of the protein. We next investigated whether nutlin-3A–induced p53 stabilization is actually due to decreased MDM-2–mediated protein degradation in our *in vitro* system by inhibiting nuclear export and proteasome function, both of which are essential for p53 degradation. As shown in Fig. 6A, treatment of MDA-V cells with leptomycin B, an inhibitor of nuclear export, stabilized p53 with an expression level comparable with that of nutlin-3A treatment. Combined treatment of HRS cells with nutlin-3A and leptomycin B resulted in a slight additional increase of total p53 level due to significant inhibition of p53 degradation (Fig. 6A).

Similarly, treatment of MDA-V cells with MG132, a specific 26S proteasome inhibitor, resulted in a substantial increase of total p53 at a level comparable with that of nutlin-3A treatment (Fig. 6B), suggesting that p53 stabilization is due to decreased degradation through the ubiquitin-proteasome system in HL cells. Furthermore, treatment of MDA-V cells with nutlin-3A in the presence of the proteasome inhibitor resulted in no additional increase of total p53 (Fig. 6B), again most likely due to significant inhibition of p53 degradation by nutlin-3A.

Taken together, these results show that nutlin-3A–induced stabilization of p53 in HRS cells is attributable to decreased degradation of the protein.

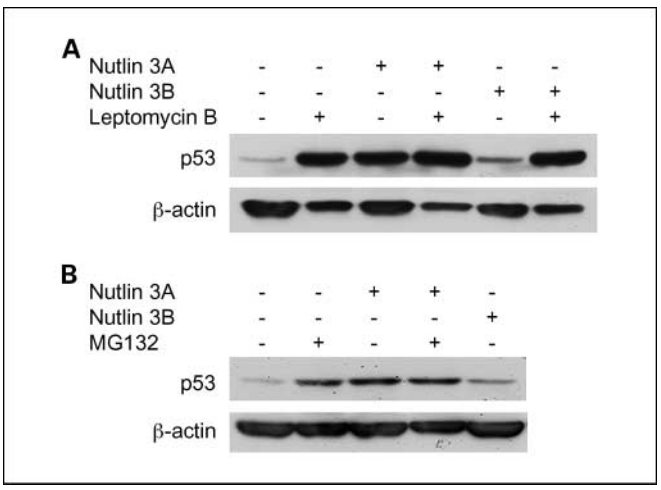


Fig. 6. Nutlin-3A–induced stabilization of p53 is due to decreased degradation of the protein in Hodgkin's lymphoma. *A,* to investigate whether nutlin-3A–induced p53 stabilization results from decreased MDM2-mediated degradation, nuclear export was inhibited with 10 nmol/L leptomycin B for 24 h. Leptomycin B treatment stabilized p53 at expression level comparable with that of nutlin-3A treatment in MDA-V cells. Combined treatment with nutlin-3A and leptomycin B resulted only in a very slight additional increase of total p53 level due to significant inhibition of p53 degradation. *B,* similarly, treatment of MDA-V cells with MG132, a specific 26S proteasome inhibitor, resulted in p53 stabilization at an expression level comparable with that of nutlin-3A treatment, suggesting that p53 stabilization is due to decreased degradation through the ubiquitin-proteasome system. Treatment of MDA-V cells with nutlin-3A in the presence of the proteasome inhibitor (pretreatment for 16 h) resulted in no additional increase of total p53, indicating that p53 degradation already was significantly inhibited by nutlin-3A.

Discussion

Although classic HL is considered to be a curable disease, approximately one third of patients fail standard therapy and late complications of long-term survivors represent a serious problem (18, 19–22). The mainstay therapeutic approach to HL is still based on the accumulative empirical knowledge of combined chemotherapy and radiotherapy despite the expanding understanding of the biology of HL (34, 35). There is need to apply new knowledge to the investigation of novel strategies targeting key molecules that control survival of HRS cells in HL.

Several previous studies, including single-cell analysis of HRS cells, have shown that the *p53* tumor suppressor gene is not mutated in most HL tumors; therefore, p53 dysfunction does not seem to be an initiating event in oncogenesis of HL (23, 24, 28, 29). However, most of the studies indicate that p53, along with its physiologic inhibitor MDM2, is overexpressed in neoplastic HRS cells, implying that the p53 pathway is somehow blocked (25, 26, 36, 37). It has also been shown, in a subset of cases, that *MDM2* gene amplification might be a mechanism for inhibition of the p53 pathway (28). Therefore, HL seems to be an ideal candidate for inhibition of p53-MDM2 interaction and subsequent stabilization and activation of wt *p53*. In this study, using the MDM2-specific antagonist nutlin-3A, we show that p53 is functional in HRS cells harboring wt *p53*. Thus, disruption of the interaction between MDM2 and p53 by nutlin-3A, through stabilization of p53 protein levels and activation of its transcriptional activity, resulted in substantial cell cycle arrest at G_1-S checkpoint, which was mediated largely by up-regulation of p21, a transcriptional target of p53.

Our data provide evidence that nutlin-3A–induced stabilization of p53 in HRS cells is largely attributable to decreased degradation of the protein. Interestingly, total p53 protein expression in HRS cells after nutlin-3A treatment seams to reach saturated levels comparable with those resulting from post-transcriptional stabilization of p53, due to significant inhibition of nuclear export or the ubiquitin-proteasome degradation system.

We also show that in HL cell lines with wt *p53*, inhibition of p53-MDM2 interaction by nutlin-3A resulted in significant apoptotic cell death, which was associated with up-regulation of two well-established transcriptional targets of p53, the proapoptotic proteins Bax and Puma. Interestingly, down-regulation of the antiapoptotic protein Bcl-2 was found after treatment with nutlin-3A only in KM-H2 cells. In agreement with previous studies using nutlin-3A in other tumor types, whereas the effect on cell cycle arrest is immediate and evident in the first 24 h of treatment, apoptosis induction is more pronounced after 48 h (10, 11). Of note, no effects of nutlin-3A on cell cycle progression or apoptosis were found in HRS cells with mt *p53*, indicating the specificity of nutlin-3A in activation of the p53 pathway. This is the first preclinical study to show antitumor activity of a potent MDM2 antagonist in HL cells with wt *p53*. However, our findings agree with recent studies in other hematologic malignancies, including multiple myeloma, B-cell chronic lymphocytic leukemia, and acute myeloid leukemia showing that nutlin-3a–induced cell cycle arrest and apoptosis is limited to neoplastic cells harboring wt *p53* (10–16).

It is known that the antineoplastic effect of genotoxic chemotherapeutic agents, many of which are used in the treatment of classic HL, is mediated largely through apoptosis induction, due in part to genotoxic activation of the p53 pathway (38, 39). Indeed, treatment of HL cells harboring wt *p53* with 0.1 μmol/L doxorubicin, a dose comparable with that used clinically, resulted in stabilization of total p53 protein at levels almost equivalent to those induced by treatment with low doses of nutlin-3a. However, doxorubicin-induced cell death was much lower compared with cell death induced by nutlin-3A. In addition, we show that chemotherapy-induced p53 is, in large part, Ser^{15} phosphorylated (^{Ser15}p-p53), most likely due to the genotoxic effect (4). In contrast, treatment of HRS cells with nutlin-3A alone stabilizes p53, which is largely unphosphorylated. These results are in accordance with previously published data showing that nongenotoxic activation of the p53 pathway by nutlin-3A is adequate for p53-induced cell death of neoplastic cells (16, 40). Because the genotoxicity of the chemotherapeutic agents is a source not only of immediate side effects but also serious late complications, the nongenotoxic mode of action of nutlin-3A might be an important advantage for the treatment of HL patients.

Combined treatment of HL cells harboring wt *p53* with doxorubicin and nutlin-3A revealed enhanced antitumor activity, a finding that has been reproduced in studies of other hematologic malignancies, indicating that nutlin-3a could be used not only as monotherapy but also as part of combined regimens for the treatment of HL, including cases with chemorefractory phenotype (12–16). Notably, recent studies suggest that the effect of nutlin-3A in normal cells differs from that in neoplastic cells (10, 41). Although both neoplastic and normal cells respond with cell cycle arrest after treatment with nutlin-3A, normal cells seem to be much more resistant to nutlin-3a–induced apoptosis compared with neoplastic cells (10, 41). In addition, the good tolerance of nutlin-3A in xenograft animal models and the almost normal phenotype of transgenic mice with overactive p53 and no alteration in the p53 isoforms are encouraging for the potential use of nutlin-3A or similar MDM2 antagonists in clinical trials (8, 10, 11).

Besides the cell autonomous function of p53 in the regulation of the cell cycle, apoptosis, DNA damage repair, senescence, and metabolism, recent findings expand its role to non-cell autonomous functions, including the regulation of angiogenesis (1, 42). Studies using animal carcinoma cells have shown that intact p53 function in the nonneoplastic supportive tissue could have an inhibitive effect on cancer growth (43). Although the effect of nutlin-3A on non-cell autonomous functions of p53 in animal models has not been studied yet, such a mechanism could be an important variable especially for classic HL, where the nonneoplastic cell population plays a role in the disease (35). The effects, if any, of nutlin-3A on the reactive cells of HL were not assessed in this study.

In conclusion, our data suggest that inhibition of p53-MDM2 interaction by a potent MDM2 antagonist, such as nutlin-3A, can lead to nongenotoxic activation of the p53 pathway resulting in G_1-S cell cycle arrest and apoptosis in cultured HRS cells carrying wt *p53*. Targeting MDM2 function to up-regulate fully functional p53 might represent a novel therapeutic strategy for patients with HL.

References

1. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408:307–10.
2. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science 1991;253:49–53.
3. Schuler M, Green DR. Transcription, apoptosis, and p53: catch-22. Trends Genet 2005;21:182–7.
4. Brooks CL, Gu W. p53 ubiquitination: Mdm2 and beyond. Mol Cell 2006;21:307–15.
5. Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. Oncogene 2005;24:2899–908.
6. Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B. Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature 1992;358:80–3.
7. Zhang R, Wang H, Agrawal S. Novel antisense anti-MDM2 mixed-backbone oligonucleotides: proof of principle, *in vitro* and *in vivo* activities, and mechanisms. Curr Cancer Drug Targets 2005;5:43–9.
8. Poyurovsky MV, Prives C. Unleashing the power of p53: lessons from mice and men. Genes Dev 2006;20:125–31.
9. Vassilev LT. p53 Activation by small molecules: application in oncology. J Med Chem 2005;48:4491–9.
10. Vassilev LT, Vu BT, Graves B, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004;303:844–8.
11. Tovar C, Rosinski J, Filipovic Z, et al. Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. Proc Natl Acad Sci U S A 2006;103:1888–93.
12. Stuhmer T, Chatterjee M, Hildebrandt M, et al. Nongenotoxic activation of the p53 pathway as a therapeutic strategy for multiple myeloma. Blood 2005;106:3609–17.
13. Kojima K, Konopleva M, Samudio IJ, et al. MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy. Blood 2005;106:3150–9.
14. Secchiero P, Barbarotto E, Tiribelli M, et al. Functional integrity of the p53-mediated apoptotic pathway induced by the nongenotoxic agent nutlin-3 in B-cell chronic lymphocytic leukemia (B-CLL). Blood 2006;107:4122–9.
15. Coll-Mulet L, Iglesias-Serret D, Santidrian AF, et al. MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. Blood 2006;107:4109–14.
16. Kojima K, Konopleva M, McQueen T, O'Brien S, Plunkett W, Andreeff M. Mdm2 inhibitor nutlin-3a induces p53-mediated apoptosis by transcription-dependent and transcription-independent mechanisms and may overcome Mdm2 and Atm-mediated resistance to fludarabine in chronic lymphocytic leukemia. Blood 2006;108:993–1000.
17. Stein H. Hodgkin lymphomas. In: Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. Tumours of haematopoietic and lymphoid tissues. Lyon (France): IARC Press; 2001. p. 239.
18. Canellos GP, Anderson JR, Propert KJ, et al. Chemotherapy of advanced Hodgkin's disease with MOPP, ABVD, or MOPP alternating with ABVD. N Engl J Med 1992;327:1478–84.
19. Behringer K, Josting A, Schiller P, et al. Solid tumors in patients treated for Hodgkin's disease: a report from the German Hodgkin Lymphoma Study Group. Ann Oncol 2004;15:1079–85.
20. Chronowski GM, Wilder RB, Levy LB, et al. Second malignancies after chemotherapy and radiotherapy for Hodgkin disease. Am J Clin Oncol 2004;27:73–80.
21. Bhatia S, Meadows AT, Robison LL. Second cancers after pediatric Hodgkin's disease. J Clin Oncol 1998;16:2570–2.
22. Linch DC, Gosden RG, Tulandi T, Tan SL, Hancock SL. Hodgkin's lymphoma: choice of therapy and late complications. Hematology Am Soc Hematol Educ Program 2000;205–21.
23. Montesinos-Rongen M, Roers A, Kuppers R, Rajewsky K, Hansmann ML. Mutation of the p53 gene is not a typical feature of Hodgkin and Reed-Sternberg cells in Hodgkin's disease. Blood 1999;94:1755–60.
24. Trumper LH, Brady G, Bagg A, et al. Single-cell analysis of Hodgkin and Reed-Sternberg cells: molecular heterogeneity of gene expression and p53 mutations. Blood 1993;81:3097–115.
25. Xerri L, Parc P, Bouabdallah R, Camerlo J, Hassoun J. PCR-mismatch analysis of p53 gene mutation in Hodgkin's disease. J Pathol 1995;175:189–94.
26. Elenitoba-Johnson KS, Medeiros LJ, Khorsand J, King TC. P53 expression in Reed-Sternberg cells does not correlate with gene mutations in Hodgkin's disease. Am J Clin Pathol 1996;106:728–38.
27. Sanchez-Beato M, Piris MA, Martinez-Montero JC, et al. MDM2 and p21WAF1/CIP1, wild-type p53-induced proteins, are regularly expressed by Sternberg-Reed cells in Hodgkin's disease. J Pathol 1996;180:58–64.
28. Kupper M, Joos S, von Bonin F, et al. MDM2 gene amplification and lack of p53 point mutations in Hodgkin and Reed-Sternberg cells: results from single-cell polymerase chain reaction and molecular cytogenetic studies. Br J Haematol 2001;112:768–75.
29. Maggio EM, Stekelenburg E, Van den Berg A, Poppema S. TP53 gene mutations in Hodgkin lymphoma are infrequent and not associated with absence of Epstein-Barr virus. Int J Cancer 2001;94:60–6.
30. Drexler HG. Recent results on the biology of Hodgkin and Reed-Sternberg cells. II. Continuous cell lines. Leuk Lymphoma 1993;9:1–25.
31. Hubinger G, Muller E, Scheffrahn I, et al. CD30-mediated cell cycle arrest associated with induced expression of p21 (CIP1/WAF1) in the anaplastic large cell lymphoma cell line Karpas 299. Oncogene 2001;20:590–8.
32. Rassidakis GZ, Thomaides A, Wang S, et al. p53 gene mutations are uncommon but p53 is commonly expressed in anaplastic large-cell lymphoma. Leukemia 2005;19:1663–9.
33. Guenova M, Rassidakis GZ, Gorgoulis VG, et al. p16INK4A is regularly expressed in Hodgkin's disease: comparison with retinoblastoma, p53 and MDM2 protein status, and the presence of Epstein-Barr virus. Mod Pathol 1999;12:1062–71.
34. Glossmann JP, Josting A, Diehl V. New treatments for Hodgkin's disease. Curr Treat Options Oncol 2002;3:283–90.
35. Re D, Kuppers R, Diehl V. Molecular pathogenesis of Hodgkin's lymphoma. J Clin Oncol 2005;23:6379–86.
36. Chen WG, Chen YY, Kamel OW, Koo CH, Weiss LM. p53 mutations in Hodgkin's disease. Lab Invest 1996;75:519–27.
37. Garcia JF, Villuendas R, Sanchez-Beato M, et al. Nucleolar p14(ARF) overexpression in Reed-Sternberg cells in Hodgkin's lymphoma: absence of p14(ARF)/Hdm2 complexes is associated with expression of alternatively spliced Hdm2 transcripts. Am J Pathol 2002;160:569–78.
38. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957–67.
39. Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis 2000;21:485–95.
40. Thompson T, Tovar C, Yang H, et al. Phosphorylation of p53 on key serines is dispensable for transcriptional activation and apoptosis. J Biol Chem 2004;279:53015–22.
41. Carvajal D, Tovar C, Yang H, Vu BT, Heimbrook DC, Vassilev LT. Activation of p53 by MDM2 antagonists can protect proliferating cells from mitotic inhibitors. Cancer Res 2005;65:1918–24.
42. Teodoro JG, Parker AE, Zhu X, Green MR. p53-mediated inhibition of angiogenesis through up-regulation of a collagen prolyl hydroxylase. Science 2006;313:968–71.
43. Kiaris H, Chatzistamou I, Trimis G, Frangou-Plemmenou M, Pafiti-Kondi A, Kalofoutis A. Evidence for nonautonomous effect of p53 tumor suppressor in carcinogenesis. Cancer Res 2005;65:1627–30.

Clinical Cancer Research

Inhibition of p53-Murine Double Minute 2 Interaction by Nutlin-3A Stabilizes p53 and Induces Cell Cycle Arrest and Apoptosis in Hodgkin Lymphoma

Elias Drakos, Athanasios Thomaides, L. Jeffrey Medeiros, et al.

Clin Cancer Res 2007;13:3380-3387.