

# Enhanced tumor cell kill by combined treatment with a small-molecule antagonist of mouse double minute 2 and adenoviruses encoding p53

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## Abstract

Strategies to treat cancer by restoring p53 tumor suppressor functions are being actively investigated. These approaches range from expressing an exogenous p53 gene in p53 mutant cancers to antagonizing a p53 inhibitor in p53 wild-type (WT) cancer cells. In addition, exogenous p53 is used to strengthen the anticancer efficacy of oncolytic adenoviruses. Many cancers express high levels of the major negative regulator of p53, mouse double minute 2 (MDM2) protein. Recently, a novel class of highly potent and specific MDM2 antagonists, the Nutlins, was identified. We envisioned that Nutlins could protect both endogenous and exogenous p53 from MDM2-mediated inactivation. We therefore investigated treating human cancer cells with a combination of adenovirus-mediated p53 gene therapy and Nutlin. Combination treatment resulted in broadly effective cell kill of p53 WT and p53-negative cancer cells. Cytotoxicity was associated with profound cell cycle checkpoint activation and apoptosis induction. We also tested Nutlin in combination with oncolytic adenoviruses. Nutlin treatment accelerated viral progeny burst from oncolytic adenovirus-infected cancer cells and caused an estimated 10- to 1,000-fold augmented eradication of p53 WT cancer cells. These findings suggest

that Nutlins are promising compounds to be combined with p53 gene therapy and oncolytic virotherapy for cancer. [Mol Cancer Ther 2007;6(5):1552–61]

## Introduction

The tumor suppressor p53 is the central coordinator of cellular responses to danger signals, such as oncogene activation, aberrant growth signals, and DNA damage. In response to these danger signals, p53 triggers cell cycle arrest, DNA repair, and apoptosis (1). Abrogation of the p53 pathway is the most common change in human cancers. A dysfunctional p53 pathway seems critical in the pathogenesis and progression of tumors, as it fails to protect against sustaining mutations culminating into malignant transformation. In the majority of cancer cells, p53 function is lost due to mutation of the p53 gene (2). In most other cases, p53 function is compromised by inhibition of the p53 protein. Inhibitors of p53 include several cellular ubiquitin ligases as well as oncogenic viral proteins. In most cell types, the endogenous ubiquitin ligase mouse double minute 2 (MDM2) is regarded as the major negative regulator of p53 function (3, 4). MDM2 binds to p53, which inhibits p53 transcriptional activity, induces p53 nuclear export, and targets p53 for proteasomal degradation. High expression of MDM2, inactivating p53, is associated with tumor development (5, 6). MDM2 is amplified in ~7% of cancers, with the highest frequencies found in soft-tissue sarcomas, osteosarcomas, and esophageal carcinomas (7). Tumors may also express MDM2 at high levels without gene amplification (8, 9).

Several anticancer therapeutic strategies aim to restore normal p53 functions. In cells expressing mutant p53, this includes introduction of the normal p53 gene or reactivating mutant p53 with molecules capable of stabilizing p53 protein folding. In p53 wild-type (WT) cells, this is done by inhibiting expression of p53 inhibitors or disrupting the interaction between p53 and its inhibitors (10). The central role of the p53-MDM2 interaction in regulating p53 activity has directed much attention to developing compounds that interfere with this interaction. Such molecules were derived from the p53 peptide interacting with the p53-binding site in MDM2 and were synthesized by computer-aided design or identified by screening libraries of microbial extracts or synthetic chemicals (11, 12). Recently, a class of small molecules, the Nutlins, was identified that inhibits the interaction between MDM2 and p53 with particular potency and selectivity (13). Nutlins showed antitumor activity *in vitro* and *in vivo* against cancer cells with WT p53 gene. Obviously, in tumors with mutated p53 where the p53-MDM2 interaction is redundant, treatment with a Nutlin has no effect. In these cells, p53 function can best be

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**Conflict of interest:** L.T. Vassilev is employed at Hoffman-La Roche, whose potential product was studied in the present work.

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restored by introduction of exogenous WT p53 (e.g., by adenovirus vector-based gene transfer). This treatment is already in advanced clinical development phase for a variety of cancer types. However, high MDM2 expression was shown to hamper the anticancer effect of p53 gene therapy (14, 15), suggesting that this treatment is less suitable for MDM2-overexpressing cancers.

Oncolytic adenoviruses, which selectively replicate in cancer cells, are also effective gene delivery agents. Moreover, their capacity to lyse cancer cells (referred to as oncolysis) with subsequent spread of viral progeny to neighboring tumor cells makes them particularly attractive anticancer agents (16). Importantly, we found previously that exogenous p53 expression enhanced the cancer cell killing potency of an oncolytic adenovirus (17). Recently, however, we found that high expression of MDM2 limited the anticancer effect of exogenous p53 expressed by oncolytic adenoviruses (18).

Hence, high MDM2 expression is a determinant of resistance against p53 gene therapy and p53-enhanced oncolytic virus therapy. Here, we test the hypothesis that these therapies could be made more effective against cancer cells with p53 WT or mutant genotype by concomitant disruption of the p53-MDM2 interaction using the MDM2 antagonist Nutlin-3.

## Materials and Methods

### Cell Lines

SJSA-1 and HCT116 cells were obtained from the American Type Culture Collection. The cell lines SaOs-2, CAL-72, U2OS, and MKN45 were kindly provided by Dr. F. van Valen (Westfälische Wilhelms-Universität Münster, Münster, Germany), Dr. J. Gioanni (Laboratoire de Cancérologie, Faculté de Médecine, Nice, France), Dr. S. Lens (Dutch Cancer Institute, Amsterdam, the Netherlands), and Dr. M. Tsujii (Osaka University School of Medicine, Osaka, Japan), respectively. Adenovirus E1-complementing 911 cells were obtained from IntroGene. SJSA-1 cells were maintained in RPMI 1640 supplemented with 10% FCS, 50 infectious units (IU)/mL penicillin plus 50 µg/mL streptomycin (PS), 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, and glucose to a concentration of 4.5 g/L. HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% FCS and PS. SaOs-2, CAL-72, and U2OS cells were cultured in F-12-supplemented DMEM with 10% FCS and PS. MKN45 cells were grown in RPMI 1640 with 10% FCS and PS. All cells were cultured at 37°C in a humidified, 5% carbon dioxide atmosphere.

### Nutlins and Recombinant Adenoviruses

The small-molecule antagonist of MDM2 Nutlin-3a and its inactive enantiomer Nutlin-3b, which serves as a control for nonspecific effects, were obtained from Hoffmann-La Roche. Nutlins were dissolved in DMSO at a concentration of 10 mmol/L and further diluted for experiments in complete medium.

The replication-deficient adenovirus vector Adwtp53 that harbors a human p53 gene driven by a SV40 virus early

promoter in place of the E1 region has been described previously (19). The conditionally replicative adenovirus AdΔ24 that lacks 24 bp corresponding to amino acids 122 to 129 in the CR2 domain of E1A necessary for binding to Rb protein (20) and has a deleted E3 region and its derivative AdΔ24-p53 that carries a SV40 virus early-p53 expression cassette inserted in place of the deleted E3 region were described before (17).

### Western Blot Analysis

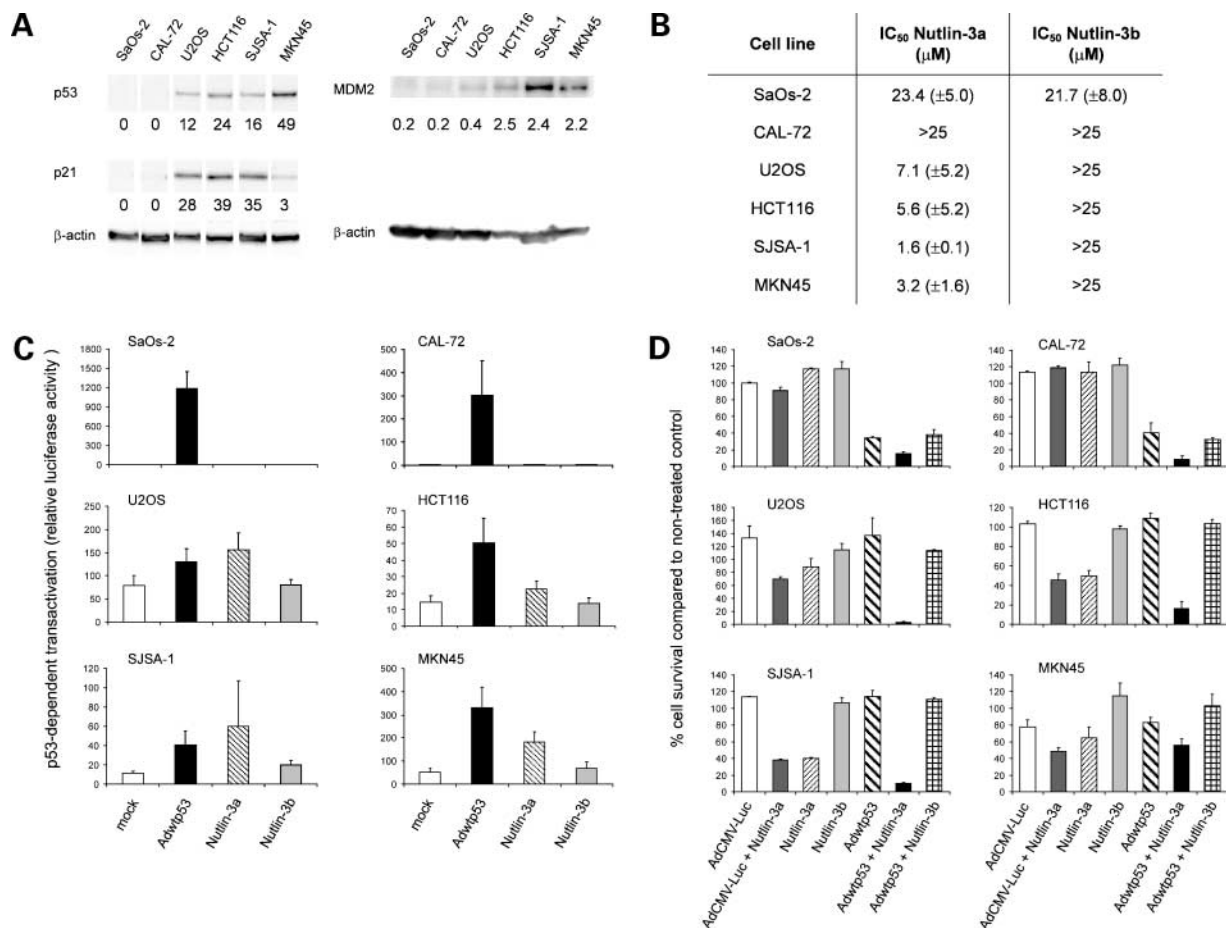
Cells were harvested and lysed in 200 µL radioimmuno-precipitation buffer (50 mmol/L Tris-HCl, 1% Igepal, 0.5% sodium deoxycholate, 150 mmol/L NaCl, 0.05% SDS) supplemented with 1 µL of 200 mmol/L Pefabloc (Roche Diagnostics) and incubated for 30 min on ice. Lysates were cleared by centrifugation and protein concentrations were determined using bicinchoninic acid protein assay kit (Pierce). For detection of p21 or p53, 15 µg protein was fractionated on a NuPAGE 10% Bis-Tris gel (Invitrogen Life Technologies) and transferred to a polyvinylidene difluoride membrane (Bio-Rad). For detection of MDM2 protein, 35 µg protein was loaded on a 7% Tris-acetate gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane. Proteins were detected by enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences) after incubation with primary antibodies for p53 (DO7, DAKO), p21 (SX118, DAKO), MDM2 (SMP14, DAKO), or β-actin (AC-15, Sigma) followed by secondary antibody anti-immunoglobulin G-horse radish peroxidase conjugate (DAKO). Protein levels were quantified by densitometric scanning on a VersaDoc 4000 imaging system using Quantity One software (Bio-Rad Laboratories).

### p53 Reporter Assay

Cells were plated at a density of 50% to 70% confluency in a 96-well plate and transfected either with the p53-dependent reporter plasmid PG13-Luc or with negative control construct MG15-Luc as described previously (17). After 3 h, cells were treated with or without adenovirus (Adwtp53, AdΔ24, or AdΔ24-p53) at a multiplicity of infection of 100 IU/cell, Nutlin-3a or Nutlin-3b (8 µmol/L), or a combination of virus and Nutlin. Forty-eight hours after transfection, cells were harvested and luciferase expression in cells was measured using the Luciferase Chemiluminescent Assay System (Promega). p53 transcriptional activity is expressed as the relative luciferase activity of PG13-Luc-treated cells over MG15-Luc-treated cells.

### Colorimetric WST-1 Cell Viability Assay

Cells were seeded in a 96-well plate at a density of  $5 \times 10^3$  cells per well. The next day, cells were inoculated with Adwtp53 at 100 IU/cell (SaOs-2 and U2OS) or 300 IU/cell (CaL-72, HCT116, SJSA-1, and MKN45), Nutlin-3a or Nutlin-3b (1 µmol/L for SJSA-1 and MKN45; 3 µmol/L for HCT116; and 8 µmol/L for SaOs-2, CAL-72, and U2OS), or a combination of both. Five days after treatment, culture medium was replaced by 100 µL of 10% WST-1 (Roche Diagnostics) in culture medium and cell viability was determined by colorimetric WST-1 conversion assay. Formazan dye formation was measured



**Figure 1.** Effects of Adwtp53 and Nutlin-3 on a panel of human cancer cell lines with different p53 and MDM2 phenotypes. **A**, Western blot analysis of p53 and p21, MDM2, and  $\beta$ -actin (loading control) expression in six human cancer cell lines. Relative expression compared with the loading control is given below each lane. **B**, IC<sub>50</sub> values of Nutlin-3a or Nutlin-3b on cancer cell lines with different p53 and MDM2 levels (**C**), p53 activation by Adwtp53, and Nutlin-3a treatment. Cancer cells were transfected with the plasmids PG13-Luc and MG15-Luc and subsequently treated with Adwtp53, Nutlin-3a, or Nutlin-3b. Forty-eight hours later, luciferase expression was measured. To determine p53-dependent transcriptional activation, relative PG13-Luc/MG15-Luc expression was calculated. *Columns*, mean of a representative experiment done in triplicate; *bars*, SD. **D**, analysis of the cytotoxic effect of Adwtp53 and Nutlin-3 on cancer cells. Cells were treated with Adwtp53, Nutlin-3a, or a combination of Adwtp53 and Nutlin-3a. As controls, AdCMV-Luc and Nutlin-3b were used. Five days after start of the treatment, cell viability was assessed using WST-1 conversion assay and expressed as percentage viability compared with nontreated control cells. *Columns*, mean of a representative experiment done in triplicate; *bars*, SD.

at 450 nm using a Bio-Rad model microplate reader. Relative WST-1 conversion in treated cells compared with untreated control cells was calculated after subtraction of WST-1 conversion in the absence of cells. Dose response graphs of Nutlin treatment were analyzed using the GraphPad Prism 4 program (GraphPad Software, Inc.). The IC<sub>50</sub> values of Nutlin treatment on different cell lines are given as mean values of three independent experiments with SD.

#### Cell Counting Assay

Cells ( $5 \times 10^3$ ) were seeded per well in a 96-well plate and cultured overnight. The next day, cells were treated with Adwtp53 or the control vector AdCMV-Luc (SaOs-2 and U2OS at 50 IU/cell and CAL-72, HCT116, SJSA-1, and MKN45 cells at 300 IU/cell) for 1.5 h. Subsequently, cells were treated with Nutlin-3a or Nutlin-3b (SaOs-2, CAL-72, and U2OS at 8  $\mu$ mol/L; HCT116 at 3  $\mu$ mol/L; and

SJSA-1 and MKN45 at 1  $\mu$ mol/L). On days 1 to 5 after infection, cells were harvested. Cell suspensions are mixed 1:1 with 0.4% trypan blue solution (Sigma) and viable cells were counted using a cell counting chamber (Marienfeld). The detection limit in this assay was 289 viable cells per culture.

#### Flow Cytometry Analysis

SaOs-2 and U2OS cells were seeded at  $1 \times 10^5$  cells per well in a six-well plate and cultured overnight. The next day, cells were treated with Adwtp53 (100 IU/cell), Nutlin-3a (8  $\mu$ mol/L), Nutlin-3b (8  $\mu$ mol/L), or a combination of virus and Nutlin. Forty-eight hours after treatment, adherent and nonadherent cells were collected. Cells were washed in PBS and fixed in 70% ethanol and stored for a minimum of 1 h at  $-20^\circ\text{C}$ . Before analysis, cells were washed in PBS again. The pellet was resuspended in propidium iodide solution [50  $\mu$ g/mL propidium iodide

and 1 mg/mL RNase A (Sigma) in PBS] for 1 h and analyzed by flow cytometry on a FACScan (Becton Dickinson). Cell cycle distribution was analyzed by ModFitLT cell cycle analysis software (Verity Software House). This analysis excludes the sub-G<sub>1</sub> cell population before calculating the percentage cells in G<sub>0</sub>-G<sub>1</sub>, S, and G<sub>2</sub>-M phases of the cell cycle.

#### Virus Propagation Assay

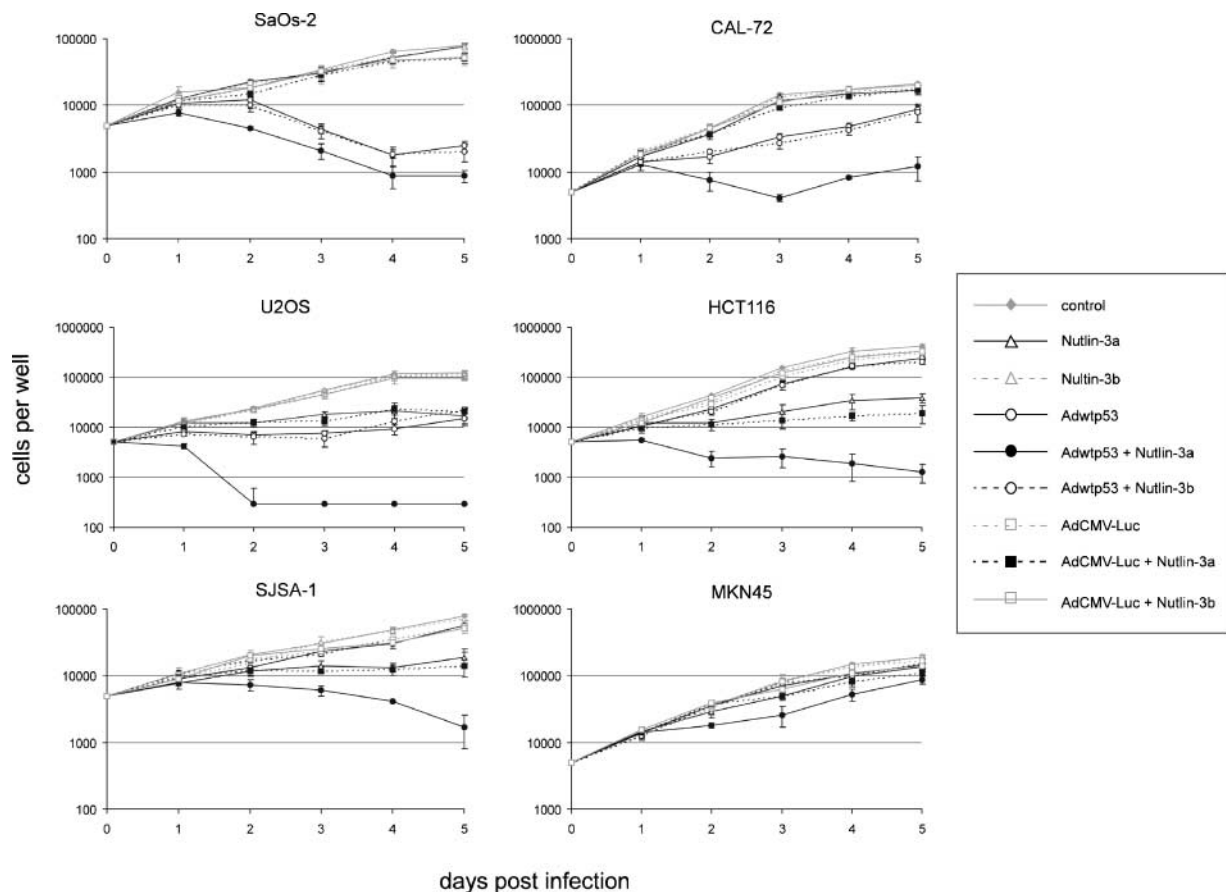
U2OS cells ( $5 \times 10^3$ ) were seeded per well in a 96-well plate. The next day, cells were infected with Ad $\Delta$ 24-p53 at 100 IU/cell for 1.5 h at 37°C after which infection medium was replaced with fresh medium with or without Nutlin-3a or Nutlin-3b (8  $\mu$ mol/L). Cells and supernatant were harvested separately 1 to 8 days after infection. Virus was released via three freeze-thaw cycles. The number of infectious particles produced in the cells and in the medium was determined by 3-fold limiting dilution infection on 911 cells in triplicate. After 48 h, cells were fixed in methanol and stained for hexon expression using the mouse anti-hexon antibody and rat anti-mouse immunoglobulin (horseradish peroxidase conjugate) from the AdenoX rapid titer kit (BD Biosciences).

#### Assessment of Conditionally Replicative Adenovirus Oncolytic Potency on Cancer Cells

Cells were seeded at a density of  $5 \times 10^3$  cells per well for most cell lines and  $5 \times 10^2$  cells per well for SJS-1 cells in a 96-well plate. The next day, cells were infected with Ad $\Delta$ 24 or Ad $\Delta$ 24-p53 at the indicated multiplicity of infection in medium containing 2.5% FCS for 1.5 h at 37°C. Subsequently, complete medium with or without Nutlin-3a or Nutlin-3b (8  $\mu$ mol/L for SaOs-2, CAL-72, and U2OS; 3  $\mu$ mol/L for HCT116; and 1  $\mu$ mol/L for SJS-1 and MKN45 cells) was added. Cells were harvested 8 to 15 days after infection with 50% medium (with or without Nutlin) refreshment once or twice weekly. After removal of the culture medium, cells were fixed in 4% formaldehyde in PBS and stained using 1% crystal violet dye in 70% ethanol. After several washes, culture plates were air dried and scanned on a Bio-Rad GS-690 imaging densitometer.

#### Statistical Analysis

The Student's *t* test was used for statistical evaluation of differences between treatment groups. Differences were considered to be significant when  $P < 0.05$ .



**Figure 2.** Effect of combination treatment with Adwtp53 and Nutlin-3a on viable cell number. Cells were treated with Nutlin-3 and/or adenovirus vector and viable cells were counted on days 1 to 5 after start of the treatment. Points, mean of an experiment done in triplicate; bars, SD. Values shown for U2OS cells 2 to 5 d after start of Adwtp53 plus Nutlin-3a treatment represent the detection limit of the assay.

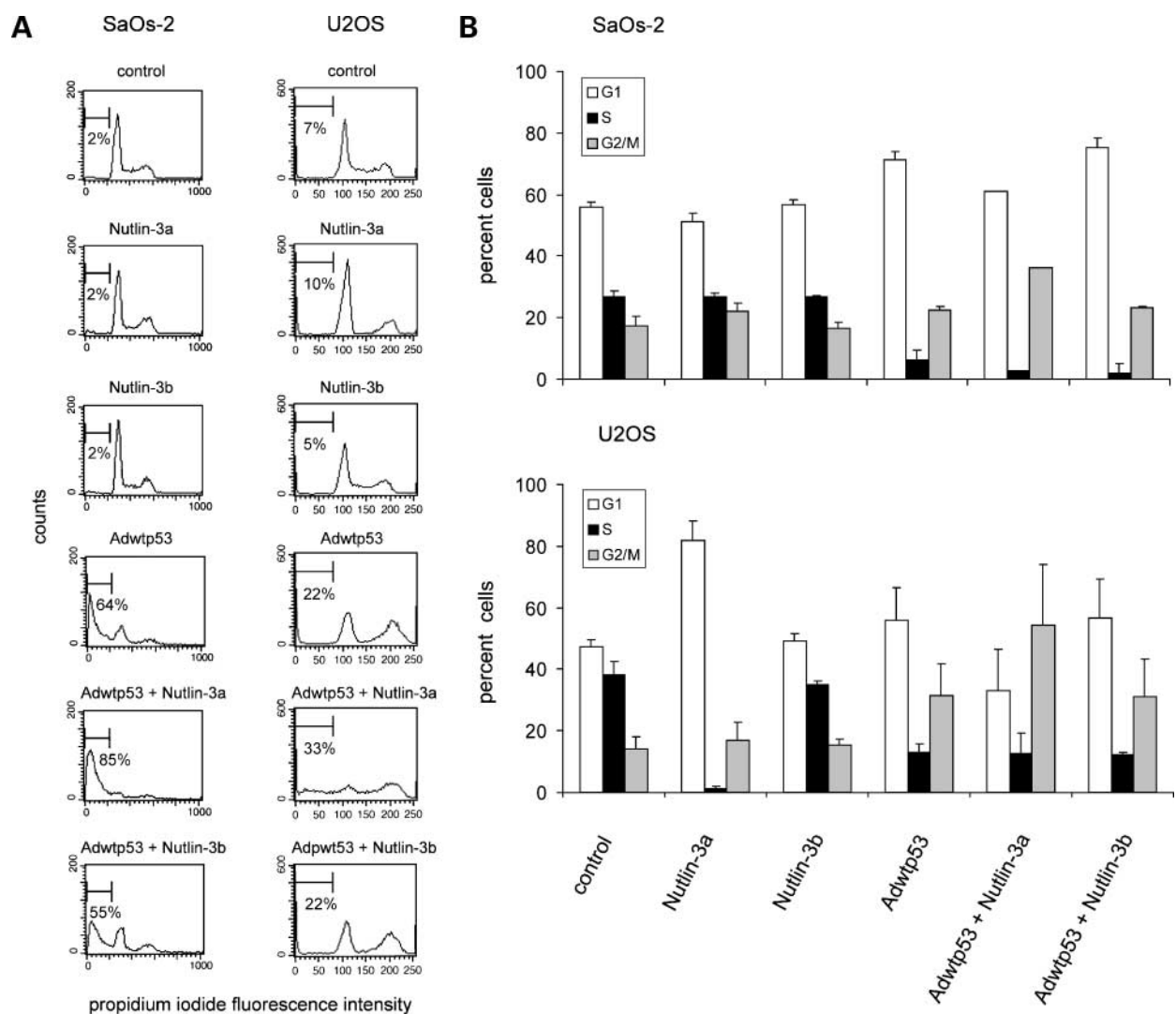
## Results

### Susceptibility of Human Cancer Cell Lines to Treatment with Nutlin-3

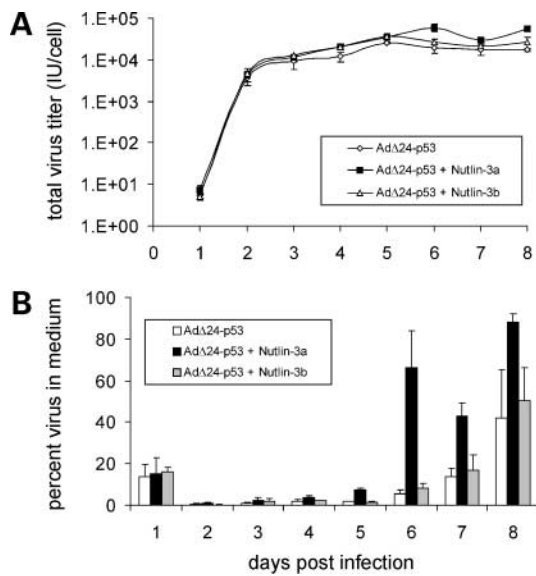
For our studies, we selected six human cancer cell lines representing different p53/MDM2 phenotypes. Figure 1A shows Western blot analysis for expression of p53, p21, and MDM2 in these cells. Expression levels were normalized by that of  $\beta$ -actin. As expected, in p53-null SaOs-2 and CAL-72 osteosarcoma cells, p53 was absent and expression levels of its transcriptional targets p21 and MDM2 were low or undetectable. The other four cell lines (i.e., SJSA-1 and U2OS osteosarcoma cells, MKN45 gastric carcinoma cells, and HCT116 colorectal carcinoma cells) all expressed p53,

consistent with their documented p53 WT genotypes. The p53 target p21 was expressed in all four cell lines, albeit at low level in MKN45 cells. MDM2 expression was lower in U2OS cells than in HCT116, SJSA-1, and MKN45 cells.

We then subjected these cancer cells to treatment with the specific small-molecule antagonist of the p53-MDM2 interaction Nutlin-3. This *cis*-imidazoline derivative was synthesized as racemic mixture from which the enantiomers 3a and 3b were separated with the use of a chiral column. Nutlin-3b is 150 times less potent in binding MDM2 than Nutlin-3a (13). Therefore, Nutlin-3b was included in all experiments to serve as control for non-MDM2-related activities. Cells were incubated with



**Figure 3.** Cell cycle analysis of SaOs-2 and U2OS cells treated with Adwtp53 and Nutlin. Cells were treated for 48 h with Adwtp53, Nutlin-3a or Nutlin-3b, or a combination of virus and Nutlin. Cells were then harvested, stained with propidium iodide, and analyzed by flow cytometry. **A**, DNA histograms from a representative experiment. Percentages of cells in the sub-G<sub>1</sub> area are indicated in each histogram. Average percentages from the three experiments are given in the text. **B**, cell cycle distribution was determined by ModFitLT analysis. *Columns*, mean values of three independent experiments; *bars*, SD. For the combination of Adwtp53 and Nutlin-3a on SaOs-2 cells, the columns represent the average of only two experiments because massive apoptosis in the third experiment precluded cell cycle distribution analysis.



**Figure 4.** Effect of Nutlin-3a on AdΔ24-p53 propagation. U2OS cells were treated with AdΔ24-p53 at 100 IU/cell alone or in combination with Nutlin-3a or Nutlin-3b at a concentration of 8  $\mu$ mol/L. Cells and medium were harvested separately at the indicated time points and infectious virus titers were determined. **A**, total amount of adenovirus harvested from the cultures. **B**, proportion of AdΔ24-p53 in the medium as percentage of the total amount of virus. *Points and columns*, mean values of a representative experiment done in triplicate; *bars*, SD.

Nutlin-3a or Nutlin-3b for 5 days and  $IC_{50}$  values were then determined (Fig. 1B). Consistent with its mode of action, Nutlin-3a exerted selective toxicity against p53 WT cells only. Nutlin-3a-specific  $IC_{50}$  values were not reached for p53-deficient SaOs-2 and CAL-72 cells, where antagonizing the p53-MDM2 interaction should be irrelevant.

#### Restoring p53 Function in Cancer Cells by Adwtp53 or Nutlin-3 Treatment

Next, we investigated the effects of p53 gene therapy using replication-defective adenovirus vector Adwtp53 or MDM2 inhibition using Nutlin-3 on p53 activity in cancer cells. We subjected the six cancer cell lines to Adwtp53, Nutlin-3a, or Nutlin-3b and measured p53 transcriptional activity using a p53-dependent reporter construct. As can be seen in Fig. 1C, all cell lines displayed p53 activities that were consistent with their genetic status and with the results from the Western blot analysis. WT p53 cells had p53-specific transactivation values ranging from 10 to 80, whereas in p53-deficient cells, these values were <2. Transduction of p53-deficient cells with Adwtp53 resulted in very high p53 activities. Adwtp53 also increased p53 transcriptional activity in p53 WT cells, but on average to a lesser extent than in p53-deficient cells. This suggested that p53 inhibitors including MDM2 that are expressed in p53 WT cancer cells also inhibited exogenous Adwtp53-encoded p53 to some extent. Cells were also infected with the control virus AdGFP, a replication-defective adenovirus vector expressing green fluorescence protein. This did not affect p53 transactivation (data not shown). As

expected, Nutlin-3a induced p53 activity in p53 WT cells (by at least 2-fold) but not in p53-deficient cells. The absolute increase in p53 activity was substantially lower in HCT116 cells than in the other three cell lines. In addition to MDM2, several other endogenous p53 inhibitors have been identified (21). Perhaps, multiple p53 inhibitors are expressed in HCT116 cells, in which case disrupting the p53-MDM2 interaction would only partially restore p53 activity. This would be in line with the previous observation that silencing MDM2 in HCT116 cells by RNA interference did not stabilize p53 (22). Taken together, Nutlin-3a and Adwtp53 each contributed to restoring p53 functions in cancer cells in their own way (i.e., by reactivating endogenous p53 and by providing exogenous p53 activity, respectively).

#### Combination Treatment with Adwtp53 and Nutlin-3 Augments Cancer Cell Kill

Because MDM2 seemed to inhibit both endogenous and exogenous p53 and because exogenous p53 may induce MDM2 expression in p53-deficient cells with an intact MDM2 gene, we hypothesized that combined treatment of cancer cells with Adwtp53 and Nutlin could be more effective than either treatment alone. Cancer cells were treated with Adwtp53, Nutlin-3a, or a combination of both agents. As controls, AdCMV-Luc, a replication-deficient adenovirus vector expressing firefly luciferase, and Nutlin-3b were used. Five days later, cell viabilities were measured by WST-1 conversion assay (Fig. 1D). As single agents, Adwtp53 decreased viability only of p53-null SaOs-2 and CAL-72 cells and Nutlin-3a decreased viability only of p53 WT cell lines. Most importantly, combination treatment with Adwtp53 plus Nutlin-3a was more effective than the best of either single treatment on SaOs-2, CAL-72, U2OS, HCT116, and SJSA-1 cells ( $P < 0.05$ ). The increased cell killing by combination treatment was dependent on introduction of exogenous p53 and disruption of the p53-MDM2 interaction by Nutlin-3a because neither AdCMV-Luc with Nutlin-3a nor Adwtp53 with Nutlin-3b caused similar combination effects.

These findings were corroborated by counting viable cells 1 to 5 days after start of treatment (Fig. 2). Nutlin-3a alone did not affect expansion of SaOs-2 cells and had only minor effect on CAL-72 and MKN45 cells (2%, 21%, and 29% reduction in viable cell numbers on day 5, respectively). In contrast, it strongly reduced U2OS, HCT116, and SJSA-1 viable cell output compared with controls (86%, 91%, and 76% reduction, respectively; all  $P < 0.005$ ). However, also in these cultures, absolute cell numbers increased over time, suggesting that Nutlin-3a delayed cell proliferation rather than killed cells. Adwtp53 treatment as monotherapy killed SaOs-2 cells (absolute loss of viable cells) and delayed proliferation of CAL-72 ( $P < 0.001$ ) and U2OS ( $P < 0.005$ ) cells. In line with the observations in the WST-1 assay experiment, Adwtp53 with Nutlin-3a was the most effective treatment on all six tested cell lines ( $P$  values compared with the best single agent treatment:  $P < 0.05$  for SaOs-2, U2OS, HCT116, SJSA-1, and MKN45;  $P < 0.01$  for CAL-72). On most cell

lines, combination treatment reduced the absolute viable cell count, indicating that cells were killed. Again, we did not observe similar effects when treated with AdCMV-Luc+Nutlin-3a or Adwtp53+Nutlin-3b.

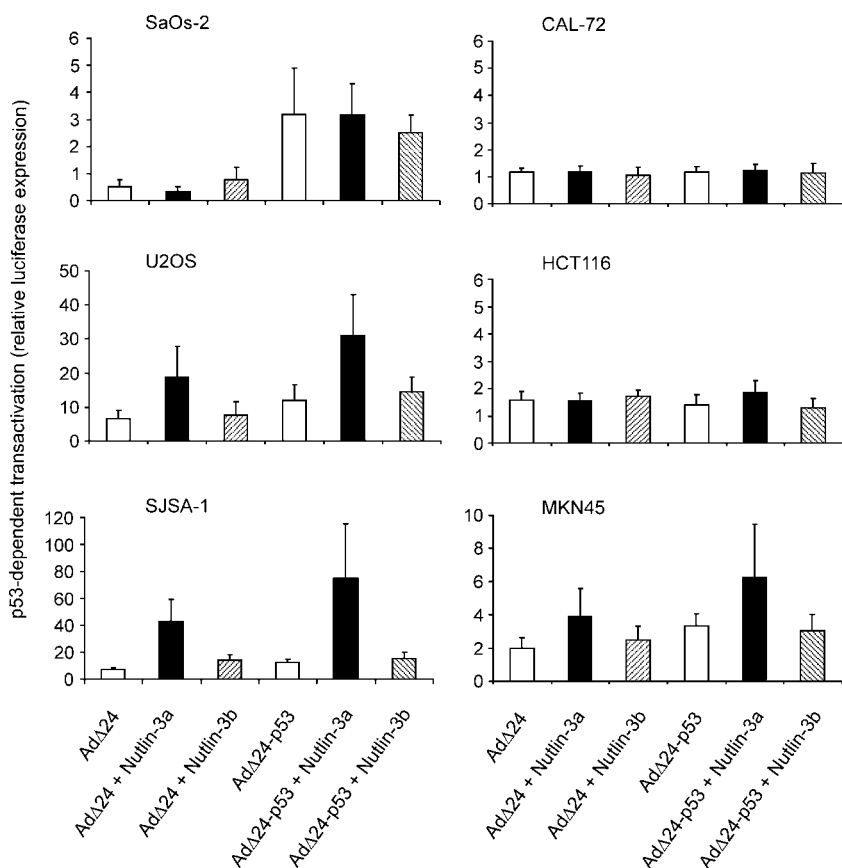
#### Combination Treatment with Adwtp53 and Nutlin-3 Induces Cell Cycle Arrest and Apoptosis in Cancer Cells

To explain the combined effect of activating the p53 pathway in cancer cells with Adwtp53 and Nutlin-3a, we investigated the effect of this treatment on cell cycle arrest and apoptosis induction. SaOs-2 and U2OS cells were subjected for 2 days to 100 IU/cell Adwtp53 or 8  $\mu$ mol/L of the active or inactive Nutlin-3 enantiomer or to a combination of Adwtp53 plus Nutlin-3. Cell cycle distribution analysis (Fig. 3) showed that Adwtp53 treatment decreased the S phase population ( $P < 0.005$ ) in both cell lines, indicating activation of cell cycle checkpoints. As single agent, Nutlin-3a, but not 3b, caused a substantial shift in cell cycle populations only in p53 WT U2OS cells. Accumulation of cells in G<sub>1</sub> phase ( $P < 0.01$ ) and depletion of cells in S phase ( $P < 0.005$ ) indicated arrested cell cycle progression mainly at the G<sub>1</sub>-S cell cycle checkpoint. Combined treatment with Adwtp53 and Nutlin-3a caused the highest accumulation in G<sub>2</sub>-M phase in both cell lines, although this did not differ significantly from single agent treatments. As measure for cells in apoptosis, we analyzed sub-G<sub>1</sub> events (Fig. 3A). Due to variation between experi-

ments, differences were not significant. However, whereas Nutlin-3a-treated cells did not show more apoptosis than controls, Adwtp53 consistently increased the number of sub-G<sub>1</sub> events in both cell lines (average, 7–38% in SaOs-2; 4–10% in U2OS). Combination treatment further increased the fraction of cells in apoptosis (average, 59% in SaOs-2; 25% in U2OS). Thus, protecting Adwtp53-encoded p53 from MDM2-mediated inactivation seemed to augment apoptosis induction.

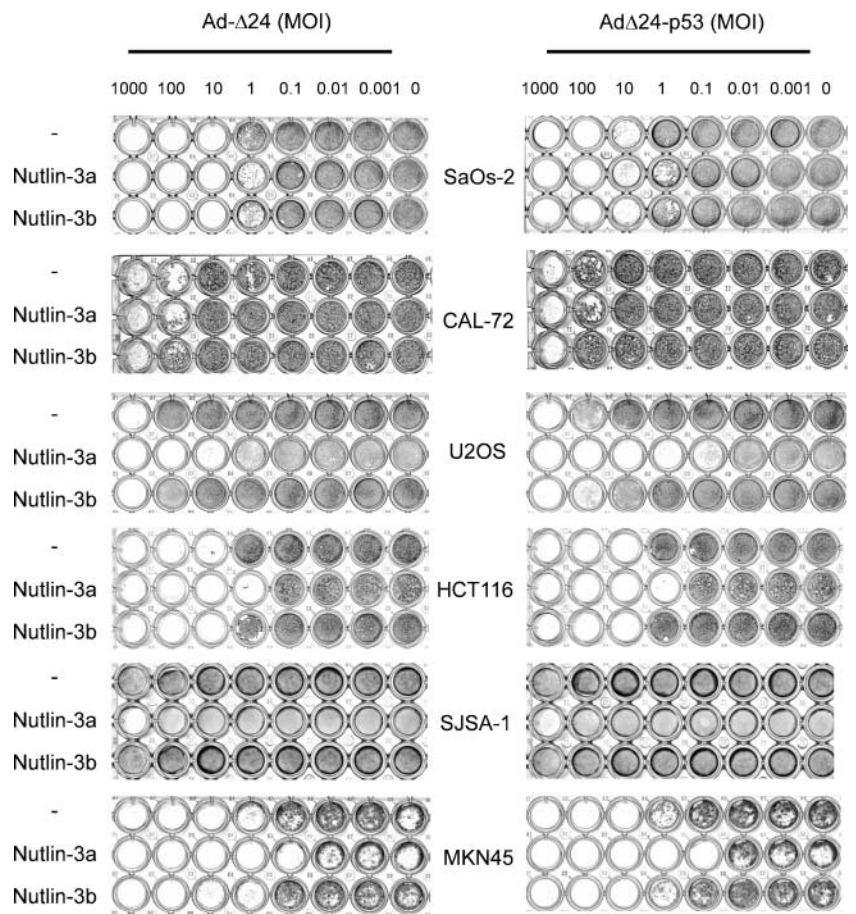
#### Nutlin-3 Accelerates Productive Oncolytic Adenovirus Replication in Cancer Cells

An effective way to kill cancer cells is by means of oncolytic adenoviruses. Previously, we observed profound anticancer effects by the p53-expressing oncolytic adenovirus Ad $\Delta$ 24-p53 (17, 23). Ad $\Delta$ 24-type oncolytic adenoviruses encode E1A proteins that are incapable of sequestering pRb from E2F-1, due to a deletion of eight amino acids in their pRb-binding CR2 domain (20). This deprives the virus of its capacity to induce cell cycle progression in normal cells with intact pRb control. Consequently, Ad $\Delta$ 24-type adenoviruses exhibit selective replication in cells with dysfunctional pRb control, where E2F-1 mediates cell cycle progression through the G<sub>1</sub>-S phase checkpoint. Because Nutlin-3 activated this checkpoint (see Fig. 3) and could thus potentially inhibit replication of Ad $\Delta$ 24-type oncolytic adenoviruses, we investigated if Nutlin treatment affected



**Figure 5.** p53 transcriptional activity in cancer cells treated with Ad $\Delta$ 24 or Ad $\Delta$ 24-p53 with or without Nutlin-3. Cells were transfected with PG13-Luc or MG15-Luc and subsequently cultured in medium containing Ad $\Delta$ 24 or Ad $\Delta$ 24-p53 or a combination of virus with Nutlin-3a or Nutlin-3b. After 48 h, luciferase expression was measured and relative PG13-Luc/MG15-Luc expression was calculated. Columns, mean of a representative experiment done in triplicate; bars, SD.

**Figure 6.** Effect of Nutlin treatment on oncolytic potency of Ad $\Delta$ 24 and Ad $\Delta$ 24-p53 against cancer cells. Cancer cells were infected with Ad $\Delta$ 24 or Ad $\Delta$ 24-p53 at various multiplicities of infection (MOI) in IU/cell. *Top rows*, cells were subjected to oncolytic adenovirus only; *middle rows*, cells were subjected to virus and Nutlin-3a; *bottom rows*, cells were subjected to virus and Nutlin-3b. After 8 to 15 d, the remaining viable cells were stained with crystal violet. Data are from representative experiments of at least two independent experiments done on each cell line.



replication of Ad $\Delta$ 24-p53. U2OS cells were cultured in the presence of 8  $\mu$ mol/L Nutlin-3a or Nutlin-3b and infected with 100 IU/cell Ad $\Delta$ 24-p53. At various time points after infection, the amount of infectious virus particles produced was determined. Figure 4A shows that over a period of 8 days, the titer of Ad $\Delta$ 24-p53 rose to a final yield of  $\sim 2 \times 10^4$  IU/cell. The virus production profiles in the presence of the active or the inactive Nutlin enantiomer were almost identical. Thus, Nutlin-3 did not inhibit oncolytic adenovirus replication. To investigate if Nutlin-3 affected oncolysis and release of progeny virus, we measured Ad $\Delta$ 24-p53 titers in cells and culture medium separately. Figure 4B shows that the release of Ad $\Delta$ 24-p53 progeny into the medium of infected U2OS cells was accelerated in the presence of Nutlin-3a compared with Nutlin-3b. Taken together, these data showed that in the presence of Nutlin-3a, the production of infectious Ad $\Delta$ 24-p53 in U2OS cells was not changed, but Ad $\Delta$ 24-p53 progeny release was induced sooner after infection.

#### Nutlin-3 Protects Exogenous p53 Expressed by an Oncolytic Adenovirus from Inactivation

We next sought to determine if Nutlin-3 protects oncolytic adenovirus-encoded p53 from MDM2-mediated inactivation. In adenovirus-infected cells, adenovirus proteins including E1B-55K and E4orf6 interact with p53. This

is associated with p53 redistribution over cellular compartments and degradation (24, 25). In the face of adenovirus proteins interacting with p53, the binding of p53 to MDM2 might therefore become bypassed. To investigate if Nutlin-3 would still affect p53 activity in this context, cells were infected with Ad $\Delta$ 24 or Ad $\Delta$ 24-p53 and treated with Nutlin-3a or Nutlin-3b. Two days later, p53 transcriptional activity was measured. As can be seen in Fig. 5, Ad $\Delta$ 24 infection severely decreased p53 activity in p53 WT cells (compare with Fig. 1C). In three of these cell lines, Nutlin-3a protected endogenous p53 at least partially from adenovirus protein-mediated inactivation. Nutlin-3a also increased p53 transcriptional activity in Ad $\Delta$ 24-p53-infected p53 WT cell lines, to levels exceeding those in Ad $\Delta$ 24/Nutlin-3a-treated cells. This suggested that also in the face of oncolytic adenovirus replication, Nutlin-3a protected exogenous p53 from inactivation. However, Ad $\Delta$ 24-p53-encoded p53 transcriptional activity remained low (compare with Adwt-p53-encoded p53; see Fig. 1C), suggesting that most exogenous p53 interacted with Ad $\Delta$ 24-encoded or induced proteins.

#### Nutlin-3 Augments Oncolytic Adenovirus Replication in p53 WT Cancer Cells

Finally, we explored if combined treatment with oncolytic adenovirus and Nutlin-3a would accomplish a more



effective eradication of cancer cells. The six cancer cell lines were inoculated with Ad $\Delta$ 24 or Ad $\Delta$ 24-p53 in a dilution titration. The viruses were allowed to replicate for up to 15 days before remaining cells were stained with crystal violet (Fig. 6). Ad $\Delta$ 24 and Ad $\Delta$ 24-p53 exhibited dose-dependent cytotoxicity on all cancer cell lines. Oncolytic efficacies against SJS-1 cells, which express low levels of the primary adenovirus receptor CAR (data not shown), were less than against the other cell lines. Nutlin-3a did not clearly augment oncolytic adenovirus efficacy against the two p53-deficient cell lines. In contrast, treatment with Nutlin-3a, but not with Nutlin-3b, augmented oncolytic propagation of Ad $\Delta$ 24 and Ad $\Delta$ 24-p53 on all four WT p53 cell lines. Under the experimental conditions used, enhancement of Ad $\Delta$ 24 oncolytic efficacy by Nutlin-3 treatment ranged from an estimated 10-fold on HCT116, SJS-1, and MKN45 cells to 100-fold on U2OS cells. Combined Ad $\Delta$ 24-p53 with Nutlin-3a treatment was somewhat more effective, with efficacy increases up to 100-fold on MKN45 cells and 1,000-fold on U2OS cells (see Fig. 6).

## Discussion

We investigated if restoring p53 functions by concomitant p53 gene delivery and disruption of the p53-MDM2 interaction would provide an effective means to kill cancer cells irrespective of their p53 status. Separately, adenovirus-encoded p53 and MDM2 antagonist Nutlin-3 increased functional p53 expression and induced cancer cell death in line with their known modes of action. Together, exogenous p53 and Nutlin-3 elicited the most effective death of p53-positive and p53-negative cancer cells. Nutlin-3 thus enhanced endogenous as well as exogenous p53-mediated cell kill. Interestingly, the effect of Nutlin-3 on exogenous p53 seemed somewhat different from its documented effect on endogenous p53. Endogenous WT p53 reactivated by Nutlin-3 treatment arrested U2OS cells only in G<sub>1</sub>, whereas addition of exogenous p53 also activated the G<sub>2</sub>-M cell cycle checkpoint. Moreover, although U2OS did not exhibit noticeable apoptosis in response to Nutlin-3 Adwtp53/Nutlin-3 combination treatment induced extensive apoptosis, killing all cells within 2 days. Thus, p53-dependent apoptotic signaling was not impaired in U2OS cells and could be exploited to kill these cells by Adwtp53/Nutlin-3 combination treatment. It remains to be elucidated which factors govern differential cell cycle and apoptosis responses to endogenous versus exogenous p53.

Nutlin-3 also markedly enhanced the potency of oncolytic adenoviruses against WT p53 cell lines. It accelerated cell death and viral burst, culminating in a more effective eradication of these cells. This is in line with previous observations that p53 promotes oncolytic adenovirus propagation in cancer cells (17, 23, 26, 27). Nutlin-3 thus presumably augments oncolytic adenovirus propagation by relieving p53 from MDM2 inhibition. Interestingly, in this context, reactivation of endogenous p53 seemed more important than protecting exogenous p53 from degrada-

tion. Recently, it was found that adenovirus E1A protein stabilizes p53 in the nucleus through forming a tricomplex with the structural MDM2 homologue MDMX (28). Notably, Nutlin-3 does not inhibit the MDMX-p53 interaction (29). This suggests that combined treatment with Nutlin-3 will not interfere with this potentially important p53 regulation by oncolytic adenovirus.

The precise mechanism by which p53 augments adenovirus-induced killing of cancer cells remains poorly understood. We found that Nutlin-3 reproducibly augmented Ad $\Delta$ 24 and Ad $\Delta$ 24-p53 propagation on HCT116 cells, without increasing p53 transcriptional activity. This could suggest that the oncolysis-enhancing effect of p53 is independent of its transcriptional activity. Interestingly, it was reported recently that during early stages of adenovirus infection, a fraction of p53 accumulates in mitochondria (25), where p53 is known to exert direct transcription-independent proapoptotic activity (30). It could thus be speculated that oncolysis enhancement by p53 relies primarily on its apoptogenic effects at mitochondria, rather than on nuclear p53 driving target gene expression. However, Nutlin-3 also increased Ad $\Delta$ 24 oncolytic efficacy on U2OS cells, where endogenous p53 reactivation did not promote apoptosis. This would point at a p53-dependent effect on adenovirus propagation entirely distinct from apoptosis induction. Clearly, these hypotheses await experimental verification.

In the aggregate, we have shown herein that restoration of p53 functions in cancer cells by concomitant exogenous p53 gene delivery and MDM2 inhibition provides efficacious cancer cell kill by increased induction of cell death and augmented adenovirus-induced oncolysis.

## References

1. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer* 2002;2:594–604.
2. Chang F, Syrjanen S, Syrjanen K. Implications of the p53 tumor-suppressor gene in clinical oncology. *J Clin Oncol* 1995;13:1009–22.
3. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. *Nature* 1997;387:299–303.
4. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997;387:296–9.
5. Bond GL, Hu W, Bond EE, et al. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell* 2004;119:591–602.
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. Momand J, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. *Nucleic Acids Res* 1998;26:3453–9.
8. Polsky D, Bastian BC, Hazan C, et al. HDM2 protein overexpression, but not gene amplification, is related to tumorigenesis of cutaneous melanoma. *Cancer Res* 2001;61:7642–6.
9. Eymann B, Gazzeri S, Brambilla C, Brambilla E. Mdm2 overexpression and p14(ARF) inactivation are two mutually exclusive events in primary human lung tumors. *Oncogene* 2002;21:2750–61.
10. Lane DP, Lain S. Therapeutic exploitation of the p53 pathway. *Trends Mol Med* 2002;8:S38–42.
11. Klein C, Vassilev LT. Targeting the p53-2 interaction to treat cancer. *Br J Cancer* 2004;91:1415–9.
12. Chene P. Inhibiting the p53-2 interaction: an important target for cancer therapy. *Nat Rev Cancer* 2003;3:102–9.

13. 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.
14. Lane D. Anthony Dipple Carcinogenesis Award. p53 from pathway to therapy. *Carcinogenesis* 2004;25:1077–81.
15. Tang HJ, Qian D, Sondak VK, Stachura S, Lin J. A modified p53 enhances apoptosis in sarcoma cell lines mediated by doxorubicin. *Br J Cancer* 2004;90:1285–92.
16. Post DE, Khuri FR, Simons JW, Van Meir EG. Replicative oncolytic adenoviruses in multimodal cancer regimens. *Hum Gene Ther* 2003;14:933–46.
17. van Beusechem VW, van den Doel PB, Grill J, Pinedo HM, Gerritsen WR. Conditionally replicative adenovirus expressing p53 exhibits enhanced oncolytic potency. *Cancer Res* 2002;62:6165–71.
18. van Beusechem VW, van den Doel PB, Gerritsen WR. Conditionally replicative adenovirus expressing degradation-resistant p53 for enhanced oncolysis of human cancer cells overexpressing murine double minute 2. *Mol Cancer Ther* 2005;4:1013–8.
19. Ameyar M, Shatrov V, Bouquet C, et al. Adenovirus-mediated transfer of wild-type p53 gene sensitizes TNF resistant MCF7 derivatives to the cytotoxic effect of this cytokine: relationship with c-myc and Rb. *Oncogene* 1999;18:5464–72.
20. Fueyo J, Gomez-Manzano C, Alemany R, et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect *in vivo*. *Oncogene* 2000;19:2–12.
21. Brooks CL, Gu W. p53 ubiquitination: Mdm2 and beyond. *Mol Cell* 2006;21:307–15.
22. Kaeser MD, Pebernard S, Iggo RD. Regulation of p53 stability and function in HCT116 colon cancer cells. *J Biol Chem* 2004;279:7598–605.
23. Geoerger B, Vassal G, Opolon P, et al. Oncolytic activity of p53-expressing conditionally replicative adenovirus AdΔ24-53 against human malignant glioma. *Cancer Res* 2004;64:5753–9.
24. Berk AJ. Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. *Oncogene* 2005;24:7673–85.
25. Lomonosova E, Subramanian T, Chinnadurai G. Mitochondrial localization of p53 during adenovirus infection and regulation of its activity by E1-19K. *Oncogene* 2005;24:6796–808.
26. Hall AR, Dix BR, O'Carroll SJ, Braithwaite AW. p53-dependent cell death/apoptosis is required for a productive adenovirus infection. *Nat Med* 1998;4:1068–72.
27. Sauthoff H, Pipiya T, Heitner S, et al. Late expression of p53 from a replicating adenovirus improves tumor cell killing and is more tumor cell specific than expression of the adenoviral death protein. *Hum Gene Ther* 2002;13:1859–71.
28. Li Z, Day CP, Yang JY, et al. Adenoviral E1A targets Mdm4 to stabilize tumor suppressor p53. *Cancer Res* 2004;64:9080–5.
29. Patton JT, Mayo LD, Singhi AD, Gudkov AV, Stark GR, Jackson MW. Levels of HdmX expression dictate the sensitivity of normal and transformed cells to Nutlin-3. *Cancer Res* 2006;66:3169–76.
30. Marchenko ND, Zaika A, Moll UM. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J Biol Chem* 2000;275:16202–12.