Nongenotoxic p53 Activation Protects Cells against S-Phase–Specific Chemotherapy

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Abstract

Mutations in the tumor suppressor gene *TP53* represent the most frequent genetic difference between tumor cells and normal cells. Here, we have attempted to turn this difference into an advantage for normal cells during therapy. Using the Mdm2 antagonist nutlin-3, we first activated p53 in U2OS and HCT116 cells to induce cell cycle arrest. These arrested cells were found to be resistant to subsequent transient treatment with the nucleoside analogue gemcitabine, as revealed by clonogenic assays following drug removal. In contrast, isogenic cells lacking functional p53 continued to enter S phase regardless of nutlin-3 pretreatment and remained highly susceptible to gemcitabine-mediated cytotoxicity. The sequential treatment with nutlin-3 alone, followed by transient exposure to nutlin-3 plus gemcitabine, efficiently compromised the clonogenicity of tumor cells with deletions or mutations of p53 but largely spared the proliferation of nontransformed human keratinocytes. Nutlin-3 pretreatment also conferred protection of p53-proficient cells against cytosine arabinoside but not against doxorubicin or cisplatin. We propose that the cell cycle arrest function of p53 can be used to convert p53 from a killer to a protector of cells, with the potential to reduce unwanted side effects of chemotherapy. (Cancer Res 2006; 66(21): 10274-80)

Introduction

The most frequent known genetic difference between normal and cancerous cells consists of mutations within the tumor suppressor gene *TP53*, resulting in malfunction of its product, p53 (1). It would therefore be highly desirable to use wild-type (WT) p53 as a positive discriminator, allowing the protection of normal cells but not tumor cells in the course of cancer therapy. Such a concept seems counterintuitive at first glance because p53 is activated by genotoxic drugs, capable of inducing apoptosis, and widely regarded as a mediator of chemotherapy-induced cell death. However, it should be noted that chemotherapy can be effective despite tumor-associated p53 mutations, although p53 does not easily cause apoptosis in normal cells (2). Furthermore, WT p53 can be activated to induce cell cycle arrest in virtually any cell type and by much weaker stimuli than required for cell death (3). We propose here that this cell cycle arrest function can be used for the protection of normal cells because many available chemothera-peutic drugs specifically eliminate actively cycling cells. For instance, the effects of inhibitors of mitotic spindle formation are restricted to dividing cells, and activating p53 can partially protect fibroblasts against paclitaxel (4). On the other hand, nucleoside analogues, such as gemcitabine or cytosine-arabinoside (Ara-C), are widely used in chemotherapy and their effects are mediated through their false incorporation into replicating DNA. Resting cells do not undergo large-scale DNA replication and are therefore hardly affected by such nucleoside analogues. Deliberate induction of cell cycle arrest through p53 can therefore be expected to have a protective effect in this situation. The small-molecule nutlin-3 was developed to inhibit the p53 antagonist Mdm2, thereby activating p53 in a nongenotoxic fashion (5). The original purpose of these investigations was the activation of p53 in malignant cells that do not carry a *TP53* mutation, resulting in direct killing or chemo-sensitization. Here, we tested the idea of using nutlin-3 to protect normal cells (with WT p53) against nucleoside analogues in a scenario where tumor cells carry deletions or mutations of *TP53*. Indeed, pharmacologic inhibition of Mdm2 protected cells lacking *TP53* mutations against treatment with nucleoside analogues. We propose that this approach has the potential to provide sharper therapeutic discrimination between normal cells and tumor cells with p53 mutations.

Materials and Methods

Cell culture and drug treatment. U2OS cells were maintained in DMEM; HCT116 cells were maintained in McCoy's medium. Media were supplemented with 10% fetal bovine serum. Immortalized keratinocytes [EPC2-hTERT; generous gift of O.G. Opitz (University of Freiburg, Freiburg, Germany); ref. 6] were maintained in serum-free medium supplemented with 0.2 ng/mL epidermal growth factor and 25 μg/mL BPE (all from Invitrogen). Nutlin-3 (Alexis Biochemicals) was dissolved in DMSO as a stock solution of 25 mg/mL (43 mmol/L). Gemcitabine was stored as an aqueous solution. Cytosine β-D-arabinofuranoside (Ara-C), doxorubicin hydrochloride, and *cis*-diammineplatinum(II) dichloride (cisplatin) were purchased from Sigma and dissolved in DMSO as stock solutions of 20 mg/mL (Ara-C) or 10 mg/mL (doxorubicin and cisplatin). DMSO concentrations were kept constant in all experiments. In experiments involving drug combinations, the cells were treated with nutlin-3 alone for the first 24 hours followed by another incubation for 24 hours with a combination of nutlin-3 and a chemotherapeutic drug.

Immunoblots. Proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose followed by incubation with antibodies in PBS containing 5% milk powder and Tween 20 (0.05%). The following primary antibodies were used for detection: Ab-1 for p21/cip1/waf1 (Oncogene), Ab-6 DO-1 (Oncogene) and Ab-2 pAb1801 (Calbiochem) for p53, and AC-15 (Abcam) for β-actin. The monoclonal antibody 2A10 against Mdm2 was a generous gift of A.J. Levine. Primary antibodies were detected by chemiluminescence (Pierce), using a peroxidase-coupled secondary antibody (Jackson).

Flow cytometry. After standard fixation and propidium iodide staining, 10,000 cells per assay were analyzed by a FACScan Flow Cytometry System (Becton Dickinson), using the WinMDI software.
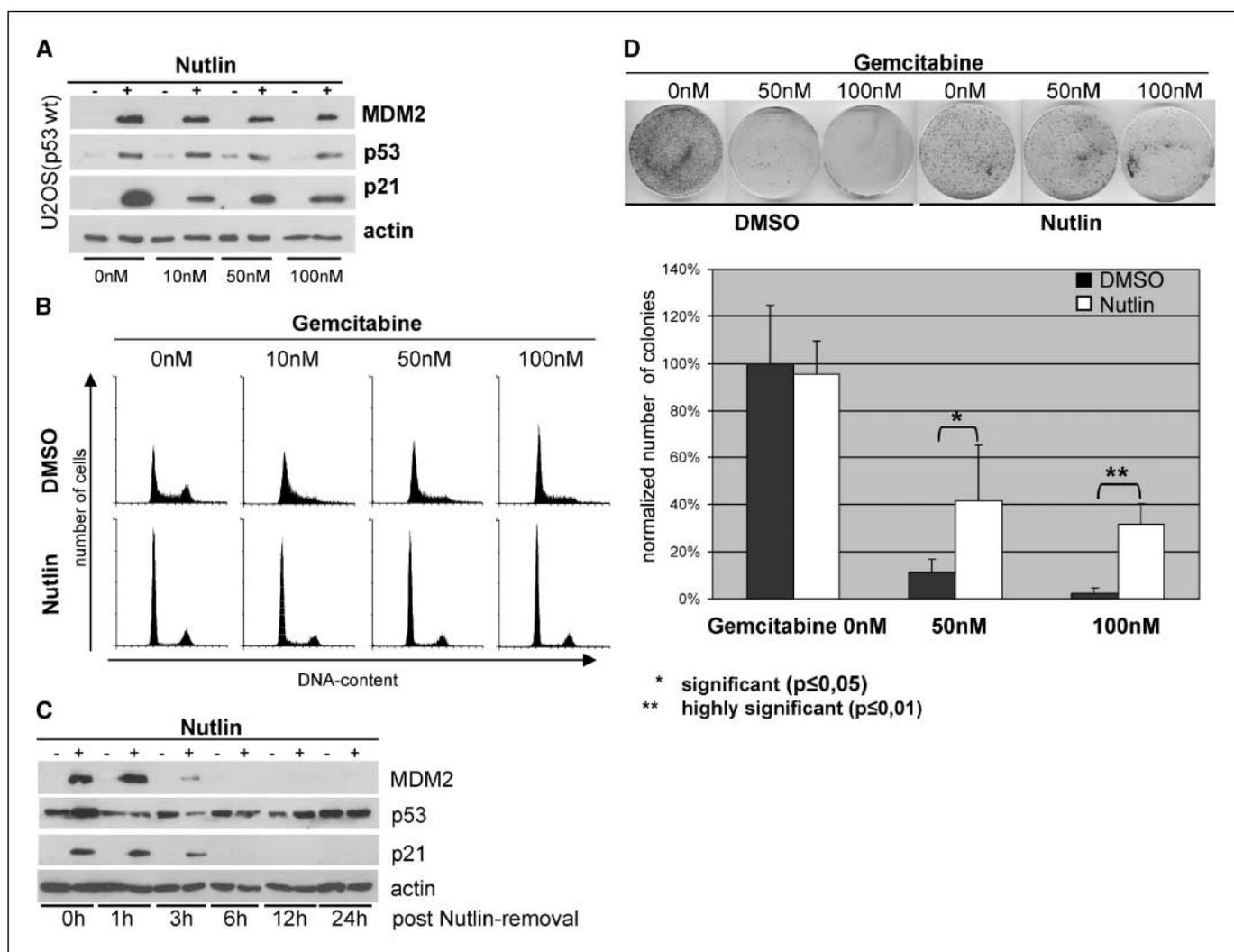
Figure 1. Effect of Mdm2 inhibition on p53 activity, cell cycle distribution, and clonogenic survival in response to gemcitabine. *A,* p53 levels and activity in U2OS cells after nutlin-3 and gemcitabine treatment. Cells were incubated for 24 hours with 8 μmol/L nutlin-3 or the solvent DMSO. Then, for another 24 hours, different concentrations of gemcitabine (0, 10, 50, and 100 nmol/L) were applied in combination with 8 μmol/L nutlin-3 or DMSO. The levels of p53, p21, and Mdm2 were assessed by immunoblot analysis. β-Actin staining was used as a loading control. *B,* cell cycle distribution of U2OS cells after nutlin-3 and gemcitabine treatment. Cells were incubated with drugs as described in (*A*), and their DNA content was determined by propidium iodide labeling and flow cytometry. *C,* p53 levels and activity after removal of nutlin-3. U2OS cells were treated with 4 μmol/L nutlin-3 or the solvent DMSO for 24 hours. Subsequently, nutlin-3 was removed and cells were cultivated further in medium without drugs. The cells were harvested for immunoblot analysis at the indicated time points, and proteins were detected as in (*A*). *D,* effect of nutlin-3 on the long-term survival of cells. U2OS cells were treated with 8 μmol/L nutlin-3 or DMSO for 24 hours followed by another incubation with gemcitabine (0, 50, 100 nmol/L) together with nutlin-3 or DMSO. After 48 hours in total, the cells were trypsinized and counted. To do clonogenic survival assays, 5,000 cells of the control (DMSO, without gemcitabine) were seeded in a 10-cm cell culture dish, and for the other samples, the equivalent volume of cell suspension was seeded. After 8 to 12 days, colonies were stained with crystal violet. The plates were scanned and colonies were counted by automated image analysis. Three independent experiments were subjected to statistical analysis for each cell line. *Columns,* mean; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$. The *t* test for two samples, assuming equal variances, was used to determine the levels of significance.

Clonogenic survival assays. Cells were seeded at a number of 5,000 per 10-cm dish, as counted for control cells (DMSO without gemcitabine). Equivalent volumes of cell suspension were used for plating the other cell samples. After 8 to 14 days, the emerging colonies were fixed and stained with crystal violet. The cell culture dishes were scanned to obtain digital images. The colonies were counted using the ImageQuant software (Amersham), and three independent experiments were carried out in each case. The mean number of the DMSO-treated cells was arbitrarily set to 100%, and all other numbers were normalized accordingly. For statistical analysis, the Student *t* test for two samples was used, assuming equal variances.

Results

Antagonizing Mdm2 induces reversible cell cycle arrest, protecting U2OS cells against gemcitabine treatment. Gemcitabine

(2′,2′-difluorodeoxycytidine) is a nucleoside analogue with specific activity during the S phase of the cell cycle, leading to strand termination of newly synthesized DNA by several mechanisms (7). To investigate the effect of nongenotoxic p53 activation on the efficacy of gemcitabine, we used nutlin-3, a specific inhibitor of the interaction between p53 and Mdm2 (5). We preincubated U2OS cells (carrying WT p53) with nutlin-3 for 24 hours and then added gemcitabine in a range of different concentrations together with nutlin-3. As expected (5), the application of nutlin-3 resulted in increased protein levels of p53 and its target gene products, Mdm2 and p21 (Fig. 1*A*). The application of gemcitabine had no additional effect on the protein levels, and gemcitabine alone did not detectably increase the levels of p53 and its target gene products. This is in accordance with the notion that triggering the intra-S

phase checkpoint does not increase the ability of p53 to induce *CDKN1A/p21* (8).

Next, we used flow cytometry to determine the cell cycle–dependent DNA content in response to the same treatment scheme (Fig. 1*B*). The application of gemcitabine led to a DNA content slightly higher than 2N, corresponding to arrest within early S phase (9). In contrast, cells treated with nutlin-3 alone were arrested in G_1 and G_2-M but not in S phase. Following nutlin-3 pretreatment, the addition of gemcitabine did not further affect the distribution of the DNA content.

We then asked whether nutlin-3–induced p53 activation is reversible in this system. To this end, nutlin-3 was removed after treating cells for 24 hours, and the levels of p53 and its target gene products were monitored by immunoblot analysis in a time course. As shown in Fig. 1*C*, the accumulation of Mdm2 and p21

diminished at 3 hours after nutlin-3 removal and was completely abolished after 6 hours, suggesting that cells might be capable of resuming proliferation on withdrawal of nutlin-3.

To assess the possibility that nutlin-3 can protect cells against a chemotherapeutic treatment, we did clonogenic survival assays. After pretreatment with nutlin-3 (or DMSO alone) and subsequent incubation with gemcitabine (together with DMSO or nutlin-3, respectively), all drugs were washed away. The cells were then seeded and allowed to form colonies. As shown in Fig. 1*D*, gemcitabine treatment alone strongly reduced the number of colonies. A moderate decrease was also observed on treatment with nutlin-3 alone, perhaps as a result of delayed cell proliferation during the incubation period before seeding. However, when cells were pre-incubated with nutlin-3, the number of colonies remaining after subsequent gemcitabine treatment was enhanced up to 10-fold.
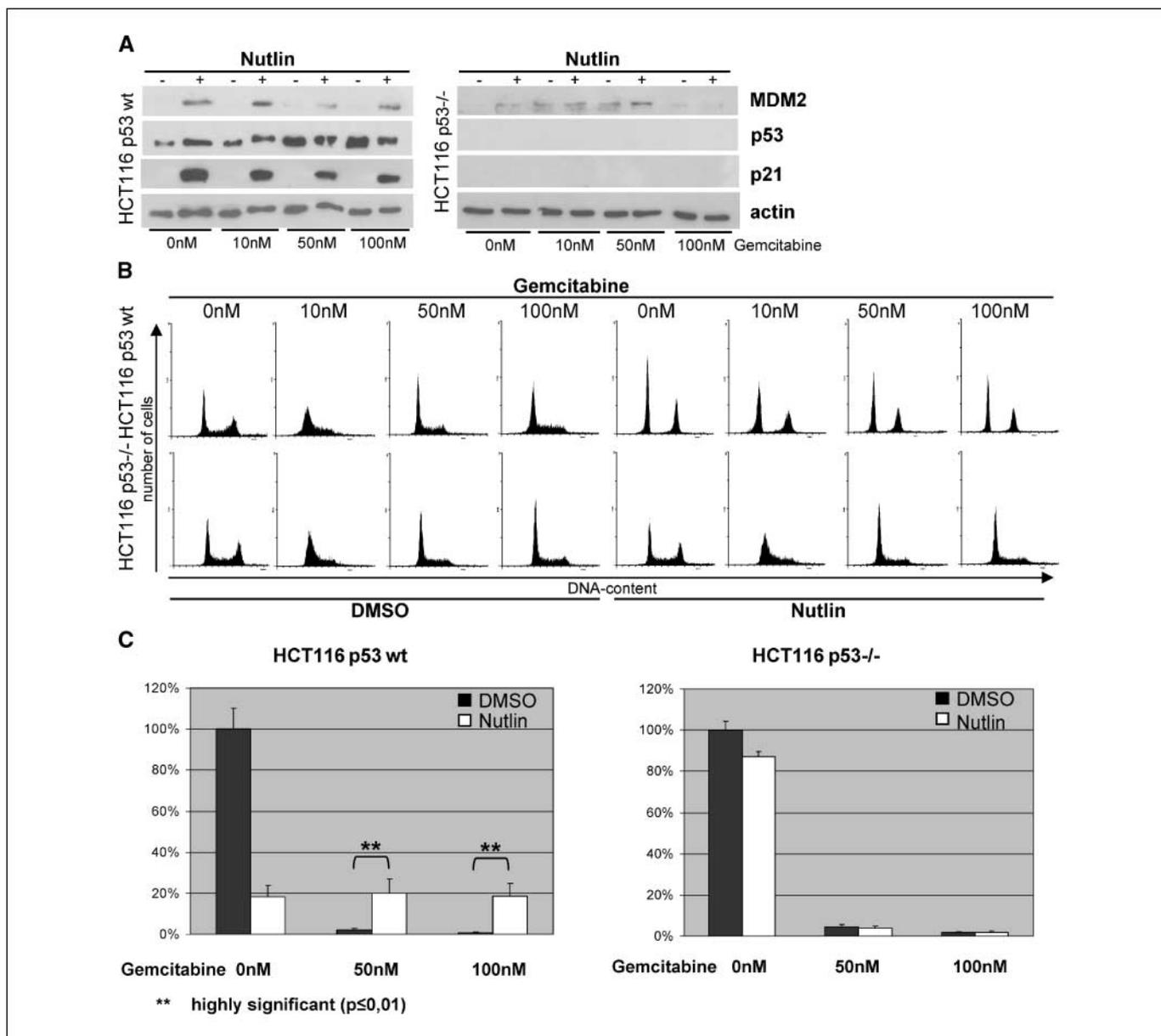
Figure 2. Protection by nutlin-3 as a function of the p53 status. *A,* effect of nutlin-3 on p53 levels and activity in an isogenic pair of cell lines. HCT116p53wt and HCT116p53$^{-/-}$ were treated sequentially with nutlin-3 and gemcitabine followed by immunoblot analysis as in Fig. 1*A*. *B,* flow cytometry was done as described for Fig. 1*B*. *C,* clonogenic survival assays were done as in Fig. 1*D*.
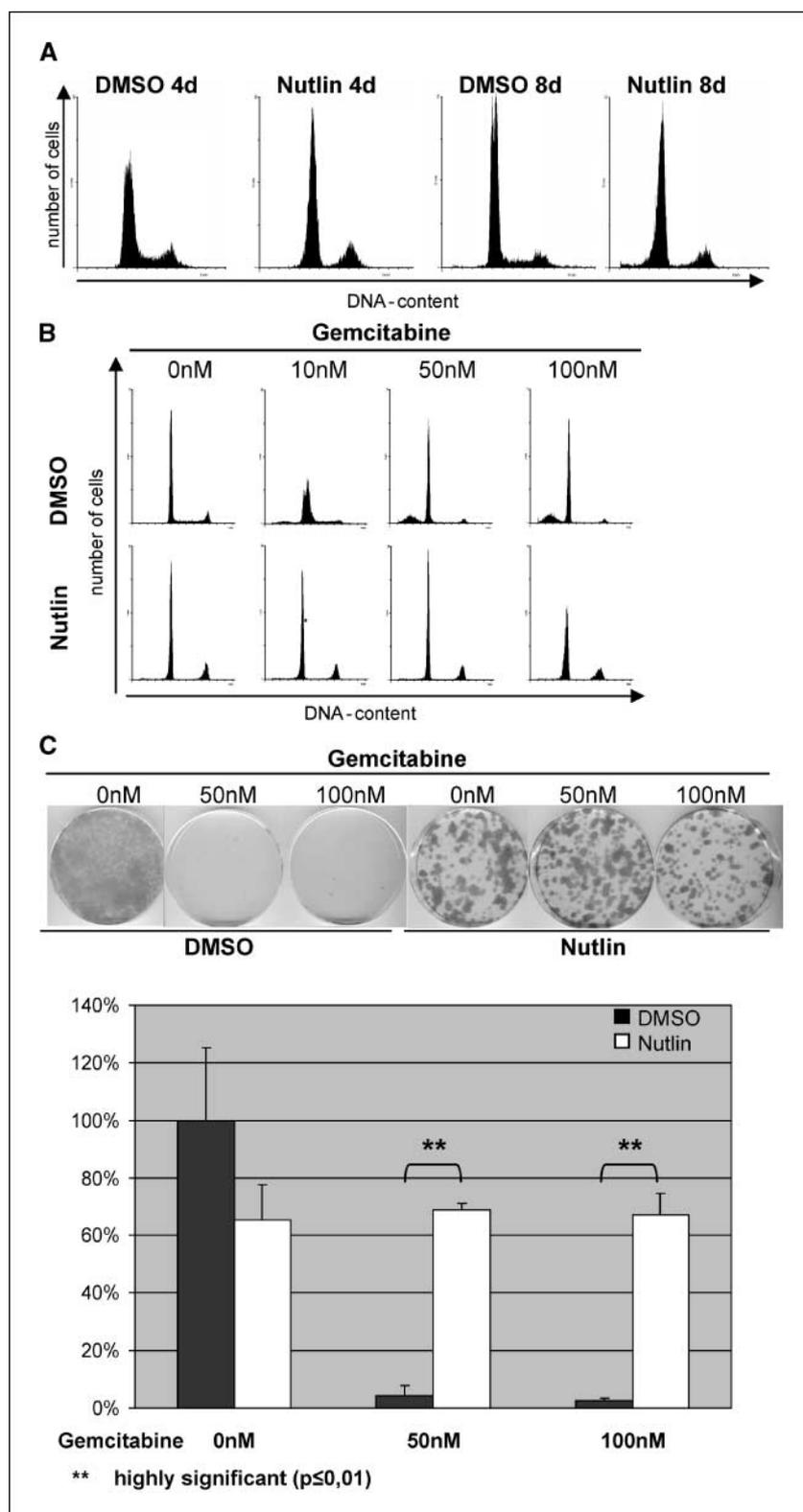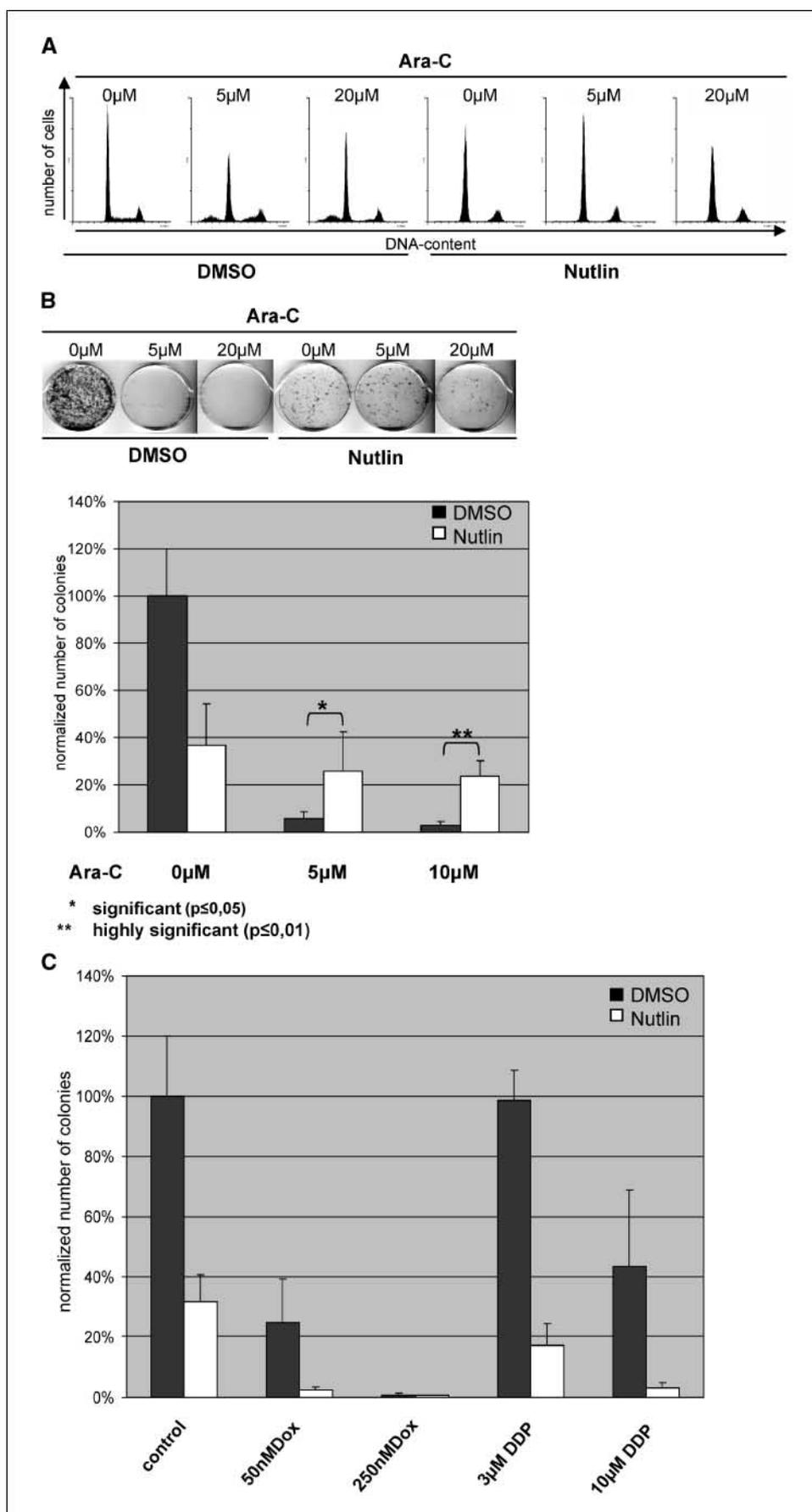
Figure 3. Protection of nontransformed cells by nutlin-3. *A,* cell cycle distribution after long-term treatment of EPC2-hTERT cells with nutlin-3. The cells were treated with nutlin-3 or DMSO for 4 and 8 days and then subjected to flow cytometry. *B,* cell cycle distribution after combined treatment with nutlin-3 and gemcitabine in EPC2-hTert cells. Cells were treated and subjected to flow cytometry as in Fig. 1*B*. Note that gemcitabine alone induced a sub-G_1 peak in these cells. *C,* effect of nutlin-3 on the long-term survival of EPC2-hTert cells. The cells were treated and clonogenic survival assays were done as in Fig. 1*D*.

This indicates that nutlin-3-treated cells are at least partially protected against the effects of gemcitabine.

WT p53 is required for protection by nutlin-3. To assess whether the protective effect of nutlin-3 was actually dependent on the presence of p53, we used an isogenic pair of cell lines, derived from the human colon carcinoma cell line HCT116 (10). HCT116 cells that contain WT p53 (HCT116p53wt) essentially showed the same phenotype as U2OS cells about p53 activation, cell cycle distribution, and colony formation (Fig. 2*A-C*). In contrast, HCT116 lacking p53 (HCT116p53$^{-/-}$) were hardly affected by nutlin-3. They contained p53 target gene products at much lower levels (Fig. 2*A*), they were trapped in S phase by gemcitabine regardless of nutlin-3

Figure 4. Protection by nutlin-3 against nucleoside analogues but not against other chemotherapeutic agents. *A,* cell cycle distribution of nontransformed keratinocytes (EPC2-hTert) after nutlin-3 and Ara-C treatment. Cells were treated with 8 μmol/L nutlin-3 or DMSO for 24 hours followed by another incubation with Ara-C plus nutlin-3 or DMSO. After 48 hours in total, the cells were analyzed by flow cytometry using propidium iodide for detection of DNA. *B,* cells were treated as in (*A*) and clonogenic survival assays were done as described in Fig. 1*D*. *C,* clonogenic survival assays after the combined treatment with nutlin-3 and doxorubicin or cisplatin. EPC2-hTert cells were treated for 24 hours with nutlin-3 or DMSO followed by another incubation with nutlin-3 plus doxorubicin (*Dox*) or cisplatin (*DDP*). After 48 hours in total, clonogenic assays were done as in Fig. 1*D*.

pretreatment (Fig. 2B), and their survival in clonogenic assays was drastically reduced by gemcitabine despite nutlin-3 pretreatment (Fig. 2C). Similarly, tumor cells with a deletion (H1299 cells) or mutation (C33A and HT-29 cells) of p53 were not protected by nutlin-3 against gemcitabine treatment (Supplementary Figs. S1 and S2). We conclude that the presence of WT p53 is strictly required to ensure the protective effect of nutlin-3 against gemcitabine.

Nontransformed human keratinocytes survive nucleoside analogue treatment in the presence but not in the absence of an Mdm2 antagonist. Next, we addressed whether nontransformed human cells can be protected by nutlin-3. We chose human keratinocytes immortalized by transduction with the catalytic subunit of telomerase (hTERT), termed EPC2-hTERT (6). First, we tested the effect of a long-term treatment of nutlin-3 on these cells. Flow cytometric analysis (Fig. 3A) revealed that these cells, even on 8 days of treatment with nutlin-3, arrested preferentially in G_1 phase but hardly underwent apoptosis, suggesting that even prolonged treatment with nutlin-3 does not lead to cell death. In contrast, gemcitabine readily induced the accumulation of cells with a sub-G_1 DNA content, indicative of cell death (Fig. 3B). Nutlin-3 pretreatment abolished this sub-G_1 peak. Correspondingly, more than half of the cells survived and caused colonies when pretreated with nutlin-3 before addition of gemcitabine (Fig. 3C). We conclude that the outlined strategy is suitable, at least in principle, to spare normal cells from the effects of gemcitabine.

Nutlin-3 protects against nucleoside analogues but not against other chemotherapeutic agents. Based on the observed results, we asked whether nutlin-3 could also protect EPC2-hTert cells against cytosine β-D-arabinofuranoside (Ara-C), a pyrimidine analogue. As depicted in Fig. 4A and B, pretreatment with nutlin-3 largely abolished the effects of Ara-C on cell cycle distribution and colony formation. Protection of cells against Ara-C by nutlin-3 was also observed in HCT116 cells and was strictly dependent on the presence of WT p53 (data not shown). Hence, nutlin-3 confers protection against more than one nucleoside analogue. To test whether nutlin-3 is able to protect against other types of chemotherapeutic agents, we chose doxorubicin and cisplatin. Doxorubicin causes DNA damage by inducing double-strand breaks, whereas cisplatin forms intrastrand and interstrand cross-bridges. Survival after the combined treatment of either nutlin-3 and doxorubicin or nutlin-3 and cisplatin was assessed by clonogenic assays in EPC2-hTert cells. As outlined in Fig. 4C, combination treatment with nutlin-3 enhanced the cytotoxicity of doxorubicin in this assay. This is in accordance with the observed synergism of nutlin-3 and doxorubicin in B-cell leukemia cells (11). A similar cooperation was observed on treatment with cisplatin and nutlin-3. In conclusion, nutlin-3 enhances the cytotoxicity of cisplatin and doxorubicin, whereas it largely reduces that of the nucleoside analogues examined.

Discussion

Our results suggest a strategy that exploits p53 for the chemoprotection of normal cells. This strategy is potentially applicable when a tumor contains a mutation or deletion of *TP53*. Given the high frequency of such mutations in malignant

tumors, it would be attractive to translate this or a similar approach into clinical applications. However, although blocking unwanted side effects on nontumor cells through Mdm2 inhibition would presumably increase the tolerated dose of nucleoside analogues, it will be important to determine whether an increase in gemcitabine or Ara-C dose would actually lead to improved treatment efficacy toward tumor cells. We consider this study as a proof of principle that supports the general feasibility of chemoprotection by p53-mediated cell cycle arrest, only representing a first step toward possible clinical applications.

Recent results suggest that the effects of nutlin-3 on p53 activity are more pronounced in cancer cells than in normal cells (12), possibly due to constitutive activation of a DNA damage signaling pathway (13). On the other hand, results using human keratinocytes (this report) or human diploid fibroblasts (4) indicate that normal cells can still respond to nutlin-3. It seems that Mdm2 inhibition causes a weaker but effective activation of p53 in normal as opposed to cancerous cells. Together with the generally lower susceptibility of normal cells to apoptotic stimuli (2), this might further ensure that nutlin-3 and similar drugs can induce cell cycle arrest but only low frequencies of apoptosis in normal cells.

The approach described here is in seeming contrast to more traditional attempts to target p53 for chemoprotection. Although p53 can be used to protect cells from drugs that specifically act in the S or M phase of the cell cycle, the drug candidate pifithrin, for instance, was reported to inhibit p53 activity and to prevent apoptosis of normal cells on genotoxic stress induced by γ irradiation (14). In such a case, it is conceivable that, on γ irradiation, which induces DNA double-strand breaks regardless of the cell cycle stage, the absence or inhibition of p53 avoids apoptosis and promotes the survival of certain cell species.

Finally, our study raises the question whether sequential combinations of conventional drugs might compromise the effects of gemcitabine, especially when they prevent the onset of DNA replication. This is of special relevance about tumor cells with WT p53 (e.g., when gemcitabine application follows the administration of a drug that blocks cell cycle progression). Indeed, gemcitabine efficacy was decreased when administered immediately after a doxorubicin-paclitaxel combination but re-established when pretreated cells were first allowed to enter S phase (15). Thus, especially when treating tumors with a WT p53 status using drug combinations, premature cell cycle arrest should be avoided to ensure the efficacy of nucleoside analogues.

Acknowledgments

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