Activation of p53 by MDM2 Antagonists Can Protect Proliferating Cells from Mitotic Inhibitors

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Abstract

Recent studies have shown that activation of cell cycle checkpoints can protect normal proliferating cells from mitotic inhibitors by preventing their entry into mitosis. These studies have used genotoxic agents that act, at least in part, by activation of the p53 pathway. However, genotoxic drugs are known also to have p53-independent activities and could affect the sensitivity of tumor cells to antimitotic agents. Recently, we have developed the first potent and selective small-molecule inhibitors of the p53-MDM2 interaction, the nutlins, which activate the p53 pathway only in cells with wild-type but not mutant p53. Using these compounds, we show that p53 activation leads to G1 and G2 phase arrest and can protect cells from mitotic block and apoptosis caused by paclitaxel. Pretreatment of HCT116 and RKO colon cancer cells (wild-type p53) or primary human fibroblasts (1043SK) with nutlins for 24 hours followed by incubation with paclitaxel for additional 48 hours did not increase significantly their mitotic index and protected the cells from the cytotoxicity of paclitaxel. Cancer cells with mutant p53 (MDA-MB-435) responded to the same treatment with mitotic arrest and massive apoptosis. These results have two major implications for cancer therapy. First, p53-activating therapies may have antagonistic effect when combined with mitotic poisons. Second, pretreatment with MDM2 antagonists before chemotherapy of tumors with mutant p53 may offer a partial protection to proliferating normal tissues. (Cancer Res 2005; 65(5): 1918-24)

Introduction

Cell cycle is a highly ordered set of biochemical events leading to the faithful duplication and segregation of eukaryotic chromosomes (1, 2). This process is tightly controlled by checkpoint mechanisms evolved to prevent cells from entering any cell cycle phase before the completion of the previous one (3). Therefore, agents that interfere with processes specific for any particular cell cycle phase will be inactive if the cells are prohibited from entering their targeted phase. This phenomenon is in the basis of the recently proposed strategy for protection of normal cells from cytotoxicity of chemotherapeutic agents (4–6). Normal proliferating cells possess fully functional checkpoint controls and respond to treatment with cytotoxic agents, most of which are genotoxic, by cell cycle arrest in G1 and/or G2 phase. At the same time, most cancer cells that have suffered multiple genetic alterations and frequently lack effective checkpoint regulation may continue cycling through their apoptotic death. Thus, one can achieve partial protection of normal cells by pretreatment with low doses of common genotoxic drugs (7). This strategy is most applicable to chemotherapy with mitotic inhibitors, which are strictly dependent for their activity on the ability of the cells to enter mitosis. Following this approach, pretreatment of cancer cells with low doses of the genotoxic drug doxorubicin have shown cell cycle arrest and partial protection from mitotic poisons (e.g., paclitaxel, epothilone, and vinblastine). This effect is mostly due to induction of the p53 pathway and is mediated by p21Waf1/CIP1 because p53-null or p21-null cancer cells cannot be protected (7). However, doxorubicin and other DNA-damaging agents can trigger multiple molecular events including activation of p53-independent checkpoints and thus may partially protect the cancer cells during chemotherapy. This can be avoided by using agents targeted specifically at the p53 pathway.

The tumor suppressor p53 plays a pivotal role in cellular response to genotoxic damage or other forms of stress by inducing cell cycle arrest or apoptosis and thus preventing the propagation of DNA damage that may lead to malignant cell transformation (8, 9). p53 is a potent transcription factor that controls the activity of multiple target genes involved in the control of cell cycle or apoptosis. One of the main functions of activated p53 is induction of the G1-S and G2-M checkpoints of the cell cycle. This function is mediated by the product of the immediate downstream gene p21Waf1/CIP1 (8, 10). In proliferating cells that are not subjected to stress, p53 level is tightly controlled by its negative regulator MDM2, which binds p53 and modulates its transcriptional activity and stability (11–15). Recently, we have identified the first potent and selective small-molecule inhibitors of p53-MDM2 binding, the nutlins (16). These compounds can release p53 from negative control and activate the p53 pathway, leading to cell cycle arrest and apoptosis only in cancer cells that have retained wild-type p53 but not in cells in which p53 is deleted or disabled by mutation. Cellular activity of nutlins is derived solely from activation of the p53 pathway and therefore they represent unique tools for p53-dependent modulation of the cell cycle in proliferating cells.

Due to its high importance in protection from tumor development, p53 is mutated in ~50% of all human tumors rendering them practically insensitive to p53-activating agents (9, 17). This offers the opportunity to develop an improved protective strategy for protection of normal proliferating tissues without affecting the sensitivity of tumors with mutant p53 to antimitotic agents. Here, we use nutlins as p53-activating agents and show that they can induce G1-S and G2-M checkpoints and can protect normal proliferating cells but not cancer cells with...
mutant p53 from cytotoxicity of paclitaxel. Thus, MDM2 antagonists may offer a new modality for partial protection of normal proliferating tissues during antimitotic chemotherapy of p53-deficient tumors.

Materials and Methods

Cells and Drug Treatment. RKO cells were a gift from Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD) and MDA-MB-435 were provided by Dr. P. Steeg (National Cancer Institute, Bethesda, MD). HCT116, HT29, LOX, SW480, SJSA-1, and 104SSK cells were purchased from American Type Culture Collection (Rockville, MD) and grown in the recommended medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, San Diego, CA) in a humidified environment with 5% CO2. Drugs were dissolved in DMSO and kept as 10-mmol/L stock solutions in small aliquots at –20°C. For staining of cells, acridine orange and propidium iodide (Sigma Chemical Co., St. Louis, MO) were dissolved in PBS at 0.3 mg/mL and added to culture medium at 1 μg/mL. For 5- to 10-minute incubation at 37°C, cells were examined under fluorescence microscope and images were taken by SPOT digital camera. Cell viability was determined either by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (18) or by the Guava personal cell analyzer using the Guava ViaCount kit (Guava Technologies, Hayward, CA).

Cell Cycle Analysis. For analysis of cell cycle distribution, 106 cells were seeded in 75-cm2 flasks 24 hours before drug treatment. They were labeled with 20 μmol/L bromodeoxyuridine (Sigma) added to the culture medium for 2 hours before harvesting. The cells were washed twice by PBS and collected by trypsinization. They were fixed with 70% ethanol and stored overnight at –20°C. After thawing, cells were washed twice in cold PBS and resuspended in 1 mL of 2N HCl and 0.5% Triton X-100 in PBS for 30 minutes at room temperature. Cells were collected by centrifugation and the cell pellet was neutralized with 1 mL of 0.1 mol/L sodium tetraborate (pH 8.5), washed with PBS and resuspended in 0.1 mL of 0.5% Tween 20 and 1% bovine serum albumin in PBS. Anti-bromodeoxyuridine FITC-conjugated monoclonal antibody (20 μL, Becton Dickinson, San Jose, CA) was added and incubated for 30 minutes in the dark at room temperature. After one wash with Tween 20/bovine serum albumin/PBS buffer, the cell pellet was resuspended in 0.5 mL of PBS with propidium iodide (50 μg/mL), filtered through filter caps, and bromodeoxyuridine incorporation was analyzed by dual-color flow cytometric DNA techniques in the FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software. Cell number in each phase of the cell cycle was determined and calculated as a percentage of the total cell population.

Quantitative PCR. Cells were seeded in 96-well plates (104 cells per well) 24 hours before treatment. They were lysed and total RNA extracted using the ABI 6700 robotic workstation (Applied Biosystems, Foster City, CA). Aliquots containing 5 μg total RNA were converted to cDNA using the TaqMan RT reagents kit (Applied Biosystems). The relative quantity of the p21 mRNA was determined by TaqMan using gene-specific primer/probe sets and 18S RNA as a normalization control. The sequence of the p21 primers and probe were F: CTG-AGA-CTC-CTA-GGG-TCG-AA; R: CGG-CGT-TTG-GAG-TGG-TAG-AA; and probe: TTG-GCT-CAC-TGC-AAG-CTC-GCC-CCT-TT.

Results

MDM2 Antagonists Activate p53 and Arrest Cell Cycle Progression. Induction of cell cycle arrest at the G1-S and G2-M border is one of the main functions of activated p53. This arrest is mediated predominantly by the product of the p53 target gene p21waf1/cip1 (8, 10) encoding a potent cyclin-dependent kinase (CDK) inhibitor. Inhibition of CDK2/cyclin E and CDK4/cyclin D activity is thought to block cell cycle progression in late G1 phase just before entering into S phase. Inhibition of CDK1/cyclin B kinase activity leads to a block at the G2-M phase border of the cell cycle. Recently, we developed a class of specific small-molecule antagonists of p53-DMG2 interaction, the nutlins, which can release p53 from negative control and activate the p53 pathway (16). These cis-imidazoline compounds represent valuable tools for modulation of the p53 pathway in living cells. We showed previously that they can effectively arrest cell cycle progression in cells with wild-type p53 but not in cells in which p53 is missing or disabled as a transcription factor. Each of these compounds can be separated postsynthetically into two enantiomers, arbitrarily called (a) and (b). Enantiomer (a) binds to MDM2 protein with ~150-fold higher potency than enantiomer (b). Thus, the inactive enantiomer (b) offers an excellent control for cellular activities unrelated to MDM2 inhibition (16).

To show that p21 activation is involved in nutlin-induced cell cycle arrest, we incubated two human cancer cell lines with wild-type p53, HCT116 (colon cancer) and SJSA-1 (osteosarcoma) with nutlin-1 (racemic), or the active or inactive enantiomer of nutlin-3 for 24 hours and measured the expression of p21 by quantitative real-time PCR (Fig. 1A). In cells treated with nutlin-1 and the active enantiomer of nutlin-3 a dose-dependent elevation in p21 expression was observed. At 10 μmol/L, both nutlins induced p21 transcription 10-fold in HCT116 and 20-fold in SJSA-1 cells. Differences in the level of induction reflect differences in the basal level of p21 expression because the induced levels were similar between the two cell lines (data not shown). The inactive enantiomer did not show detectable elevation in p21 expression confirming the high selectivity of MDM2 antagonists.

Next, we analyzed cell cycle distribution in HCT116 and SJSA-1 cells treated with nutlin-1 for 24 hours (Fig. 1B). Nutlin-1 at 2 μmol/L showed a substantial decrease in the S phase population in both cell lines and essentially depleted the S phase pool at 4 μmol/L. At the same time, G1 and G2-M fractions increased. SJSA-1 cells showed a relatively higher G2-M fraction than HCT116 cells. Examination of the cell morphology by staining with DNA-binding dyes (acridine orange and 4′,6-diamidino-2-phenylindole) did not reveal an increase in the mitotic figures compared with untreated controls indicating that the G2-M fraction is composed of G2 cells (data not shown). Cell cycle analyses of three additional cancer cell lines with wild-type p53 (RKO, HT29, and LOX) confirmed the G1 and G2 arrest observed in HCT116 and SJSA-1 cells (data not shown). In contrast, two cell lines with mutant p53 (MDA-MB-435 and SW480) showed a cell cycle profile indistinguishable from untreated controls (data not shown). These studies confirmed that nutlins induce effective cell cycle arrest in G1 and G2 phases. This arrest is reversible within the first 24 hours of treatment but becomes increasingly irreversible thereafter as a substantial fraction of the population undergoes p53-dependent apoptosis (16).

Activation of p53-Regulated G1 and G2 Checkpoints Protect Cancer Cells from Paclitaxel. The activation of G1-S and G2-M checkpoints by p53 is a mechanism evolved to protect proliferating cells from propagation of DNA damage that may occur during chromosome replication and segregation. Cells prevented from entering mitosis should be protected effectively from mitotic antagonists of p53-MDM2 interaction, the nutlins, which can induce apoptosis and cell death. Taxanes are one of the most widely used classes of chemotherapeutic agents that block cell cycle progression in metaphase of mitosis by interfering with microtubule dynamics (19, 20). Mitotic block is followed by induction of apoptosis and cell death. Taxanes and other mitotic poisons (e.g., vinblastine and vincristine) are among the most...
potent cytotoxic drugs. However, due to their M phase–specific mechanism of action, mitotic inhibitors do not induce apoptosis in nonproliferating cells or cells blocked in cycle phases other than mitosis.

We tested the ability of nutlins to protect proliferating cancer cells from the most widely used mitotic inhibitor, paclitaxel. SJSA-1 cells were incubated with nutlin-3 or paclitaxel for 24 hours, or nutlin-3 for 24 hours followed by paclitaxel for another 24 hours. Paclitaxel (250 nmol/L) treatment caused a massive accumulation of cells with rounded morphology and condensed chromosomes typical for metaphase (Fig. 2). Pretreatment with nutlin-3 (4 μmol/L) for 24 hours reduced dramatically mitotic figures from 93% (paclitaxel alone) to 5% (nutlin-3 + paclitaxel), although significant percentage of the cells showed deterioration due to induction of p53-dependent apoptosis in this cell line (16). Similar observations were made with HCT116 and RKO cells (data not shown). These experiments show that nutlins can effectively protect cancer cells from entering mitosis, thus making them much less sensitive to paclitaxel.

To quantify the protective effect of MDM2 antagonists against paclitaxel, we pretreated HCT116 and RKO cells with 6 μmol/L of either the active or inactive enantiomers of nutlin-3 for 24 hours and measured cell viability after additional 2-day incubation of the cells in the presence of increasing paclitaxel concentrations (Fig. 3). These experiments show that nutlins can effectively protect cancer cells from entering mitosis, thus making them much less sensitive to paclitaxel.

To assess the protective role of MDM2 antagonists on normal proliferating fibroblasts that have seemed less sensitive to MDM2 antagonists than cancer cells (16, 21) and have been shown previously to undergo reversible p53-dependent growth arrest (22, 23).

MDM2 Antagonists May Protect Normal Proliferating Cells during Chemotherapy with Antimitotic Agents. As a model for normal proliferating tissues, we chose the primary skin fibroblasts, 1043SK. Similar to cancer cells with wild-type p53, fibroblasts responded to nutlin-1 treatment with activation of p21 as manifested by the induction of p21 transcripts (Fig. 4A) and cell cycle arrest predominantly in G1-S (not shown). In addition, cell cycle arrest in 1043SK cells can be partially reversed upon drug removal for up to 7 days (16, 21). To assess the protective role of nutlins, we treated 1043SK cells for 24 hours with nutlin-1 followed by paclitaxel for 48 hours and then continued incubation in fresh, drug-free medium for additional 5 days. This regimen was established to evaluate the protective effect of nutlin on the viability of the cells and on the ability to

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1 Unpublished data.
recover and restore their proliferative capacity. Similar to the results with cancer cells, nutlin-1 showed a remarkable ability to protect fibroblasts from paclitaxel (Fig. 4B and C). Fibroblasts pretreated with 6 \mu mol/L nutlin-1 for 24 hours followed by 1 \mu mol/L paclitaxel for 48 hours (2.65 \times 10^5 cells per well) retained \sim 50\% of the viability of paclitaxel-free controls (5.1 \times 10^5 cells per well) after additional 5 days' incubation in drug-free medium. Unprotected fibroblasts treated with the same paclitaxel concentration (5.5 \times 10^5 cell/well) were \sim 7\% of the viable cells in their respective controls (Fig. 4B). These data indicated that MDM2 antagonists may protect proliferating normal tissues against mitotic inhibitors and suggested a potential clinical use.

The fact that \sim 50\% of all human tumors harbor disabling mutations or deletions in the p53 gene (16) and that these tumors are not sensitive to MDM2 antagonists (16) offers a possibility to protect selectively normal tissues without affecting the response of cancer cells to mitotic poisons. One possible chemoprotection scenario is shown in Fig. 5. Pretreatment of patients for 24 hours with doses of MDM2 antagonists adequate to effectively arrest cell cycle progression should take the majority of proliferating normal cells out of cycling mode before administration of paclitaxel. Additional 48 hours of exposure to MDM2 antagonists will help to maintain arrest and resistance to the mitotic inhibitor until paclitaxel-induced mitotic block in cancer cells triggers irreversible apoptosis. In the absence of drugs, normal cells may resume proliferation. The pretreatment with MDM2 antagonists could be applied before each paclitaxel chemotherapy cycle.

To confirm that cancer cells with mutant p53 are not protected by nutlins, we applied the treatment regimen used on 1043SK fibroblasts to the breast cancer cell line MDA-MB-435 in which p53 is disabled by mutation in its DNA-binding domain. We have shown previously that nutlin treatment does not affect cell cycle progression or cause cytotoxicity in MDA-MB-435 cells (16). Pretreatment of these cells with nutlin-1 for 24 hours did not alter their sensitivity to paclitaxel (Fig. 6). Both nutlin-treated and control cells lost their viability at paclitaxel doses higher than 10 \mu mol/L. This result suggests that MDM2 antagonists should not affect the sensitivity to mitotic inhibitors of cancer cells with mutant or deleted p53.

**Discussion**

Using MDM2 antagonists as selective p53 activators, we have shown that induction of cell cycle arrest in both normal proliferating fibroblasts (1043SK) or cancer cells with wild-type p53 (HCT116, RKO) can protect partially the cells from the cytotoxicity of paclitaxel. Partial protection of HCT116 cells against mitotic inhibitors has been reported previously by using doxorubicin as a genotoxic inducer of the p53 response (7). However, the usefulness of doxorubicin and any other DNA-damaging agent is limited by their ability to activate p53-independent checkpoint mechanism in cancer cells with mutant p53 (24, 25). The protection by MDM2 antagonists, which work solely through stabilization and activation of p53, is strictly dependent on the p53 status of the cells (16). Half of all human tumors have lost their ability to activate the p53 pathway mostly

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**Figure 2.** Pretreatment with nutlins can protect cancer cells with wild-type p53 from mitotic arrest by paclitaxel. SUJA-1 osteosarcoma cells were incubated with 4 \mu mol/L of the active enantiomer nutlin-3a for 24 hours (A), 250 \mu mol/L paclitaxel for 24 hours (B), and 4 \mu mol/L nutlin-3a for 24 hours followed by 250 \mu mol/L paclitaxel for another 24 hours (C). Live cells were stained with acridine orange and examined by fluorescence microscopy. Bars, 50 \mu m.

**Figure 3.** Nutlins protect cancer cells with wild-type p53 from paclitaxel. HCT116 and RKO cells (2,500 cells per well, 96-well plates) were grown for 24 hours before treatment with nutlin-3 (6 \mu mol/L) for another 24 hours followed by 48 hours treatment with paclitaxel. Cell viability was determined 48 hours after paclitaxel addition using the MTT assay.
due to mutations in the DNA-binding domain that render p53 inactive as a transcription factor (9, 17). These tumors will be insensitive to p53-activating agents that rely exclusively on the ability of p53 to induce downstream signaling in the p53 pathway (e.g., nutlins). Therefore, using MDM2 antagonists, one could design a chemoprotective strategy that does not have the potential to compromise the response of targeted cancer cells. One example of such strategy includes pretreatment of patients with relatively low doses of MDM2 antagonists that can induce cell cycle arrest in normal proliferating tissues before administration of antimitotic chemotherapeutics (Fig. 5). Although the experiments described in this report use paclitaxel as a mitotic inhibitor, this approach is principally applicable to any agent that derives its cytotoxicity solely from interference with the cell division phase of the cell cycle. Examples include vinblastine, vincristine, epothilones, and many of the new members of the taxane family (20) as well as novel therapeutic agents currently in development that target mitotic motors (e.g., kinesin Eg5; ref. 26) or regulators (e.g., aurora kinase; ref 27).

The experiments described in this study use primary fibroblasts as a model system for proliferating normal human cells. Fibroblasts may not represent the most sensitive tissues adversely affected during cytotoxic chemotherapy (e.g., proliferating cells from gut epithelium or hematopoietic system). However, treatment of nude mice with nutlin-3 doses that effectively inhibit the growth of tumor xenografts for 3 weeks...
did not reveal overt toxicity, thus suggesting that normal tissues may have higher tolerance to p53 activation (16, 21). Additional experiments in which the protective role of MDM2 antagonists is assessed on multiple normal tissues in vivo may help to better understand the clinical potential of this approach.

Another implication of our studies concerns combination therapy with anti-mitotic agents. Our data on the protective role of MDM2 antagonists in cancer cells with wild-type p53 suggest that these agents should be used with caution when combined with mitotic poisons. Paclitaxel and other mitotic chemotherapeutics are frequently used together with genotoxic drugs (e.g., DNA binders, alkylating agents, topoisomerase inhibitors, etc.). These agents can activate the p53 pathway in tumors with wild-type p53 via genotoxic stress. Therefore, if used before, or in combination with, mitotic drugs they could protect the cancer cells from the mitotic agents. This has been shown in vitro and in vivo by the decreased sensitivity of HCT116 colon cancer cells to paclitaxel compared with their isogenic p53-null or p21-null variants (28). Taken together, these observations suggest that p53-activating agents should be used with caution in combination therapy with mitotic poisons in patients whose tumors harbor wild-type p53.

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