Wild-Type p53 Protein Potentiates Cytotoxicity of Therapeutic Agents in Human Colon Cancer Cells

Bin Yang, James R. Eshleman, Nathan A. Berger, and Sanford D. Markowitz

Department of Molecular Biology and Microbiology [B. Y., S. D. M.], Department of Medicine [B. Y., N. A. B., S. D. M.], and Department of Pathology, Case Western Reserve University [J. R. E., J. R. E., N. A. B., S. D. M.], Cleveland, Ohio 44106

Abstract

Wild-type p53 is induced by DNA damage. In different cell types, this induction is suggested either to facilitate DNA repair by inducing a cell cycle pause or to potentiate cell death via apoptosis. Wild-type p53 in different cell types has similarly been associated with either enhancement of or increased resistance to the cytotoxicity of many cancer therapeutic agents. We have constructed a colorectal cancer cell line bearing, in addition to endogenous mutant p53 alleles, an exogenous wild-type p53 allele that is under the regulatable control of the lac repressor. Induction of wild-type p53 by isopropyl-β-thiogalactopyranoside in these cells induces a reversible growth arrest but does not induce cell death. However, we find that the induction of wild-type p53 powerfully potentiates the cytotoxicity of both irradiation and 5-fluorouracil, two agents that are used clinically in the treatment of colorectal cancer. We also find that induction of wild-type p53 potentiates the cytotoxicity of topotecan, a member of the camptothecin family of drugs that also has clinical activity against colon cancer. These findings suggest that the common loss of wild-type p53 in many colorectal cancers may play a role in the clinical resistance of these tumors to anticancer agents. Although some cancer cells may not be directly killed by p53 gene therapy, our findings suggest that genetic alteration of some cancers to induce wild-type p53 may increase their sensitivity to cytotoxic gene therapy.

Introduction

The p53 tumor suppressor gene is the most commonly altered genetic locus in human cancers (1, 2) and is, by a combination of deletion and/or mutation, specifically lost in 70% of human colorectal tumors (3). The p53 gene encodes a nuclear phosphoprotein that increases in level in response to DNA damage (4–6). In some cell types, elevated p53 induces cell growth arrest in the G1 phase of the cell cycle (5, 7, 8), and via this G1 arrest, wild-type p53 has been suggested to facilitate DNA repair (5, 9). In contrast, restoration of wild-type p53 function can directly induce apoptosis in some transformed hematopoietic cells (10–12), in some virally transformed cells (13–15), and in some solid tumors (16). By enhancing DNA repair, wild-type p53 could potentially enhance the resistance of cells to killing by agents that induce DNA damage. Alternatively, by potentiating apoptosis, wild-type p53 could potentially increase the sensitivity of cells to killing by agents that induce DNA damage. Both of these effects have been supported by findings in different cell line systems (10, 17–24).

The present study examines the role of both wild-type and mutant p53 in modulating resistance of colorectal cancers to two therapeutic agents, 5-FU and radiation, that are commonly used to treat these malignancies in clinical practice (25). Many previous studies examining the role of p53 in modulating cell responses to DNA damage have relied on inactivating wild-type p53. In contrast, we have chosen to examine the effects on responses to DNA damage of restoring wild-type p53 in transformed colon epithelial cells in which p53 is mutant. We have adopted this course because it is possible that the functions of a wild-type p53 allele will be different in tumors in which the presence of the wild-type allele is tolerated compared with tumors in which the wild-type allele has been eliminated. We have adopted this course also because strategies for successful p53 gene therapy would likely depend on the effects of such reintroduction of wild-type p53 into cancers in which the endogenous alleles are mutant. In addition, restoring inducible wild-type p53 in a cell line with mutant p53 allows experimental comparison of the effects of the wild-type and mutant alleles in otherwise isogenic cell lines.

We have used as a model the SW480 colon cancer cell line in which p53 alleles are mutant (26). Stable reintroduction of wild-type p53 has generally not been possible in tumors in which the endogenous alleles are mutant (27). Accordingly, we have performed our studies by deriving from the SW480 cell line a daughter line bearing a wild-type p53 allele under the inducible control of the lac repressor (28). In the basal state, the wild-type allele is silent, and upon addition of IPTG, the wild-type allele is expressed. We have demonstrated previously that expression of this wild-type p53 allele in SW480 induces a reversible growth arrest in the G1 phase of the cell cycle, and does not induce apoptosis or cell death even after several days of wild-type p53 expression (28). We now show that although wild-type p53 reexpression does not, by itself, trigger death of transformed colon epithelial cells, it strongly enhances the sen-
sitivity of these cells to killing by both radiation and chemotherapy.

Materials and Methods

Cell Lines and Culture. Derivatives of the SW480 colon cancer cell line (ATCC CCL 228) used in this study are: GH2, bearing an empty control expression vector; MT2, bearing an IPTG-inducible mutant p53 cDNA; and WTd, which bears an IPTG-inducible wild-type p53 cDNA (28). These cell lines were constructed by transfection of SW480 with a plasmid constitutively expressing the lac repressor protein, along with a second plasmid containing, under control of the lac operator, either wild-type p53 (WTd), mutant p53 (codon 143 Val→Arg; MT2), or no cDNA insert (GH2; Ref. 28). To maintain homogeneity of the WTd cell line, it was recloned. Two subclones, WTd8 and WTd19, which both demonstrate tight regulation of the inducible wild-type p53, were used for subsequent studies of sensitivity to cytotoxic agents. All the cell lines were cultured in DMEM with 10% heat-inactivated calf bovine serum at 37°C in a humidified atmosphere with 5% CO₂.

Drug Treatment and Colony Formation Assay. For all of these experiments, exponentially growing cells were seeded in 100-mm tissue culture dishes 12 h before IPTG treatment. At time 0, the cells were incubated with 5 mm IPTG for 48 h. Radiation was delivered at the 24-h time point at doses of 25, 100, 200, and 400 cGy in a Shepherd Mark I model 68 ¹³⁷Cs γ-iradiator at a rate of ~50 cGy/min in the presence of IPTG. Topotecan (10-hydroxyl-9-dimethylaminomethyl-(S)-camptothecin; Smith Kline Beecham Pharmaceuticals, King of Prussia, PA) was delivered as 1-h exposure also commencing at the 24-h time point. 5-FU (Smith Kline Beecham Pharmaceuticals) was commenced at the zero time point concurrently with IPTG, and 5-FU exposure was maintained continuously to 24 h. Thus, in all the experiments, IPTG treatment was continued for 24 h after exposure to the DNA damaging agent. After 2 weeks, surviving colonies were fixed and stained with methylene blue in 50% ethanol. Surviving colonies from triplicate dishes of each experiment were counted.

Immunoprecipitation. Exponentially growing cells were transferred to methionine-free DMEM for 2 h and then incubated with 100 μCi/ml L-¹³⁵S]methionine (specific activity >1000/mmol) at 37°C for 2 h. The cells were lysed with 1 ml of lysis buffer (PBSTD) containing 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100 for 10 min, and soluble extracts were collected. The incorporated radioactivity was measured by scintillation counting after trichloroacetic acid precipitation. Equal counts from different labeled protein lysates were incubated with 20-μl agarose beads conjugated to staphylococci G and A (Oncogene Science, Uniondale, NY) and 2 μg/ml (20 μl) pAb1620 (Ab-5; Oncogene Science) at 4°C overnight. The immunoprecipitates were resolved by 8% SDS-PAGE and visualized by autoradiography.

Protein Extraction and Western Blot Analysis. Exponentially growing cells incubated with IPTG at selected time points were washed three times with cold PBS. Cells were lysed in cold PBSTD lysis buffer for 10 min and collected by scraping. One hundred mg of protein lysate were separated by electrophoresis on 12.5% Laemmli gradient gel, transferred to 0.2 μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and blotted with antibodies specific to WAF1 (Oncogene Science). After application of horseradish peroxidase-conjugated secondary antibodies, reactive proteins were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Assay of Apoptosis. Apoptosis was determined by electrophoretic determination of the presence of DNA fragmentation. Approximately 1 × 10⁶ cells were harvested after treatment with either IPTG or 5-FU, or both. DNA was isolated by a modification of the method of Compton and Cidlowski (29). Cell pellets were lysed in a solution containing 10 mm EDTA, 50 mm Tris (pH 8.0), 0.5% N-lauryl-sarkosine (Sigma, Poole, UK), and 0.5 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN), and incubated for 1 h at 50°C. Then, 0.5 μg/ml RNase (Sigma) was added, and the mixture was further incubated for 1 h. DNA was extracted with phenol/chloroform, precipitated by ethanol and sodium acetate, analyzed on a 2% agarose gel, and visualized by ethidium bromide staining.

Results

Wild-Type p53 Does Not Induce Apoptosis in WTd Cells. We reported previously that in the SW480 daughter, WTd, induction of wild-type p53 by IPTG induces a reversible growth arrest in the G1 phase of the cell cycle (28). In this study, we used two subclones, WTd8 and WTd19, that are both derived from WTd cells and that retain the same inducible regul-
Fig. 2 Potentiation of cytotoxicity of γ-irradiation by wild-type p53 expression. Clonogenic survival of colorectal cancer cells is shown at different doses of γ-irradiation delivered in the presence (●) or absence (○) of the inducing agent IPTG. Derivatives of SW480 treated are: GH2, bearing an empty control expression vector; MT2, bearing an inducible mutant p53 cDNA; and WTd8 and WTd19, both clones of WTd cells that bear an inducible wild-type p53 cDNA. The figure shows means of colony numbers obtained in triplicate experiments; bars, SE.

Induction of p53 expression as the original WTd cells. Treatment of these cells with IPTG induced expression of wild-type p53 detected by immunoprecipitation with antibody pAb1620, which specifically recognizes the wild-type p53 protein (Fig. 1A). The expression of wild-type p53 protein was repressed in the absence of IPTG, was induced as early as 6 h, and gradually increased from 6 to 72 h after addition of IPTG (Fig. 1A), reaching a level approximately equal to that of the endogenous mutant p53 protein (28).

As in the parental WTd cell line, induction of wild-type p53 induced expression of WAF1/CIP1 protein detectable by Western blot analysis (28). As shown in Fig. 1B, WAF1/CIP1 protein was evident by 12 h of IPTG treatment and remained constant between 24 and 72 h of IPTG treatment. The induction of WAF1/CIP1 protein lagged ~6 h behind the IPTG induction of p53 protein (compare Fig. 1, A and B). Control experiments demonstrate that IPTG did not induce WAF1/CIP1 in either MT2 cells that contain an inducible mutant p53 vector or in GH2 cells that contain an empty vector control plasmid (28).

Examination by electrophoresis of genomic DNA extracted...
Table 1  Wild-type p53 potentiation of WTd killing by therapeutic agents

Dose enhancement factor is the ratio of the dose required to achieve LD₉₀ in the absence versus the presence of wild-type p53. Cytotoxicity enhancement factor is the ratio of the percentage of cell survival in the absence versus the presence of wild-type p53 calculated at the highest dose of each agent tested.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose enhancement factor</th>
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<tr>
<td></td>
<td>WTd8</td>
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<tr>
<td>Radiation</td>
<td>1.8</td>
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<td>5-FU</td>
<td>4.0</td>
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<td>Topotecan</td>
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**Fig. 3** Potentiation of cytotoxicity of 5-FU by wild-type p53 expression. Clonogenic survival of colorectal cancer cells is shown at different doses of 5-FU delivered in the presence (○) or absence (○) of the inducing agent IPTG. Derivatives of SW480 treated are: GH2, bearing an empty control expression vector; MT2, bearing an inducible mutant p53 cDNA; and WTd8 and WTd19, both clones of WTd cells that bear an inducible wild-type p53 cDNA. The figure shows means of colony numbers obtained in triplicate experiments; bars, SE.
from the above cells showed that induction of wild-type p53 and of WAF1/CIP1 did not activate apoptosis (Fig. 1C). No DNA fragmentation was detected in WTd8 cells even after 72 h of induction of wild-type p53 by IPTG addition.

**Wild-Type p53 Sensitizes WTd Cells to Irradiation.** The human colon cancer cell line SW480 contains only a mutant p53 allele and exhibits no radiation-induced G1 checkpoint arrest (6, 24). To determine whether the loss of wild-type p53 has an effect on radiosensitivity in SW480, we compared by a clonogenic assay the cytotoxicity of γ-irradiation in the presence or absence of induction of wild-type p53 in the SW480 daughter WTd cells. Expression of wild-type p53 in WTd cells dramatically enhances the sensitivity to γ-irradiation (Fig. 2 and Table 1). At a 400-cGy dose, induction of wild-type p53 increases the cytotoxicity of γ-irradiation ~40-fold in WTd8 cells and ~10-fold in WTd19 cells (Fig. 2). In contrast, IPTG has no influence on the radiosensitivity of MT2 or GH2 cells that, respectively, bear an inducible mutant p53 allele or an empty vector alone (Fig. 2).

**Wild-Type p53 Sensitizes WTd Cells to 5-FU.** Although 5-FU, a thymidylate synthetase inhibitor, is the main chemotherapeutic agent currently used for the treatment of co-
Wild-Type p53 Potentiates Cytotoxicity of 5-FU

In colon cancer patients, less than one-third of colon cancers respond to such therapy (25). To determine whether induction of wild-type p53 would enhance the cytotoxicity of 5-FU in colon cancer cells, we analyzed the sensitivity of WTd cells to 5-FU in the presence and absence of wild-type p53 expression induced by IPTG. As shown in Fig. 3, induction of wild-type p53 by IPTG enhances the cytotoxicity of a 20 μM dose of 5-FU by 38-fold in WTd8 cells and by 24-fold in WTd19. IPTG-induced expression of wild-type p53 resulted in a 4-fold reduction in the 5-FU LD_{95} dose in both WTd8 and WTd19 (Fig. 3 and Table 1). IPTG induction of mutant p53 in MT2 cells did not significantly alter sensitivity to 5-FU. IPTG treatment itself also did not alter the cytotoxicity of 5-FU in GH2 cells bearing an empty vector control (Fig. 3).

Wild-Type p53 Modestly Sensitizes WTd Cells to Topotecan. Topotecan is a topoisomerase I inhibitor that inhibits DNA replication and initiates DNA damage by stabilizing a DNA enzyme complex. Topotecan is a member of the camptothecin family of drugs, which has shown modest clinical activity against colon cancer (30, 31). We therefore compared the clonogenic survival of WTd cells treated with topotecan either in the presence or absence of IPTG. At concentrations below 1 μM topotecan, there is minimal difference between topotecan-induced cytotoxicity in the presence or absence of p53 induction by IPTG. At higher concentrations of topotecan, drug cytotoxicity was enhanced by the addition of IPTG. However, this enhancement was moderate, exhibiting an increase of only 7-fold in WTd8 cells and 4-fold in WTd19 cells (Fig. 4 and Table 1).

Wild-Type p53 Potentiates Apoptotic Cell Death in WTd Cells. Several alternative mechanisms could account for the potentiation by wild-type p53 of 5-FU cytotoxicity in WTd cells. When combining 5-FU with induction of wild-type p53, decreased clonogenic survival could reflect increased cell death due to apoptosis, increased cell death due to necrosis, or an irreversible growth arrest of the treated cells. As discussed previously, introduction of wild-type p53 results in induction of apoptosis in some tumors in which the p53 gene has been deleted or mutated (11, 12). However, induction of wild-type p53 in the SW480 WTd cells does not induce apoptosis as assayed by DNA fragmentation (Fig. 1), but rather induces a reversible growth arrest at the G1 phase of the cell cycle (28). To determine the mechanism of their decreased clonogenic survival, cells treated with 5 μM 5-FU concurrently with the induction of wild-type p53 were examined immediately after 24-h exposure to 5-FU and IPTG. Fig. 5 shows that, as observed previously, 24 h of IPTG treatment alone did not induce apoptosis as determined by the absence of observable DNA fragmentation. Similarly, Fig. 5 shows that 24 h of exposure to 5 μM 5-FU induces a barely observable degree of DNA fragmentation that is consistent with our previous finding that 5 μM 5-FU corresponds to approximately an LD_{10} dose as assayed by clonogenic survival of WTd cells (Fig. 3). In contrast, Fig. 5 demonstrates marked DNA fragmentation in WTd cells examined after 24-h treatment of 5-FU combined with IPTG. These findings strongly argue that although wild-type p53 is, by itself, insufficient to induce apoptosis in WTd cells, wild-type p53 enhances the cytotoxicity of 5-FU by strongly potentiating the induction of apoptosis by this agent.

Time Dependence of Sensitization of WTd by Wild-Type p53. To investigate the optimal combination of a chemotherapeutic agent, 5-FU, and wild-type p53, we first investigated the effects of varying the relative timing of administration of these two agents. Fig. 6A shows that 5 μM 5-FU administered for 24 h accompanied by 48 h of induction of wild-type p53 by IPTG were equally cytotoxic, whether the 5-FU was initiated at the start of the IPTG treatment ("concurrent" treatment) or 24 h into the IPTG treatment (pre-IPTG). However, if instead of administering these agents together, 24 h of 5-FU is administered preceding the start of IPTG treatment (post-IPTG), only minimal potentiation of cytotoxicity was observed versus that induced by 5-FU alone. Thus, potentiation of 5-FU cytotoxicity by wild-type p53 requires that both be present in the cell simultaneously.

Fig. 6B examines the effects of combining 24 h of exposure to 5-FU along with induction of wild-type p53 when both exposures are begun simultaneously, but when the exposure to IPTG is continued for varying lengths of time. Shortening the time of wild-type p53 induction from 48 to 24 h results in a modest but demonstrable decrease in the degree of potentiation of 5-FU cytotoxicity. The magnitude of the difference between the effect of a 48-h pulse and a 24-h pulse of IPTG is similar to the slight potentiation of 5-FU cytotoxicity observed when IPTG is administered starting at the end of a 24-h pulse of 5-FU (Fig. 6A). It is likely that this effect is accounted for by the continued presence of intracellular 5-FU in the cell for a period of time after washout of the drug from the medium. In contrast, no significant difference is seen between the enhancement of cytotoxicity associated with a 48-h versus a 72-h exposure to IPTG when both were commenced simultaneously with a 24-h exposure.
exposure to 5-FU. This suggests that even prolonged exposure to wild-type p53 is without added toxicity when delivered well after exposure to 5-FU, and is consistent with our previous data that induction of wild-type p53 by itself results only in a reversible growth arrest (28).

**Discussion**

We have found that absence of wild-type p53 function plays a direct role in mediating the resistance of human colon cancer to a wide variety of cancer therapeutic agents. Of more important implication, we have shown that reintroduction of wild-type p53 expression into colon cancer cells can powerfully enhance sensitivity to killing by both radiation and chemotherapy, although wild-type p53 does not, by itself, have any cytotoxic effect on these cells and although the cells continue to express endogenous mutant p53. The potential role of p53 in modulating sensitivity of cancer cells to cytotoxic agents is confounded by the dual role of wild-type p53 in putatively promoting both DNA repair, as well as in some systems promoting apoptosis (9). One approach previous investigators have taken has been to examine the effects of inactivating endogenous wild-type p53 alleles either in normal or in transformed cell types (17, 18, 32). Our approach has been to restore wild-type p53 function in a colon cancer cell line by expressing the wild-type p53 allele under the control of an inducible promoter. Our finding that wild-type p53 is not, by itself, cytotoxic in these cells is consistent with similar observations in some gliomas, sarcomas, and lung cancer cells (8, 33, 34, 35). Our finding that restoring wild-type p53 nonetheless sensitizes these colon cells to killing by radiation and chemotherapy demonstrates that, in these cells, absence of wild-type p53 function plays a direct role in resistance to cancer therapeutic agents. Our findings contrast with one report that introduction of wild-type p53 induced resistance to doxorubicin in an ovarian cancer cell line (36). Our findings, however, are consistent with previous observations that in one lung cancer cell line and in one glioma cell line, introduction of wild-type p53 by infection with a wild-type p53 adenovirus sensitized the cells to apoptotic killing by cisplatin (33, 35). Our findings now show that induction of wild-type p53 in colon cancer cell line sensitizes these cells to several new classes of therapeutic agents, including radiation, 5-FU, and topotecan. As we have examined the survival of colon cells in the presence or absence of wild-type p53 over a wide dose range of cytotoxic agents, our findings also allow us to quantitate the magnitude of the

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**Fig. 6** Schedule dependence of potentiation of 5-FU cytotoxicity by wild-type p53. A, clonogenic survival of WTd8 cells treated with 48 h of IPTG and with 24 h of 5 μM 5-FU initiated concurrently with IPTG (Concurrent), 24 h into the exposure of IPTG (Pre-IPTG), at the completion of IPTG (Post-IPTG), or in the absence of IPTG (No IPTG). B, Clonogenic survival of WTd8 cells treated with 24 h of 5-FU at the doses shown. Simultaneously with the initiation of 5-FU, treatment also was initiated with IPTG for 24, 48, or 72 h. Control cells were treated with 5-FU only (No IPTG). A and B show means of colony numbers obtained in triplicate experiments; bars, SE.
sensitization induced by wild-type p53 both in terms of a dose enhancement factor as well as a cytotoxicity enhancement factor.

We have shown previously that induction of wild-type p53 in the WTd derivative of the SW480 colon cancer cell line results in expression of wild-type p53 protein at levels comparable to that of the endogenous mutant p53 alleles (28). Thus, our findings demonstrate that the potential efficacy of combination chemotherapy with p53 gene therapy does not depend on achieving supraphysiological levels of wild-type p53 in cancer cells. The recent demonstration that wild-type p53 function can be restored in SW480 cells by antibodies that bind to C-terminal epitopes in p53 (37) gives encouragement that it will be possible to develop novel methods for restoring some measure of wild-type p53 function in human tumor cells.

This study contrasts with previous investigations in which the effects of inactivating endogenous wild-type p53 alleles, either in normal or in transformed cell types, have been examined. Different methods used by these studies have included the use of cells from p53 knockout mice, the introduction into cells of human papilloma virus E6 genes, and the introduction into cells of mutant p53 alleles. These methods have yielded conflicting results in different cell types. Lymphocytes from p53 knockout mice were shown to be resistant to radiation-induced apoptosis (10), and fibroblasts from these animals when transformed with E1A and Ras were found to be resistant to radiation and to many chemotherapeutic agents (14). In contrast, non-transformed fibroblasts from p53 knockout mice showed no change in sensitivity to radiation or chemotherapy (24). In further contrast, inactivating wild-type p53 by introduction of human papilloma virus E6 genes was shown in human fibroblasts to sensitize cells to multiple chemotherapeutic agents (18); in the RKO colon cancer cell line to increase the sensitivity of cells to cisplatin (17), but not to radiation (24); and in the MCF-7 breast cancer cell line to increase the sensitivity of the cells to cisplatin (17). One resolution of these conflicting findings is that the functional consequence of inactivating wild-type p53 alleles may differ when compared between normal and transformed cells, or may differ when compared between transformed cells that tolerate the continued presence of wild-type p53 alleles and transformed cells in which wild-type p53 alleles have been eliminated. Our finding that wild-type p53 alleles sensitize SW480 colon cancer cells to cancer therapeutic agents is consistent with studies in naturally occurring human tumors, suggesting that the presence of mutant p53 alleles in ovarian cancers is associated with resistance to cisplatin (22, 23); in lung cancers associated with resistance to cisplatin (38); in gastric cancers is associated with resistance to multiple chemotherapeutic agents (20); and in Burkitt’s lymphomas is associated with resistance to radiation (21, 39) and to chemotherapeutic agents (39).

One reason the approach of reconstituting wild-type p53 function in tumor cells has not been more widely used is the experimental difficulty in constructing stable cell lines in which wild-type p53 function is restored. Certainly, restoration of wild-type p53 directly induces apoptosis in many cell types, including hematopoietic cells (11, 12, 40), some gliomas (41), and some squamous carcinomas of the head and neck (42). However, this property is clearly not universal, and, as mentioned above, restoring wild-type p53 induces growth arrest in some glioma cells (8) and induces only growth inhibition in some sarcoma (33) and lung cancer cells (34). Future studies in these and other cell types will be helpful in determining the generality of the efficacy of potential combination p53 gene therapy with chemotherapeutic agents.

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