

Sensitizing glioma cells to cisplatin by abrogating the p53 response with antisense oligonucleotides

Kamal Datta,^{1,2} Preeti Shah,¹ Tapasya Srivastava,¹ Srinivas G Mathur,¹ Parthaprasad Chattopadhyay,¹ and Subrata Sinha¹

¹Department of Biochemistry, All India Institute of Medical Sciences, New Delhi 110029, India.

Most gene therapy strategies related to p53 concentrate on the restoration of the activity of mutant p53, as several observations indicate that tumors and cell lines having the mutant gene are resistant to chemotherapy. However, as there is also some evidence to the contrary, we studied the relationship of the p53 status to the cellular response of glioma cells that were exposed to cisplatin. At a concentration of 2.5 µg/ml (which is about half the peak pharmacological blood level reached during chemotherapy), U373MG glioma cells, which had a mutant p53 gene, were more sensitive to the drug as compared to U87MG glioma cells (with normal p53). The U373MG cells responded with apoptosis while U87MG cells responded with a G2–M arrest. In U87MG cells, blocking the p53 response by antisense oligonucleotides also sensitized the cells to 2.5 µg/ml cisplatin, and shifted the cellular response from arrest to caspase 3-mediated apoptosis. A sensitive, p53-independent, mechanism for chemotherapy-induced apoptosis suggests that, in some cases, p53 abrogation by gene therapy or small molecule-based strategies could be a viable therapeutic strategy.

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Malignant gliomas with relatively poor prognosis pose a significant challenge to conventional treatment modalities of surgery, radiotherapy and adjuvant chemotherapy. Mutation and consequent loss of p53 function has been shown to play a crucial role in tumorigenesis and development of resistance to therapeutic regimens in gliomas and other human malignancies.^{1,2} The p53 gene is found to be mutated in more than 40% of gliomas. However, the role of p53 in determining the sensitivity of tumors to chemotherapeutic agents is still not very clear. Most studies show that normal p53 is associated with a favorable^{3–6} and mutated p53 with an unfavourable therapeutic response in gliomas as well as in other malignancies.^{2,7,8} Studies in animal models as well as *in vitro* experiments have shown that tumor cell growth is inhibited by the restoration of wild-type p53 in different cell types.^{3,4,9–12} Others have shown that a mutation in the p53 gene is not an impediment in the induction of apoptosis.^{13–15} At the other end of the spectrum, some reports have shown no significant correlation between p53 mutation and a therapeutic response.^{16–18} Nonetheless, it is largely believed that the restoration of p53 function,

whether by gene therapy or by small-molecular-weight compounds, would be therapeutically useful and would enhance the beneficial response to chemotherapeutic regimens.^{3,4,19}

The cell responds to genotoxic stress by activating cell cycle checkpoints and blocking further progression into the cell cycle, thus allowing the cell some time to repair the damage. p53 has been shown to control both the G1/S and the G2/M checkpoint via p21^{WAF1} and 14-3-3σ proteins. However, p53 activation can also lead to apoptosis. The nature of the p53-mediated response of a cell to DNA damage would depend on a number of factors, including the nature and extent of the damage and the cell type.^{20,21} While many chemotherapeutic agents could directly induce apoptosis, there are a number of reports showing that a nonfunctional or mutated p53 gene may be associated with an enhanced sensitivity of cancer cells to genotoxic agents.^{22–30}

Cisplatin is a well-studied chemotherapeutic agent that acts through DNA damage. In addition to several tumor types, it is used for adjuvant chemotherapy of gliomas.³¹ To the best of our knowledge, there are no reports on the response of glioma cells to comparatively low, but therapeutically relevant doses of cisplatin. The frequency of p53 mutations in gliomas ranges from 67% in anaplastic astrocytoma to 41% in glioblastoma.³² In this study, we investigated the possibility of sensitization of glioma cells to cisplatin by abrogation of the p53 activity. We compared the response of p53 wild-type (U87MG) and mutated (U373MG) glioma cell lines to cisplatin

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Address correspondence and reprint requests to: Dr Subrata Sinha MD, PhD and Dr Parthaprasad Chattopadhyay, MD, PhD, Department of Biochemistry, All India Institute of Medical Sciences, New Delhi-110029, India. E-mail: sub_sinha@hotmail.com

²Present address: National Institutes of Health, Bldg 10/1C401, Bethesda, MD, USA.

exposure. The response of p53 wild-type cells transfected with p53 antisense oligonucleotides was also studied. U373MG cells were more sensitive to cisplatin action at the concentration of 2.5 $\mu\text{g}/\text{ml}$. This concentration of cisplatin is pharmacologically relevant; as the peak serum level of the drug is about 5 $\mu\text{g}/\text{ml}$ ³³ and the actual drug concentration would vary with time and degree of tumor perfusion. We have also shown that disruption of p53 by antisense oligos sensitizes wild-type glioma cells to this low dose of cisplatin and shifts the cellular response from G2/M arrest to apoptosis.

Materials and methods

Cell culture

Human glioma cell lines (U87MG with wild-type p53 and U373MG with mutated p53) were procured from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 10 $\mu\text{g}/\text{ml}$ ciprofloxacin (all from Sigma) at 37°C in 5% CO₂.

Cell viability

Cells were seeded in 96-well plates (1×10^4 cells/well). Following overnight culture, cells were treated with 2.5 and 5.0 $\mu\text{g}/\text{ml}$ of cisplatin (Sigma) for various time periods and under different experimental conditions at 37°C. Response to cisplatin was studied for 24, 48 and 72 hours and subsequent studies with only cisplatin or with antisense + cisplatin was carried out at 72 and 24 hours respectively. Cell viability was determined by MTT assay as described previously.³⁴ Results were compared with untreated control and expressed as percent (%) survival. Each experiment was repeated at least three times and each point was in quadruplicate. A representative experiment is depicted in results.

Study of morphology

After exposure to cisplatin (2.5 $\mu\text{g}/\text{ml}$) for 72 hours in 24-well plates (10^5 cells/well), cells (both U87MG and U373MG) were visualized under a phase contrast microscope and compared with untreated control. Morphology was also studied following transfection with p53 antisense oligos and then exposure to cisplatin (2.5 $\mu\text{g}/\text{ml}$) for 24 hours in U87MG cells. The percentage of cells showing apoptotic features like blebbing, nuclear condensation and fragmentation has been quantified from four different fields and expressed as average percentage cell death \pm standard deviation.

Flow cytometry

Cells were analyzed by flow cytometry (Epics XL, Coulter) after cisplatin (2.5 $\mu\text{g}/\text{ml}$) treatment and propidium iodide staining as described earlier³⁴ for hypodiploidy and cell cycle status. Data were analyzed by Win MDI 2.1.3 software. The hypodiploid peak ahead of G1 represents percent of apoptotic cells relative to the total

number of cells distributed in different phases of cell cycle. Results were compared with control values. Flow cytometric analysis was also performed following antisense oligos transfection for 12 hours followed by cisplatin treatment for 24 hours in U87MG cells. In the transfection experiment, apart from random oligos + cisplatin, U87MG cells treated with 2.5 $\mu\text{g}/\text{ml}$ cisplatin alone for 24 and 72 hours as well as antisense and random oligos alone were taken as additional controls.

Antisense experiments

Transfection experiments were performed with a 15-mer (5'-pdC* pdU* pdC* pdC* pdU* pdC* pdC* A* pdU* G* G*pdC* A* G* pdU*-3') phosphorothioate oligonucleotide (*) with C5 propyne-modified pyrimidine bases (pdU and pdC) synthesized commercially (IDT Inc., USA) which was antisense to p53 translation start region.³⁵ A control oligo was also synthesized (IDT Inc., USA) with a random sequence (5'-G* pdC* pdC* pdC* pdC* pdU* pdC* A* pdU* pdU* pdC* G* G* pdU* A*-3') having the same base composition as that of p53 antisense oligonucleotides. Liposome (Transfast from Promega, USA) mediated transfection was performed as per the manufacturer's protocol. Briefly, oligonucleotides were mixed with plain DMEM, gently vortexed and then Transfast was added to a concentration of 2 μM , vortexed and incubated at room temperature for 15 minutes. Cells were incubated with oligos-Transfast-medium mixture for 12 hours at 37°C in 96-well plates (10^4 cells/well) for survival assay or in a 25² cm flask (10^6 cells/flask) for Western blot and flow cytometry. Then cells were treated with cisplatin (2.5 $\mu\text{g}/\text{ml}$) for 24 hours. Controls were put up with oligonucleotides and liposomes alone. For the initial dose-response curve, oligonucleotides were used at concentrations of 50, 100, 150 and 300 nM. Subsequent experiments were performed at an oligo concentration of 100 nM.

Western blotting

Following cisplatin treatment (in 25 cm² flask), cells were lysed in lysis buffer containing protease inhibitor cocktail (50 mM Tris-Cl, 150 mM NaCl, 0.02% sodium azide, 50 $\mu\text{g}/\text{ml}$ PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinine, 0.1% Nonidet P-40). Protein was separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane (Pall-Gelman Laboratories). Equal amount of protein was loaded in each lane. After incubation with primary antibody (p53, Bcl2 and caspase 3 from Pharmingen), the blot was developed with alkaline phosphatase conjugate system as per the manufacturer's protocol (Promega).

Statistical analysis

Statistical analysis was made by a two-tailed paired Student's *t*-test, and a value of $P \leq .05$ was taken as significant.

Results

We observed that on 72 hours exposures p53 mutated U373MG cells were significantly more sensitive to lower concentrations of cisplatin (2.5 $\mu\text{g/ml}$) than the p53 wild-type U87MG cells ($P = .02$ for 2.5 $\mu\text{g/ml}$) (Fig 1). Subsequent experiments were carried out with 2.5 $\mu\text{g/ml}$ of cisplatin. While this was done by the MTT assay, similar results were obtained by a modified colony forming assay (unpublished observations).

Phase contrast microscopy showed apoptotic morphology (blebbing, nuclear condensation and fragmentation) in U373MG cells after exposure to 2.5 $\mu\text{g/ml}$ cisplatin for 72 hours ($48.3 \pm 5.19\%$) when compared to untreated cells ($1.5 \pm 0.57\%$, $P = 1.94 \times 10^{-6}$). In contrast, no obvious morphological change was observed in U87MG cells treated with 2.5 $\mu\text{g/ml}$ cisplatin ($1.7 \pm 1.29\%$) in comparison to untreated U87MG cells ($1 \pm 0.8\%$, $P = .39$). The difference between U87MG and U373MG cells treated with 2.5 $\mu\text{g/ml}$ cisplatin was statistically significant ($P = 2.30 \times 10^{-6}$) (Fig 2.IA and B). To further analyze the nature of cell death, flow cytometric analysis was made in both the cell lines following exposure to 2.5 $\mu\text{g/ml}$ cisplatin for 72 hours (Fig 2.IIA and B). In U373MG, a significant sub-G1 hypoploid cell population, indicating apoptosis, was observed in the exposed cells as compared to controls. In contrast, cisplatin (2.5 $\mu\text{g/ml}$) treatment of U87MG resulted in cell cycle arrest at G2/M.

When U87MG cells were transfected with different concentrations of p53 antisense oligonucleotides and then treated with 2.5 $\mu\text{g/ml}$ cisplatin, a significant decrease in cell survival, as compared to cells treated with random oligos, was observed at concentrations of 50 ($P = .01$) and 100 nM ($P = .01$) (Fig 3). In subsequent experiments, a concentration of 100 nM was used for both antisense and

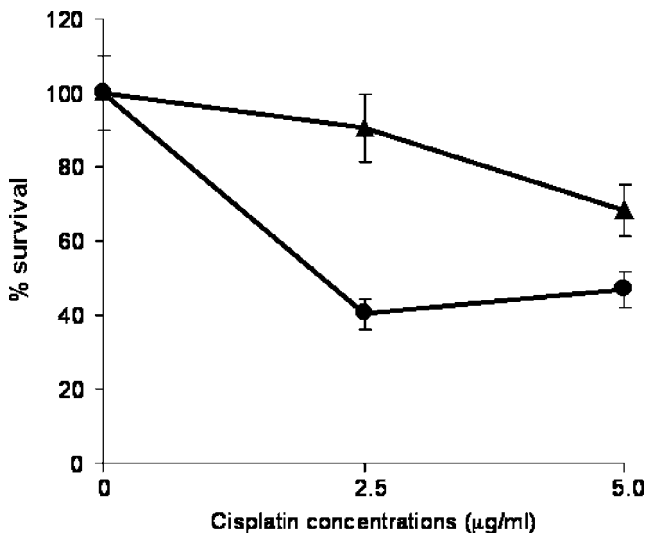


Figure 1 MTT assay showing percent survival of cells exposed to different concentrations of cisplatin (2.5 and 5.0 $\mu\text{g/ml}$) for 72 hours. U373MG cells with mutated p53 (●) were comparatively more sensitive to a low-dose cisplatin (2.5 $\mu\text{g/ml}$) than U87MG cells (▲) with wild-type p53 ($P = .02$ for 2.5 $\mu\text{g/ml}$ cisplatin between U87MG and U373MG).

random oligos. We found that the time kinetics of survival of the antisense + cisplatin-treated U87MG cells differed significantly from that of U373MG cells treated only with cisplatin. Decreased cell survival following antisense + cisplatin treatment of U87MG was observed at 24 hours. However, this differs from U373MG cells where it takes 72 hours for the toxicity of 2.5 $\mu\text{g/ml}$ cisplatin to be apparent.

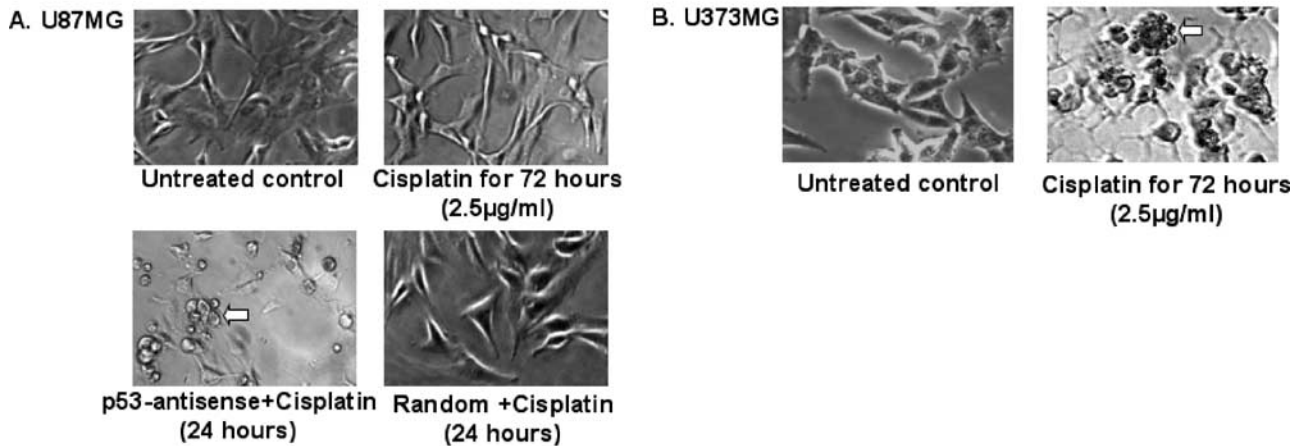
Western blot analysis shows that exposure of U87MG cells to cisplatin results in the induction of p53 protein with a peak at 18 hours (Fig 4). As expected for a cell line harboring mutant p53, the U373MG cells showed a constitutively high level of p53 protein that was not altered by cisplatin exposure (unpublished observations). For U87MG cells, the increase of p53 in response to cisplatin was abrogated by 100 nM antisense, but not by random oligos (Fig 4). Decreased levels of Bcl2 were seen in U87MG cells treated with p53 antisense oligos followed by cisplatin but not in cells treated with cisplatin alone, random oligos + cisplatin (Fig 4), or by just the antisense or random oligos (unpublished observations). The activated fragment of caspase 3 could also be detected in these cells. This suggests the activation of a p53-independent apoptotic pathway. While we have not fully worked out the apoptotic pathway of the antisense-treated U87MG cells, caspase 3 has been shown to be activated in both p53-independent and-dependent apoptosis.^{36–38}

The morphological features of the U87MG cells treated with p53 antisense oligos + cisplatin also indicated that cell death was by apoptosis ($70 \pm 2.16\%$) (Fig 2(I)A, lower panel). These features were not present in cells treated with random oligos + cisplatin ($1.9 \pm 1.7\%$, $P = 5.11 \times 10^{-5}$). Similarly, minimal apoptotic features were observed in untreated ($1 \pm 0.5\%$) and in cisplatin (2.5 $\mu\text{g/ml}$) treated U87MG cells ($1.7 \pm 1.29\%$). A flow cytometric study following propidium iodide staining of nuclei was carried out following p53 antisense transfection and 24 hours cisplatin treatment in U87MG cells. Different controls were included in the experiment — cisplatin (2.5 $\mu\text{g/ml}$) alone for 24 hours, cisplatin alone for 72 hours, random oligos + cisplatin combined for 24 hours and antisense p53 or random oligos alone for 24 hours. Analysis showed that p53 antisense transfection, followed by low-dose cisplatin treatment for 24 hours in U87MG cells, induced apoptotic cell death, as evident by an increased sub-G1 hypoploid peak (Fig 5). As seen earlier, G2/M arrest was most marked in U87MG cells treated with 2.5 $\mu\text{g/ml}$ cisplatin for 72 hours. However, some arrest was observed when treated with cisplatin alone for 24 hours, or by random oligos + cisplatin for 24 hours, suggesting the initiation of G2/M block in these cells at 24 hours.

Discussion

Despite significant advances in our understanding of molecular events and the development of novel therapeutic modalities, the overall prognosis of glioma remains

I. Morphology:



II. FACS analysis:

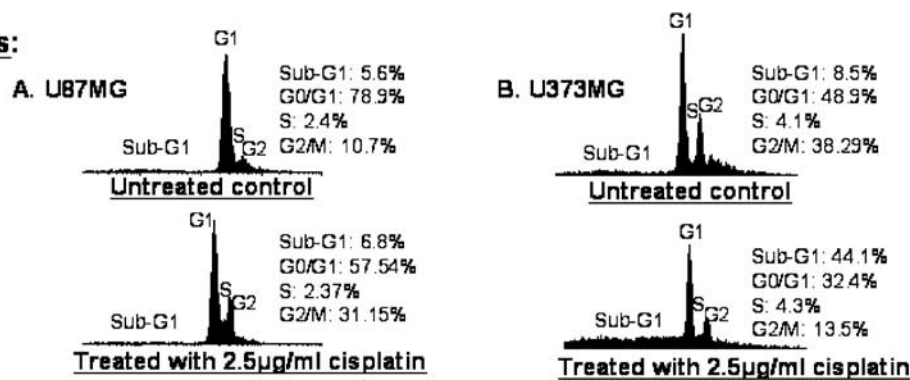


Figure 2 (I) Phase contrast microscopic study of cellular morphology after exposure to cisplatin. (A) U87MG cells showed no obvious change in morphology after exposure to cisplatin (2.5 µg/ml) for 72 hours. However, apoptotic bodies were observed (white arrow) when cells were transfected with p53 antisense oligonucleotides and then treated with the same dose of cisplatin even at 24 hours (lower panel). No obvious change was observed with random oligos. (B) In U373MG, significant apoptotic morphology was observed (white arrow) on exposure to cisplatin (2.5 µg/ml) for 72 hours. (II) Flow cytometric analysis of cells following exposure to low-dose cisplatin for 72 hours. (A) U87MG cells showed accumulation of cells (31.5%) in G2/M phase of cell cycle suggesting arrest. (B) However, at the same dose and time of cisplatin exposure, the U373MG showed significant apoptotic response (44.1%), observed as a hypoploid peak ahead of G1 peak.

poor. Cisplatin, along with radiation therapy, is used for the treatment of gliomas, although the result remains unsatisfactory due to development of resistance.³¹ Drug resistance has often been attributed to inactivation of p53 by gene mutation.³² However, the loss of wild-type p53 does not always result in a decrease in the sensitivity of human malignant cells to chemotherapeutic agents.²⁵ Massey *et al*³⁹ have shown that even with wild-type p53, the ovarian carcinoma cell line A2780 was resistant to cisplatin. This suggests that the interaction between tumor cells and therapeutic agents in relation to p53 is a complex issue that needs further elucidation.

Our study shows a differential response of the two glioma cell lines, U87MG (wild-type p53) and U373MG (mutant p53; 273 Arg to His), to a low dose of cisplatin (2.5 µg/ml). U373MG cells were more sensitive to the drug and responded by apoptosis, while U87MG cells responded by a, possibly temporary, G2/M arrest and showed no significant decrease in cell survival at this dose. This points to the influence of the p53 status of a cell in

determining sensitivity to cisplatin. At the clinically recommended dose, the peak plasma concentration of cisplatin is 5 µg/ml.³³ As many regions of the tumor are poorly perfused and exposed to drug concentrations lower than the peak serum level, a concentration of 2.5 µg/ml is pharmacologically relevant.

While a number of studies show the association of p53 mutation with resistance to chemo- and radiotherapy,^{7,8,40-42} our results are in agreement with others showing that in some cases nonfunctional/mutated p53 is associated with enhanced chemosensitivity.²²⁻³⁰ It is known that the activation of cell cycle check points by p53, leading to either G1/S arrest⁴³ or to G2/M arrest,⁴⁴ would give cells an opportunity to repair DNA damage and thus reduce the sensitivity of the cells to chemotherapeutic agents. Our results support the notion that at a low dose of a DNA-damaging agent, p53 induces cell cycle arrest and at higher concentrations induces apoptosis. We suggest that at a high concentration of cisplatin, there is increased platination of DNA leading to

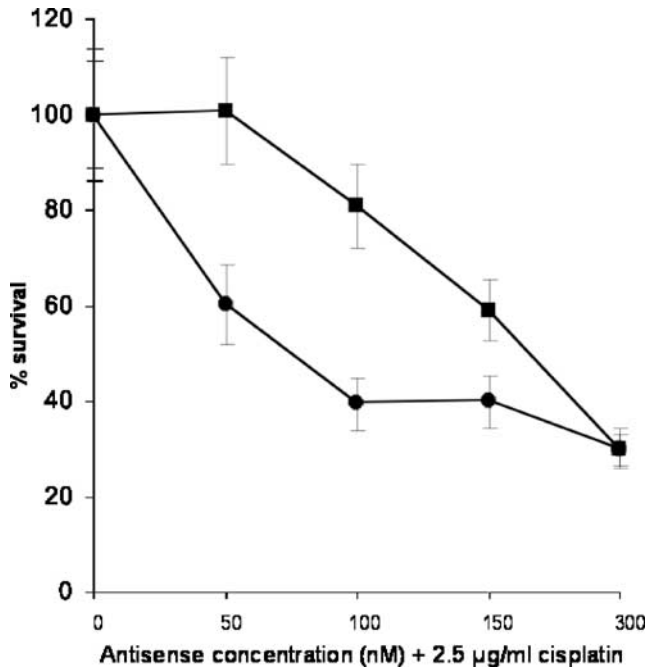


Figure 3 p53 antisense transfection sensitizes U87MG cells to low-dose cisplatin at 24 hours. When compared to random oligo-transfected cells, the antisense p53 oligonucleotide-transfected cells were significantly sensitive to 2.5 µg/ml cisplatin at lower oligo concentrations. Difference between specific antisense (●) and random (■) oligonucleotides was marked at the concentration of 50 ($P = .01$) and 100 nM ($P = .01$). At higher concentrations, no such difference was observed suggesting nonspecific toxicity of oligos. An oligo concentration of 100 nM was used for both p53 antisense and random oligos in subsequent experiments.

overwhelming of the repair machinery and activation of p53-mediated apoptosis, while at a lower concentration, p53 executes the option of cell cycle arrest at G2/M to facilitate repair of the platinated DNA. Our study highlights the importance of the activation of the G2/M checkpoint in U87MG by p53 as a means for protecting the cells from cisplatin toxicity.

G2/M arrest in response to DNA damage can occur both with and without the mediation of p53.⁴⁵ Earlier studies in p53-mutated cells have demonstrated the cytoprotective effect of the G2/M block and showed that there was enhanced sensitivity to cytotoxic drugs when it was removed by agents like pentoxifylline.⁴⁶⁻⁴⁸ We found that when U373MG cells (with mutated p53) were treated with cisplatin, they did not show a G2/M block, but responded with apoptosis. Using antisense oligos we have been able to show that the cytoprotective G2/M block in U87MG cells was p53 mediated. While the different behavior of the two cell lines suggested a role of p53 in determining the cellular response to cisplatin, the genetic background of the two cell lines differed. Hence, U87MG cells were transfected with antisense p53 oligos to further clarify the role of this molecule in low-dose cisplatin-induced cytotoxicity.

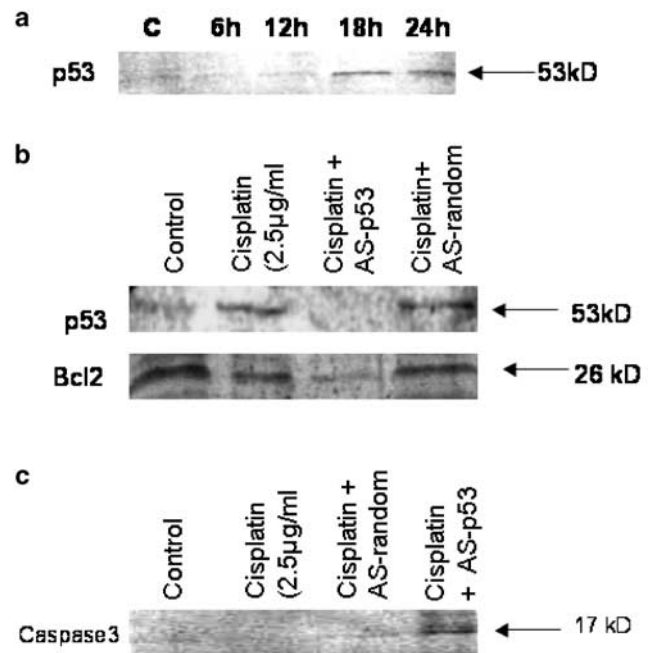


Figure 4 Induction of p53 protein by cisplatin (2.5 µg/ml) and its abrogation by p53 antisense oligos in U87MG. (a) p53 induction was observed at 12 hours with peak induction at 18 hours. Lane C represents untreated control. (b) Induction of p53 by cisplatin (2.5 µg/ml) was significantly inhibited by antisense oligos but not by random oligos at 18 hours. At the same time, Bcl2 level was decreased significantly with cisplatin + p53 antisense. (c) Activated caspase 3 was detected in cells transfected with antisense p53 and then exposed to cisplatin (2.5 µg/ml) for 18 hours. No such activation was seen in untreated control or cells treated with cisplatin alone or with random oligos + cisplatin.

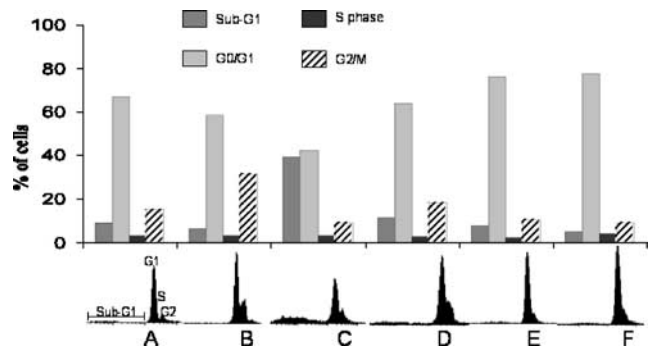


Figure 5 Flow cytometric study of U87MG cells following transfection of antisense oligonucleotides. Cells transfected with p53 antisense oligos (100 nM) and then exposed to cisplatin (2.5 µg/ml) showed significant (39.4%) apoptosis (C) replacing the G2/M arrest. No significant apoptosis was observed in controls treated with only cisplatin for 24 hours (A) and for 72 hours (B), random oligos + cisplatin (D), only p53 antisense oligos (E) and only random oligos (F). Treatment with cisplatin alone for 72 hours showed significant (31.54%) G2/M arrest (B). In all, 15.0 and 18.28% of the cells were in G2/M phase when treated with cisplatin alone for 24 hours (A) and cisplatin + random oligos (D), respectively, indicating initiation of the arrest.

Based on ratios of efficacy and nonspecific toxicity, an oligos concentration of 100 nM was used. Exposure to this concentration of p53 antisense oligos abrogated the cisplatin-induced expression of the protein, which showed a peak at 18 hours, in U87MG cells. Transfection of p53 antisense oligos followed by 2.5 µg/ml cisplatin treatment markedly increased drug-induced toxicity in these cells, and shifted the cellular response from G2/M arrest to apoptosis. Apoptosis was indicated by morphology, flow cytometry, a fall in Bcl2 protein and detection of activated caspase 3. While the increased apoptosis of U87MG cells treated with antisense + cisplatin indicated a response similar to U373MG cells, there were some differences, notably in the time kinetics of the response. Antisense p53-transfected U87MG cells responded to cisplatin treatment in 24 hours, unlike the U373MG cells, which showed cytotoxic response in 72 hours. This could be due to intrinsic differences between the cells, and justifies the use of antisense technology for studying the role of p53 in this system.

The observation, that at levels lower than the peak serum concentration of the clinically recommended dose, cisplatin induced significant toxicity in cells only when the p53 response was abrogated, suggests that the drug may be more active in tumors with an inactivated p53 gene. In gliomas, p53 mutation is a common occurrence and ranges from 41 to 67%.³² As bioavailability of chemotherapeutic agents in solid tumors like glioma varies in different regions of the tumor, it is quite likely that a subset of tumor cells is exposed to a sublethal dose of the drug. This could result in cells with a wild-type p53 gene undergoing a temporary G2/M arrest, from which they eventually recover. Exposure to sublethal doses of drugs also increases the possibility of drug resistance. Hence gene knockout and other strategies for the abrogation of wild-type p53 activity, rather than its restoration, could be worth investigating as a measure for enhancing the drug sensitivity of gliomas. The results also suggest caution when identifying conditions where gene therapy and other approaches for the restoration of p53 activity could be used for therapeutic benefit.

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