Differential Sensitivity of Malignant Glioma Cells to Methylating and Chloroethylating Anticancer Drugs: p53 Determines the Switch by Regulating xpc, ddb2, and DNA Double-Strand Breaks

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Abstract
Glioblastoma multiforme is the most severe form of brain cancer. First line therapy includes the methylating agent temozolomide and/or the chloroethylating nitrosoureas [1-(2-chloroethyl)-1-nitrosourea; CNU] nimustine [1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; ACNU], carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea; BCNU], or lomustine [1-(2-chloroethyl)-3-cyclohexyl-1-nitroso- urea; CCNU]. The mechanism of cell death after CNU treatment is largely unknown. Here we show that ACNU and BCNU induce apoptosis in U87MG [p53 wild-type (p53wt)] and U138MG [p53 mutant (p53mt)] glioma cells. However, contrary to what we observed previously for temozolomide, chloroethylating drugs are more toxic for p53-mutated glioma cells and induce both apoptosis and necrosis. Inactivation of p53 by pifithrin-α or siRNA down-regulation sensitized p53wt but not p53mt glioma cells to ACNU and BCNU. ACNU and BCNU provoke the formation of DNA double-strand breaks (DSB) in glioma cells that precede the onset of apoptosis and necrosis. Although these DSBs are repaired in p53wt cells, they accumulate in p53mt cells. Therefore, functional p53 seems to stimulate the repair of CNU-induced cross-links and/or DSBs generated from CNU-induced lesions. Expression analysis revealed an up-regulation of xpc and ddb2 mRNA in response to ACNU in U87MG but not U138MG cells, indicating p53 regulates a pathway that involves these DNA repair proteins. ACNU-induced apoptosis in p53wt glioma cells is executed via both the extrinsic and intrinsic apoptotic pathway, whereas in p53mt glioma cells, the mitochondrial pathway becomes activated. The data suggest that p53 has opposing effects in gliomas treated with methylating or chloroethylating agents and, therefore, the p53 status should be taken into account when deciding which therapeutic drug to use. [Cancer Res 2007;67(24):11886–95]

Introduction
Glioblastoma multiforme (GBM; WHO grade IV) is the most common primary malignant brain tumor in adults. Despite considerable advances during the last two decades in neurosurgical techniques, radiation, and chemotherapy, treatment of malignant gliomas remains mostly palliative. Median survival is about 1 year from the time of diagnosis, and even in the most favorable situation, most patients die within 2 years (1, 2). Standard therapy consists of surgical resection followed by radiotherapy. Adjuvant chemotherapy with carmustine [1,3-bis(2-chloroethyl)-1-nitroso- urea; BCNU] is commonly prescribed in the United States, and several clinical trials have been reported with different chemotherapeutic regimens on the basis of chloroethylating nitrosoureas (3, 4). Alternatively, methylating agents, such as temozolomide, are now more often used in glioma therapy. Because promoter methylation of the O6-methylguanine-DNA methyltransferase (MGMT) gene correlates with a benefit in temozolomide therapy (5), the current standard protocol for the treatment of GBM is temozolomide concomitant with ionizing radiation. Chloroethylating agents are often used in glioma therapy, but there is no clear rationale that determines whether methylating or chloroethylating alkylating drugs should be applied during therapy.

The chloroethylnitrosoureas that are in use in cancer therapy are nimustine [1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; ACNU], BCNU, lomustine [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; CCNU], nimustine [1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; ACNU], carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea; BCNU], or lomustine [1-(2-chloroethyl)-3-cyclohexyl-1-nitroso- urea; CCNU]. The mechanism of cell death after CNU treatment is largely unknown. Here we show that ACNU and BCNU induce apoptosis in U87MG [p53 wild-type (p53wt)] and U138MG [p53 mutant (p53mt)] glioma cells. However, contrary to what we observed previously for temozolomide, chloroethylating drugs are more toxic for p53-mutated glioma cells and induce both apoptosis and necrosis. Inactivation of p53 by pifithrin-α or siRNA down-regulation sensitized p53wt but not p53mt glioma cells to ACNU and BCNU. ACNU and BCNU provoke the formation of DNA double-strand breaks (DSB) in glioma cells that precede the onset of apoptosis and necrosis. Although these DSBs are repaired in p53wt cells, they accumulate in p53mt cells. Therefore, functional p53 seems to stimulate the repair of CNU-induced cross-links and/or DSBs generated from CNU-induced lesions. Expression analysis revealed an up-regulation of xpc and ddb2 mRNA in response to ACNU in U87MG but not U138MG cells, indicating p53 regulates a pathway that involves these DNA repair proteins.

ACNU-induced apoptosis in p53wt glioma cells is executed via both the extrinsic and intrinsic apoptotic pathway, whereas in p53mt glioma cells, the mitochondrial pathway becomes activated. The data suggest that p53 has opposing effects in gliomas treated with methylating or chloroethylating agents and, therefore, the p53 status should be taken into account when deciding which therapeutic drug to use. [Cancer Res 2007;67(24):11886–95]
largely ameliorated by functional p53. This is due to induction of apoptosis through the extrinsic pathway via Fas/CD95/Apo-1. In p53 mutant cells, temozolomide induces apoptosis via the intrinsic apoptotic pathway, which becomes less efficiently activated than the receptor-driven pathway in response to DNA methylation (16).

Given the critical role of p53 in temozolomide-induced cell death in gliomas, we wondered whether p53 would have a similar effect on cell death after treatment with chloroethylating agents. The mechanism of cell death after CNU treatment is unknown. Here we show that ACNU and BCNU induce cell death by apoptosis in p53 wild-type and p53 mutant glioma cells. However, in sharp contrast to what we observed previously for methylating agents, chloroethylating drugs are more toxic in p53 mutated than in p53 wild-type glioma cells. We also show that in p53 mutant glioma cells, chloroethylating agents trigger both necrosis and apoptosis, whereas in p53 wild-type cells necrosis was only marginally induced. Furthermore, we show that in p53 mutant glioma cells, DNA double-strand breaks (DSB) accumulate and the repair genes *xpc* and *ddb2* are not up-regulated in response to ACNU, indicating a DNA repair defect in these cells causing hypersensitivity to CNUs. In addition, the data shows that ACNU-induced apoptosis in p53 wild-type cells occurs via both the extrinsic and intrinsic apoptotic pathway, whereas in p53 mutant cells CNUs activate mainly the mitochondrial-dependent intrinsic pathway.

**Materials and Methods**

**Cell Culture**

U87MG (p53 wild-type), U138MG (p53 mutated), LN229 (p53 wild-type), and LN308 (p53 mutated) glioma cell lines were routinely grown in DMEM (Invitrogen Corporation) supplemented with 10% FCS (fetal bovine serum; Cultilab) and 1% antibiotic-antimycotic (Invitrogen Corporation), at 37°C in a humidified 7% CO2 atmosphere. Culture medium was then removed, and cells were fixed and had their DNA denatured in a one-step reaction by addition of FixDenat solution. Subsequently, samples were incubated for 90 min with anti-BrdUrd-peroxidase solution. After washing, the substrate was added for 20 min when photometric detection was performed at 570 nm in an ELISA reader. Values are expressed in relation to control samples that were considered as 100%.

**Preparation of RNA and reverse transcription-PCR**

Total RNA was isolated using the RNA II Isolation kit from Macherey-Nagel. Two micrograms of RNA were transcribed into cDNA by Superscript II (Invitrogen Corporation) in a volume of 40 and 3 µL was subjected to reverse transcription-PCR (RT-PCR). RT-PCR was performed using specific primers (MWG Biotechnology) and Red-Taq Ready Mix (Sigma).

**Preparation of Cell Extracts for Protein Analysis**

Fractionated cell extracts. Cell pellets of treated and untreated samples were suspended in fractionation buffer A (10 mmol/L HEPES-KOH (pH 7.4), 0.1 mmol/L EDTA, 1 mmol/L ethylene glycol-bis (b-aminoethyl ether), 250 mmol/L sucrose, 1 mmol/L Na2VO3, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSE), and 10 mmol/L DTT) and 100 µmol/L DTT (pH 8.5)]. This suspension was homogenized by sonication. After centrifugation at 10,000 rpm for 10 min, the supernatant containing the cytoplasmic proteins was isolated. The pellets, containing the nuclei, organelles, and membranes, were then suspended in fractionation buffer B (20 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L β-mercaptoethanol, 5% glycerine, 1 mmol/L Na2VO3, 0.5 mmol/L PMSE, and 10 mmol/L DTT (pH 8.5)]. This suspension was sonicated by sonication. After centrifugation at 10,000 rpm for 10 min, the supernatant contains the nuclear proteins and the pellet the membrane fragments. This membrane pellet was suspended in fractionation buffer B containing 1% Triton X-100. All protein concentration was determined by the method of Bradford (19).

**Western Blot Analysis**

The method used here is based on the method described by Renart et al. (20). Protein (30 µg) of cell extracts was separated in a 12% SDS-polyacrylamide gel. Thereafter, proteins were blotted onto a nitrocellulose membrane (Protran; Schleicher & Schuell) for 3 h. Membranes were blocked for 2 h at room temperature in 5% (w/v) fat-free milk powder in TBS containing 0.1% Tween 20, incubated overnight at 4°C with the primary antibody (1:500–1,000 dilution), washed thrice with 0.1% Tween 20 in TBS, and incubated for 2 h with a horseradish peroxidase-coupled secondary antibody 1:3,000 (Amersham Biosciences AB). Antibodies used were...
anti-Bax, anti-Bcl2, anti-extracellular signal-regulated kinase2 (Santa Cruz Biotechnology, Inc.), anti-p53 (Cell Signaling), and anti-Bak (Calbiochem). After final washing with 0.1% Tween 20 in TBS (trice for 10 min each), blots were developed by using a chemiluminescence detection system (Amersham Biosciences AB).

Caspace Activity
The caspase Colorimetric Assay (R&D Systems) was performed according to the manufacturer's protocol. Briefly, cells were treated with 50 µmol/L ACNU, and after particular intervals of postexposure, they were trypsinized, counted, and collected by centrifugation. Cell pellets were lysed on ice, centrifuged, and the supernatant was transferred and kept on ice. The enzymatic reactions were carried out in 96-well microplates (405 nm; 37°C; 1–2 h) with the addition of a equal volume of 2-x reaction buffer and appropriate caspase colorimetric substrate before the measurement on an ELISA reader.

Transfection of Glioma Cells with MGMT and DN-FADD
The transfection method for MGMT and DN-FADD in human glioma cells has been described in our previous work (16). Briefly, MGMT transfectants were generated by cotransfection of U87MG (p53wt) and U138MG (p53mt) cells with the mammalian expression vector (pSV2MGMT) harboring the MGMT gene described previously (7) and the pSV2neo plasmid for selection. G418-resistant clones were picked in 24-well plates and tested for MGMT expression using Western blot and MGMT activity assay. DN-FADD transfectants were generated in U87MG (p53wt) and U138MG (p53mt) cells by transfecting pcDNA3-FADD-DN (21) that already contained the neo gene. FADD-DN–positive clones were determined by Western blotting. Stably p53siRNA-transfected U87MG cells were described before (16, 22).

Immunohistochemistry
U87MG (p53wt) and U138MG (p53mt) cells were seeded on coverslips. Following treatment with 50 µmol/L ACNU and 72 h, the cells were fixed with 4% formaldehyde. A second fixation step was performed using 100% methanol (−20°C; 20 min). Cells were then blocked in 5% BSA PBS (0.3% Triton X-100). The antibodies used were anti–phosphorylated histone H2AX (γ-H2AX; Upstate) and Alexa Fluor 546 (Molecular Probes). Just before mounting, DNA was stained with 100 nmol/L 4’,6-diamidino-2-phenylindole for 15 min. Between all steps, cells were washed in PBS (0.3% Triton X-100) for 5 min thrice. Slides were mounted in antifade medium [Glycerol/PBS, 1:1; 2.5% DABCO (pH 8.6) with HCl].

Results
Cytotoxicity of ACNU and BCNU in p53wt and p53mt glioma cells. First, we examined the cytotoxic effect of ACNU and BCNU in U87MG (p53 wt) and U138MG (p53mt) glioma cells in colony-forming survival assays. As shown in Fig. 1, U138MG (p53mt) cells are clearly more sensitive to the killing effect of both ACNU (Fig. 1A) and BCNU (Fig. 1B), when compared with U87MG (p53wt) cells. This is in marked contrast to their response to methylating agents, such as N-methyl-N′-nitro-N-nitrosoguanidine, shown for comparison in Fig. 1C, for which p53wt were more sensitive than p53mt glioma cells (16).

ACNU and BCNU treatment induces both necrosis and apoptosis in human glioma cells. Next, we compared the apoptotic response of U87MG (p53wt) and U138MG (p53mt) cells after exposure to ACNU and BCNU. Analysis of sub-G1 fraction of exponentially growing cells at different times after treatment shows that both ACNU (Fig. 2A) and BCNU (Fig. 2B) were effective in inducing apoptosis in glioma cells. Apoptosis was a late response, starting at ~70 h and constantly increasing until 144 h from treatment. Interestingly, during the entire posttreatment period, U87MG (p53wt) cells displayed a lower level of apoptosis when compared with p53-mutated cells. This was also observed in a dose-response study after treatment with ACNU and BCNU (data not shown).

To substantiate these results and, further, to discriminate between apoptotic and necrotic cell death, the analysis was performed by Annexin V/propidium iodide double-staining and quantification by flow cytometry. Cells were harvested 144 h after treatment with different concentrations of ACNU. Data shown in Fig. 2C revealed that U87MG (p53wt) and U138MG (p53mt) cells undergo apoptosis upon treatment and that p53wt cells are more resistant than p53mt cells to ACNU (Fig. 2C). Surprisingly, analysis of the necrotic fraction (Fig. 2D) showed that ACNU induces a high level of necrotic cell death in U138MG (p53mt) cells, whereas U87MG (p53wt) cells do not exhibit significant induction of necrosis. This indicates that functional p53 protects not only against apoptotic but also against necrotic cell death upon chloroethylnitrosourea treatment. Similar results were obtained with BCNU (data not shown). Taken together, the results show that ACNU and BCNU induce both apoptosis and necrosis in human glioma cells and that functional p53 causes resistance to these agents.

Modulation of p53 affects ACNU-induced apoptosis. To address the question of whether p53 becomes activated in response to CNUs, the nuclear levels of p53 were investigated at different times after ACNU treatment in U87MG (p53wt) and U138MG (p53mt) cells. As shown in Fig. 3A, there is a clear stabilization of nuclear p53 in U87MG (p53wt) cells, starting as soon as 24 h after treatment. The transfection method for MGMT and DN-FADD in human glioma cells has been described in our previous work (16). Briefly, MGMT transfectants were generated by cotransfection of U87MG (p53wt) and U138MG (p53mt) cells with the mammalian expression vector (pSV2MGMT) harboring the MGMT gene described previously (7) and the pSV2neo plasmid for selection. G418-resistant clones were picked in 24-well plates and tested for MGMT expression using Western blot and MGMT activity assay. DN-FADD transfectants were generated in U87MG (p53wt) and U138MG (p53mt) cells by transfecting pcDNA3-FADD-DN (21) that already contained the neo gene. FADD-DN–positive clones were determined by Western blotting. Stably p53siRNA-transfected U87MG cells were described before (16, 22).

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treatment. In U138MG (p53mt) cells, no p53 was observed in the nuclear fraction. To delineate that p53 induction was in fact related to the resistance of U87MG (p53wt) and U138MG (p53mt) cells, analyzed by sub-G1 population scoring. B, time-response curve after 50 μmol/L BCNU of U87MG (p53wt) and U138MG (p53mt) cells, analyzed by sub-G1 population scoring. C, dose-response curve of the apoptotic fraction of U87MG (p53wt) and U138MG (p53mt) cells after 144 h ACNU treatment, analyzed by Annexin V/propidium iodide double staining. D, dose-response curve of the necrotic fraction of U87MG (p53wt) and U138MG (p53mt) cells after 144 h ACNU treatment, analyzed by Annexin V/propidium iodide double staining and flow cytometry. For C and D, similar results were obtained using BCNU.

Figure 2. Apoptosis induction by ACNU and BCNU in p53 wild-type and mutant glioma cells. A, time-response curve after 50 μmol/L ACNU of U87MG (p53wt) and U138MG (p53mt) cells, analyzed by sub-G1 population scoring. B, time-response curve after 50 μmol/L BCNU of U87MG (p53wt) and U138MG (p53mt) cells, analyzed by sub-G1 population scoring. C, dose-response curve of the apoptotic fraction of U87MG (p53wt) and U138MG (p53mt) cells after 144 h ACNU treatment, analyzed by Annexin V/propidium iodide double staining. D, dose-response curve of the necrotic fraction of U87MG (p53wt) and U138MG (p53mt) cells after 144 h ACNU treatment, analyzed by Annexin V/propidium iodide double staining and flow cytometry. For C and D, similar results were obtained using BCNU.

MGMT protects both p53wt and p53mt glioma cells against ACNU. Chloretoxylating agents induce a broad spectrum of DNA lesions; one of them is O⁶-chloroethylguanine. MGMT is thought to play the main role in defense by removing the chloroethyl group from the O⁶-position of guanine, thereby preventing secondary interstrand cross-link formation. To show that the killing effect of ACNU in glioma cells is in fact due to O⁶-chloroethylguanine, we stably transfected U87MG (p53wt) and U138MG (p53mt) cells with MGMT. The transfectants exhibit MGMT protein (Fig. 4A) and activity (data not shown), in contrast to the parental cell lines that have no detectable MGMT. Figure 4A shows that the presence of functional MGMT significantly protects against apoptosis induction in both U87MG (p53wt) and U138MG (p53mt) cells. Because MGMT specifically repairs O⁶-chloroethylguanine adducts, the reduction of apoptosis frequency almost back to control level found in MGMT expressing cells strongly suggests that O⁶-chloroethylguanine is in fact the initial DNA damage that, upon conversion into secondary lesions, triggers apoptosis in glioma cells.

p53wt cells display better recovery from DNA replication inhibition than p53mt cells after ACNU treatment. ACNU induces the formation of interstrand cross-links in the DNA by molecular rearrangement of the O⁶-chloroethyl group. Because interstrand cross-links are strong inhibitors of DNA replication, we determined the DNA synthesis in U87MG (p53wt) and U138MG (p53mt) cells at different times after treatment with ACNU. Using the BrdUrd-incorporation method, we show that 12 h after treatment DNA synthesis is blocked in both U87MG (p53wt) and U138MG (p53mt) cells (Fig. 4B). It is evident, however, that U87MG (p53wt) cells were able to recover 48 h after treatment to normal levels of DNA synthesis, whereas in U138MG (p53mt) cells, replication levels did not recover. This indicates that in U87MG (p53wt) cells, replication progression was no longer inhibited by replication blocking lesions, whereas in U138MG (p53mt) cells, lesions that block replication were obviously still present. To elucidate whether this replication inhibition was a consequence of interstrand cross-link formation due to O⁶-chloroethylguanine,
we analyzed the replication rates after ACNU treatment in MGMT-transfected glioma cells. Figure 4C shows the results for U87-MGMT (p53wt) and U138-MGMT (p53mt) cells, respectively. It is shown that in the presence of MGMT replication blockage does not occur in both cell lines, except at the highest ACNU concentration tested (200 μmol/L), which is very likely due to saturation of the MGMT repair activity.

p53-mutated glioma cells display a higher level of DSBs upon ACNU treatment. Although the repair of interstrand cross-links in mammalian cells is poorly understood, there is strong evidence supporting the formation of DSBs during lesion processing. γH2AX has widely been used as a marker for DSBs. Therefore, the levels of γH2AX in these cells after ACNU treatment was investigated. As shown in Fig. 5A, in U87MG (p53wt) cells γH2AX was induced up to 72 h from treatment, followed by a decrease after 96 and 120 h. In contrast, in U138MG (p53mt) cells the level of γH2AX induction was clearly higher and continued to increase steadily up to 120 h (Fig. 5A). γH2AX induction after ACNU treatment was also investigated by fluorescence microscopy. As shown in Fig. 5B, clearly much higher amounts of γH2AX foci were observed in U138MG (p53mt) cells than in U87MG (p53wt) cells. The data are in line with the enhanced sensitivity of U138MG (p53mt) cells to ACNUs, compared with U87MG (p53wt) cells.

Expression of DNA repair genes in glioma cells after ACNU treatment. The effect of ACNU treatment on the expression of DNA repair genes was determined by quantitative RT-PCR. U87MG (p53wt), U138MG (p53mt), U87sip53, and U87MGMT cells were used for these experiments. Data shown in Fig. 5D revealed that from all genes tested, enhanced expression was only observed for xpc and ddb2 mRNA after ACNU treatment. Most interestingly, this occurred only in U87MG (p53wt) but not in U138MG (p53mt) glioma cells, which also displayed a very low basal ddb2 expression. Xpc and ddb2 induction was clearly attenuated in U87sip53 cells, supporting the role of p53 in the regulation of XPC and DDB2.

ACNU activates both the extrinsic and intrinsic apoptosis pathway in glioma cells. Which pathway is involved in ACNU-induced apoptosis in glioma cells? This question was addressed by investigating U87MG (p53wt) and U138MG (p53mt) cells that were stably transfected with dominant-negative FADD (DN-FADD; the cell lines were designated as U87DN-FADD and U138DN-FADD, respectively), which are impaired in apoptosis signaling through the extrinsic pathway (16). Although a ~40% reduction of apoptosis rate was observed in U87MG (p53wt) cells, there was no protective effect in U138MG (p53mt) cells (Fig. 6A). This indicates that only in p53wt cells, the extrinsic pathway plays a role in apoptosis induction.
by ACNU. Further, after ACNU treatment, Bcl-2 in the mitochondrial fraction becomes transiently up-regulated and subsequently degraded in both cell lines (Fig. 6B). Bax showed up-regulation after ACNU treatment notably in U87MG (p53wt) cells, whereas Bak was not affected in either cell lines (Fig. 6B). Because the activation of the intrinsic pathway depends on the ratio between the antiapoptotic Bcl-2 and the proapoptotic Bax and Bak, the data are taken to indicate that the intrinsic pathway also becomes activated in glioma cells after ACNU treatment. As expected from the response of DN-FADD transfected cells, U87MG (p53wt) cells showed a clear activation of caspase-8, whereas U138MG (p53mt) cells did not (Fig. 6C). Both lines showed caspase-9 and caspase-3 activation, with U138MG (p53mt) cells responding slightly better (Fig. 6C). The activation of the effector caspase-7 was also determined in response to ACNU (Fig. 6D). It occurred only in U138MG (p53mt) cells (at times where the cells undergo apoptosis; see Fig. 2A), which is in line with its higher ACNU sensitivity.

Discussion

Even after recent advances in therapy, patients suffering from GBM (WHO grade IV) have a very poor prognosis. Treatment consists of surgery followed by radiation and chemotherapy. The most often used chemotherapeutic agents are methylation (such as temozolomide) and chloroethylating agents (such as ACNU, BCNU, and CCNU). In our previous work, we studied the molecular action of methylating agents in human malignant glioma cells (16). The present work was aimed at elucidating the mechanism of cell death induced by O6-chloroethylating agents in the same glioma cell system. As a first step toward this goal, we compared the effects of ACNU and BCNU on a p53 wild-type and mutant glioma cells. DNA synthesis quantification was performed by BrdUrd incorporation (see Materials and Methods) in U87MG and U138MG cells (B), and in U87MGMT6 and U138MGMT1 cells (C). For all cell lines, DNA synthesis analysis was performed 12 h and 48 h after treatment with different concentrations of ACNU (indicated in the figure). Data are presented in relation to the nontreated control.
consequences of necrosis induction upon CNU administration will be elucidated in detail in forthcoming studies.

Both colony forming experiments and the quantification of apoptosis and necrosis revealed that p53 mutant glioma cells are significantly more sensitive to ACNU and BCNU treatment than p53 wild-type cells. The higher sensitivity of the colony assay allowed for the use of lower doses. Clearly, p53 protects human glioma cells against chloroethylating agents. This was confirmed by

![Figure 5](image-url)

**Figure 5.** Induction of γ-H2AX and nucleotide excision repair (NER) genes after ACNU treatment. A, Western blot analysis of γ-H2AX in U87MG (p53wt) and U138MG (p53mt) cell lines after 50 μmol/L ACNU treatment at the indicated time points. ERK-2 was used as loading control. B, γ-H2AX foci formation determined by fluorescent microscopy in U87MG (p53wt) and U138MG (p53mt) cells untreated and treated with 50 μmol/L ACNU 72 h after treatment. C, quantification of the number of γ-H2AX foci by fluorescence microscopy in U87MG (p53wt) and U138MG (p53mt) cells at 72 h after 50 μmol/L ACNU treatment. A total number of at least 40 cells were scored for each condition (untreated and treated). D, expression of NER genes after ACNU treatment. Analysis of NER gene (csa, cab, dbd2, ercc1, xpa, xpc, xpd, and xpg) expression by PCR analysis in U87MG (p53wt), U138MG (p53mt), U87sp53, and U87MGMT6 cells 6 and 24 h after 50 μmol/L ACNU treatment. Gapdh was used as loading control.
pharmacologic inhibition of p53 by pifithrin-\(\alpha\) and by down-regulating p53 by siRNA transfection. The data obtained with MGMT-transfected cells further show that the induction of O\(^6\)-chloroethylguanine is the main signal that triggers apoptosis (and necrosis) after CNU treatment because cells expressing this DNA repair protein are protected against the toxic effects of the drug independent of their p53 status. The data further support the role of MGMT as a key node in the resistance of human glioma cells against methylating and chloroethylating agents.

How is cell death executed in response to DNA adducts generated by CNUs? It is well-established that O\(^6\)-chloroethylguanine lesions become converted into interstrand cross-links (8, 10), which are strong replication-blocking lesions (24). This was confirmed here as ACNU treatment inhibited DNA synthesis in glioma cells. This was

Figure 6. Apoptosis pathways used by U87MG (p53wt) and U138MG (p53mt) cells after ACNU treatment. A, apoptotic response of U87MG (p53wt), U138MG (p53mt), and the respective dominant-negative FADD clones U87DN-FADD and U138DN-FADD at 144 h after 50 \(\mu\)mol/L ACNU treatment. Inset, DN-FADD expression in nontransfected and transfected clones. ERK-2 was used as loading control. **, \(P < 0.01\). B, Western blot analysis of the expression of Bcl-2, Bax, and Bak at different time points after 50 \(\mu\)mol/L ACNU treatment. ERK-2 is shown as loading control. C, activation of initiator caspase-8 and caspase-9 and effector caspase-3 72 h after 50 \(\mu\)mol/L ACNU treatment. Results are shown as fold of activation in relation to control (untreated) cells. D, activation of the effector caspase-7 at different time points after 50 \(\mu\)mol/L ACNU treatment. The primary antibody used is specific for the activated form of caspase-7. ERK-2 was used as loading control.
completely abolished in MGMT-transfected cells, which shows that O\textsuperscript{6}-chloroethylguanaine or secondary lesions derived from them are responsible for DNA replication inhibition. Moreover, whereas p53wt cells recovered by returning back to normal DNA synthesis levels, p53mt glioma cells did not show any recovery from DNA synthesis blockage. This indicates that p53 wild-type cells are able to remove the DNA blocking lesions from their genome, whereas p53 mutant cells are impaired. The strong and sustained blockage of DNA synthesis is related to a high cell killing response, which was also shown for other experimental systems (25). The high sensitivity of p53 mutant glioma cells to ACNU is consistent with the hypothesis that critical lesions are not, or only incompletely, repaired or erroneously processed if p53 is functionally lacking. It is conceivable that nonrepaired interstrand cross-links originating from O\textsuperscript{6}-chloroethylguanaine adducts are converted during DNA replication into DSBs that are considered to be a most critical downstream apoptosis signal upon DNA base damage (26). In fact, we clearly observed higher levels of H2AX phosphorylation in p53 mutant glioma cells, which has been reported to be generated during interstrand cross-link processing and indicative of the presence of DSBs (27). The formation of DSBs after interstrand cross-link induction is a possible consequence of stalled replication forks during S phase (28). In line with this is the finding that replication-inhibited p53 mutant glioma cells are more resistant to apoptosis induction by ACNU than proliferating cells.\textsuperscript{3} From the increased level of DSBs in p53 mutant glioma cells along with their enhanced sensitivity, it is pertinent to conclude that p53 wild-type glioma cells are more efficient in the repair of CNU-induced interstrand cross-links or DSBs generated from them than p53-mutated glioma cells.

The findings outlined above prompted us to elucidate the expression of DNA repair genes in glioma cells upon ACNU treatment. The obtained data revealed that the basal level of expression of NER genes is nearly equal in p53 wild-type and mutant cells. However, xpc and ddb2 were found to be up-regulated after ACNU treatment, which only occurs in p53 wild-type and not p53 mutant cells. Interestingly, this up-regulation seems to be long lasting because it was observed 24 h after treatment. The long period necessary for up-regulation of these genes could be explained by the time required for the formation of interstrand cross-links after O\textsuperscript{6}-chloroethylguanaine adducts, which takes 8 to 12 h in mammalian cells (29). The up-regulation of xpc and ddb2 via p53 is well described for UV-C irradiation (30–32). In this report we show, for the first time, that xpc and ddb2 up-regulation occurs in glioma cells after CNU treatment. Recently, it has been shown that XPC is involved in the recognition of psoralen-induced interstrand cross-links (33). Although we can only speculate about the role of XPC and DDB2 in interstrand cross-link repair generated by O\textsuperscript{6}-chloroethylguanaine adducts, the data support the view that p53 mutant glioma cells are defective in the repair or processing of O\textsuperscript{6}-chloroethylguanaine generated secondary lesions, making them more sensitive to CNUs.

Another difference observed between p53 wild-type and p53 mutant glioma cells after ACNU treatment pertains to the apoptotic pathways used by these cells. In p53 wild-type cells, both the extrinsic (as shown by caspase-8 activation and protection mediated by DN-FADD transfection) and the intrinsic (as shown by Bcl-2 degradation, Bax up-regulation, and caspase-9 activation) apoptotic pathways are activated. In p53 mutant cells, however, only the intrinsic pathway (as shown by lack of caspase-8 activation and effect of DN-FADD) seems to be involved in ACNU-triggered apoptosis. This is in line with previous results obtained with temozolomide, where p53 mutant cells undergo apoptosis mainly through the intrinsic pathway. We should note that there was a significant activation of caspase-7 in p53 mutant cells, which conformed to their increased apoptotic response.

Collectively, the data presented here show for the first time that p53 protects against the killing effects of the chloroethylating anticancer drugs ACNU and BCNU in glioma cells. These data are in striking contrast to our previous findings obtained with methylating agents, including temozolomide, where it was shown that p53 greatly stimulates their killing properties (15, 16). Obviously, for temozolomide, p53 determines the switch between receptor and mitochondrial apoptotic pathway, whereas for CNUs, p53 determines the level of DNA repair. Although the mechanisms involved need further exploration, the data have important implications for glioma chemotherapy: (a) p53 seems to be a predictive marker of therapy and, therefore, the p53 status of the tumor tissue upon resection should be assessed; (b) it is recommended that p53-mutated gliomas should be treated with CNUs instead of temozolomide or other methylating drugs, provided functional MGMT is not expressed, and (c) if the p53 status switches from p53 wild-type to p53 mutant during tumor progression, the chemotherapeutic regime should switch from methylating (temozolomide) to chloroethylating agents (ACNU, BCNU, and CCNU) under MGMT-inactivated conditions. We are aware that these are presently only theoretical considerations, as the results were obtained under in vitro conditions in glioma cells. The data might, however, be useful in further exploiting new therapeutic avenues that will hopefully improve glioma therapy.

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3 L.F.Z. Bautista and B. Kaina, unpublished data.

References


Differential Sensitivity of Malignant Glioma Cells to Methylyating and Chloroethylating Anticancer Drugs: p53 Determines the Switch by Regulating xpc, ddb2, and DNA Double-Strand Breaks
