

# 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-nitrosourea; 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- $\alpha$  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-nitrosourea; 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 *O<sup>6</sup>-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-L-nitrosourea; CCNU], semustine [1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; MeCCNU], and fotemustine [1-[N-(2-chloroethyl)-N-nitrosoureido] ethylphosphonic acid diethyl ester]. Although the pharmacokinetics is slightly different, they are similar at the molecular level. The main target of these drugs is the DNA, forming upon generation of nucleophilic chloroethyl-*enium* ions a wide range of DNA lesions similar to methylating agents (6). The main killing lesion seems, however, to be *O<sup>6</sup>-chloroethylguanine* (7–9). This adduct is unstable, undergoing intramolecular rearrangement leading to an intermediary *N1-O<sup>6</sup>-ethenoguanine* and, in a second step, *N1-guanine-N3-cytosine* interstrand cross-links (8, 10). These cross-links are supposed to be the ultimate killing lesions after treatment with chloroethylating nitrosoureas (for review, see ref. 11).

The development of resistance of gliomas to radiation and chemotherapy is a major problem during the treatment of tumors. Only a few factors that determine drug resistance of gliomas have been identified thus far. One is MGMT, which rapidly removes methyl and chloroethyl groups from the *O<sup>6</sup>*-position of guanine (for review, see ref. 11, 12). Therefore, inactivation of MGMT is beneficial during therapy (13). Another factor is mismatch repair that has an impact on the killing effects of methylating but not chloroethylating agents (14). A third determinant, recently identified, of glioma cell sensitivity to methylating agents is p53 (15, 16). Temozolomide induces apoptosis in human glioma cells, which is

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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% CO<sub>2</sub> atmosphere. U87MG siPuro and U87MG sip53 cells were from the laboratory of M. Weller (Department of General Neurology, University of Tübingen, Tübingen, Germany). All cell lines were described previously (16).

### Colony Survival Assay

Colony-forming assays were performed as previously described (17). Briefly, U87MG (p53wt) and U138MG (p53mt) glioma cells growing in log phase were used. Cells were seeded in triplicate at appropriate cell numbers in 60-mm Petri dishes to yield ~100 surviving colonies after ACNU or BCNU treatment. Cells were allowed to attach and then exposed to increasing concentrations of the drugs. After 2 weeks, colonies were fixed (in acetic acid:methanol:H<sub>2</sub>O, 1:1:8), stained (in 0.01% Amido Black) and counted (>100 cells/colony). The surviving fraction was plotted on a log scale.

### Drug Treatment

Approximately 10<sup>5</sup> cells were plated in 60-mm Petri dishes 24 h before treatment with different concentrations of ACNU or BCNU (Sigma). Stocks were prepared by dissolving the drugs in sterile H<sub>2</sub>O, filtered, and stored at -20°C. Temozolomide (Schering-Plough) was dissolved in DMSO and diluted in distilled water. The p53 inhibitor Pifithrin- $\alpha$  (Calbiochem), which reversibly blocks p53-dependent transcriptional activation (18), was added 1 h before ACNU treatment. The percentage of the cells undergoing apoptosis was determined at 144 h.

### Quantification of Apoptosis

**Analysis by sub-G<sub>1</sub>-flow cytometry.** After different times (indicated for each experiment), both adherent and detached cells were collected and centrifuged at 1,000 rpm for 5 min. Pelleted cells were fixed with 70% ethanol and stored for up to 1 week at -20°C. Immediately before analysis,

cells were treated with RNase (0.03 mg/mL) and subsequently stained with propidium iodide (16.5 mg/mL) in PBS. Samples were then transferred to microtubes, and propidium iodide fluorescence was measured by flow cytometry (FACScalibur). For each sample, 10,000 cells were analyzed and the results are shown as percentage of subdiploid nuclei (WinMDI Software), which represent apoptotic cells.

**Analysis by Annexin V/propidium iodide double staining.** Approximately 10<sup>5</sup> cells were plated in 60-mm Petri dishes, and 144 h after treatment with different concentrations of ACNU and BCNU, both adherent and detached cells were collected and centrifuged at 1,000 rpm for 5 min. After washing with PBS, cells were treated with binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl<sub>2</sub> × 2 H<sub>2</sub>O, and 0.1% bovine serum albumin). Cell suspension was then transferred to microtubes where 2.5 μL of Annexin V-FITC (PharMingen) was added. After incubation on ice, in the dark, for 15 min, 430 μL of binding buffer and 10 μL of propidium iodide (50 μg/mL) were added. Samples were immediately analyzed by flow cytometry. All experiments were repeated at least thrice. Mean values ± SD are shown, and data were compared statistically using Student's *t* test.

### Quantification of DNA Synthesis

The Cell Proliferation ELISA bromodeoxyuridine (BrdUrd) colorimetric assay (Roche Applied Science) was performed according to the manufacturer's protocol. Briefly, 1.0 to 5.0 × 10<sup>4</sup> cells were plated in a 96-well microplate for 24 h before treatment with different concentrations of ACNU. Different times after treatment, BrdUrd were added directly to the cell medium for 2 h at 37°C in a humidified 7% CO<sub>2</sub> 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 370 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 (β-aminoethyl ether), 250 mmol/L sucrose, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 10 mmol/L DTT]. The cells were lysed by freeze/thaw/vortexing. The lysate was then centrifuged at 10,000 rpm for 10 min, and 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 Na<sub>3</sub>VO<sub>4</sub>, 0.5 mmol/L PMSF, and 10 mmol/L DTT (pH 8.5)]. This suspension was homogenized 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 mg) 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 (thrice for 10 min each), blots were developed by using a chemiluminescence detection system (Amersham Biosciences AB).

### Caspase Activity

The caspase Colorimetric Assay (R&D Systems) was performed according to the manufacturer's protocol. Briefly, cells were treated with 50  $\mu$ mol/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 an 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 transfecants 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 transfecants 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  $\mu$ mol/L ACNU and 72 h, the cells were fixed with 4% formaldehyde. A second fixation step was performed using 100% methanol ( $-20^{\circ}\text{C}$ ; 20 min). Cells were then blocked in 5% BSA PBS (0.3% Triton X-100). The antibodies used were anti-phosphorylated histone H2AX ( $\gamma$ -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.

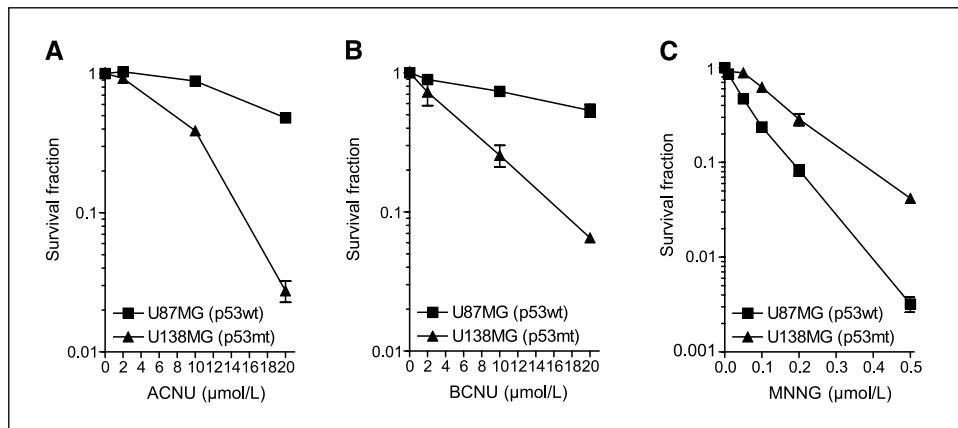
First, we examined the cytotoxic effect of ACNU and BCNU in U87MG (p53wt) and U138MG (p53mt) glioma cells in colony-

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).

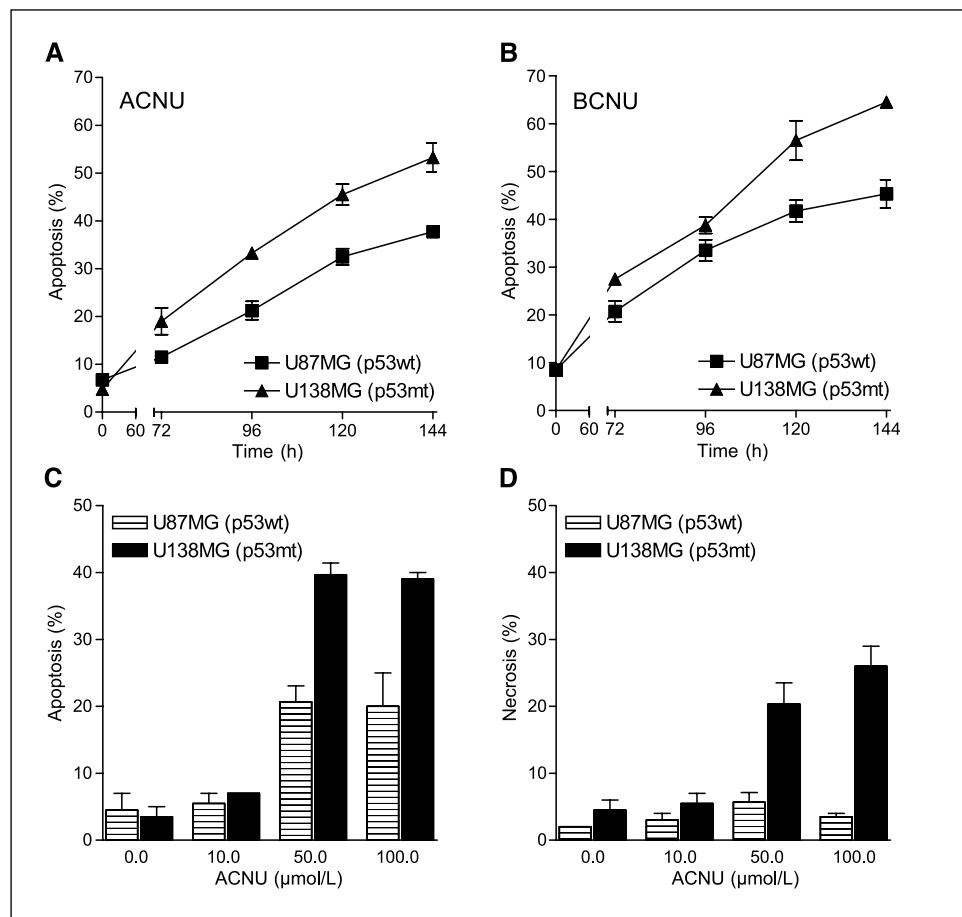
**ACNU and BCNU treatment induces both necrosis and apoptosis in human glioma cells.** Next, we compared the apoptotic response of U87MG (p53wt) and U138MG (p53mt) cells after exposure to ACNU and BCNU. Analysis of sub-G<sub>1</sub> fraction of exponentially growing cells at different times after treatment 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  $\sim 70$  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



**Figure 1.** Colony formation after ACNU (A), BCNU (B), and MNNG (C) treatment. U87MG (p53wt) and U138MG (p53mt) cells were exposed to different concentrations and cultivated for 12 d before scoring surviving colonies. Data are the mean of three independent experiments. MNNG survival curves were shown for comparison. Data of C is from Roos et al. (16).



**Figure 2.** Apoptosis induction by ACNU and BCNU in p53 wild-type and mutant glioma cells. *A*, time-response curve after 50  $\mu\text{mol/L}$  ACNU of U87MG (p53wt) and U138MG (p53mt) cells, analyzed by sub-G<sub>1</sub> population scoring. *B*, time-response curve after 50  $\mu\text{mol/L}$  BCNU of U87MG (p53wt) and U138MG (p53mt) cells, analyzed by sub-G<sub>1</sub> 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.

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) cells to ACNU, we treated U87MG (p53wt) cells stably expressing siRNA targeted to p53 (U87sip53) with different concentrations of ACNU and compared the results with U87MG (p53wt) cells transfected with an empty vector (mock). The data shown in Fig. 3*B* show that knock-down of p53 increases the sensitivity of cells toward ACNU. To further substantiate this finding, pifithrin- $\alpha$ , a specific p53 inhibitor, was added to the cell culture medium 1 h before ACNU treatment (50  $\mu\text{mol/L}$ ) of U87MG (p53wt) and U138MG (p53mt) cells. Figure 3*C* shows that inhibition of p53 by pifithrin- $\alpha$  increases the cell death levels specifically in U87MG (p53wt) cells, although having no effect in U138MG (p53mt) cells. To further confirm the role of p53 in chloroethylnitrosourea-induced cell death, we used another pair of glioma cells (Fig. 3*D*). Again, p53wt cells (the line LN-229) were more resistant to ACNU than p53mt cells (LN-308). Overall, the data supports the finding that p53 plays a protective role against cell death (executed by apoptosis and necrosis) upon treatment with chloroethylating agents.

**MGMT protects both p53wt and p53mt glioma cells against ACNU.** Chloroethylating agents induce a broad spectrum of DNA lesions; one of them is  $O^6$ -chloroethylguanine. MGMT is thought to play the main role in defense by removing the chloroethyl group from the  $O^6$ -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  $O^6$ -chloroethylguanine, we stably transfected U87MG (p53wt) and U138MG (p53mt) cells with

MGMT. The transfectants exhibit MGMT protein (Fig. 4*A*) and activity (data not shown), in contrast to the parental cell lines that have no detectable MGMT. Figure 4*A* shows that the presence of functional MGMT significantly protects against apoptosis induction in both U87MG (p53wt) and U138MG (p53mt) cells. Because MGMT specifically repairs  $O^6$ -chloroethylguanine adducts, the reduction of apoptosis frequency almost back to control level found in MGMT expressing cells strongly suggests that  $O^6$ -chloroethylguanine is in fact the initial DNA damage that, upon conversion into secondary lesions, triggers apoptosis after CNU treatment 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  $O^6$ -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. 4*B*). 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  $O^6$ -chloroethylguanine,

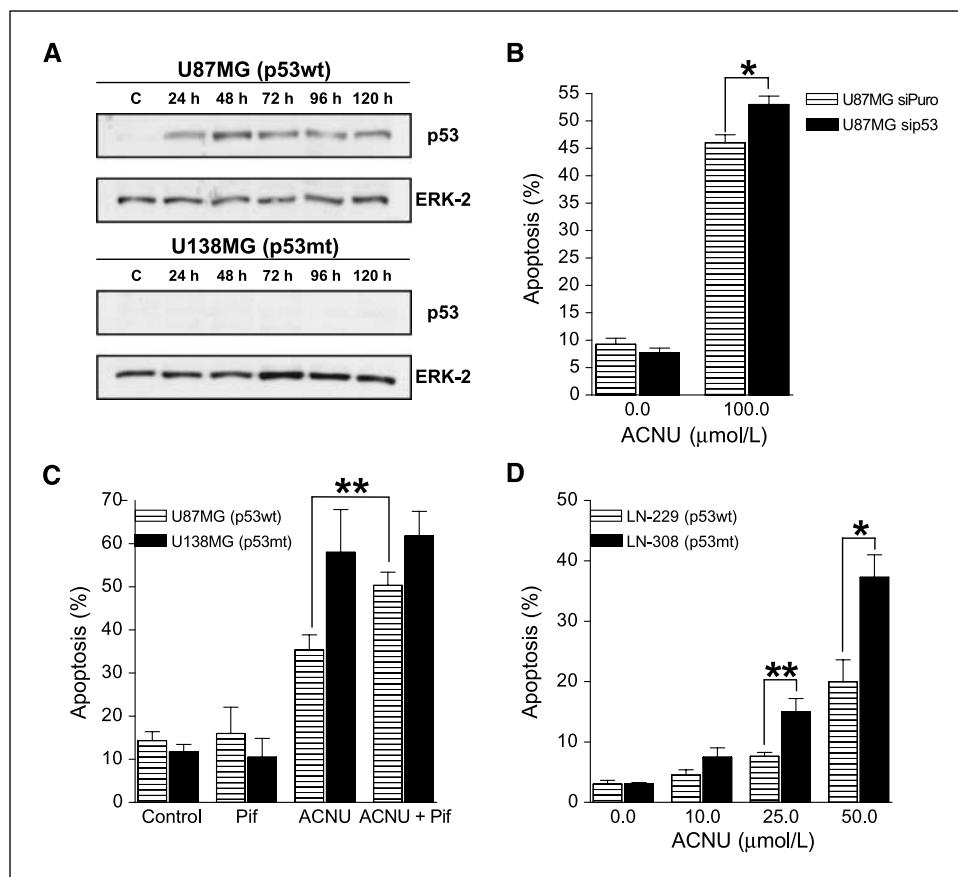
we analyzed the replication rates after ACNU treatment in MGMT-transfected glioma cells. Figure 4C shows the results for U87-MGMT (p53wt) and U138-MGMT (p53mt) cells, respectively. It is shown that in the presence of MGMT replication blockage does not occur in both cell lines, except at the highest ACNU concentration tested (200  $\mu\text{mol/L}$ ), 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.  $\gamma$ -H2AX has widely been used as a marker for DSBs. Therefore, the levels of  $\gamma$ -H2AX in these cells after ACNU treatment was investigated. As shown in Fig. 5A, in U87MG (p53wt) cells  $\gamma$ -H2AX 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  $\gamma$ -H2AX induction was clearly higher and continued to increase steadily up to 120 h (Fig. 5A).  $\gamma$ -H2AX induction after ACNU treatment was also investigated by fluorescence microscopy. As shown in Fig. 5B, clearly much higher amounts of  $\gamma$ -H2AX foci were observed in U138MG (p53mt) compared with U87MG (p53wt) cells (for quantification see Fig. 5C). The data indicate that in U138MG (p53mt) cells, more DSBs were produced from interstrand cross-link processing and left unrepaired, whereas in U87MG (p53wt) cells, less DSBs were produced and/or were subject to repair. The data are in line with the enhanced sensitivity of U138MG (p53mt) cells to CNUs, compared with U87MG (p53wt) cells.

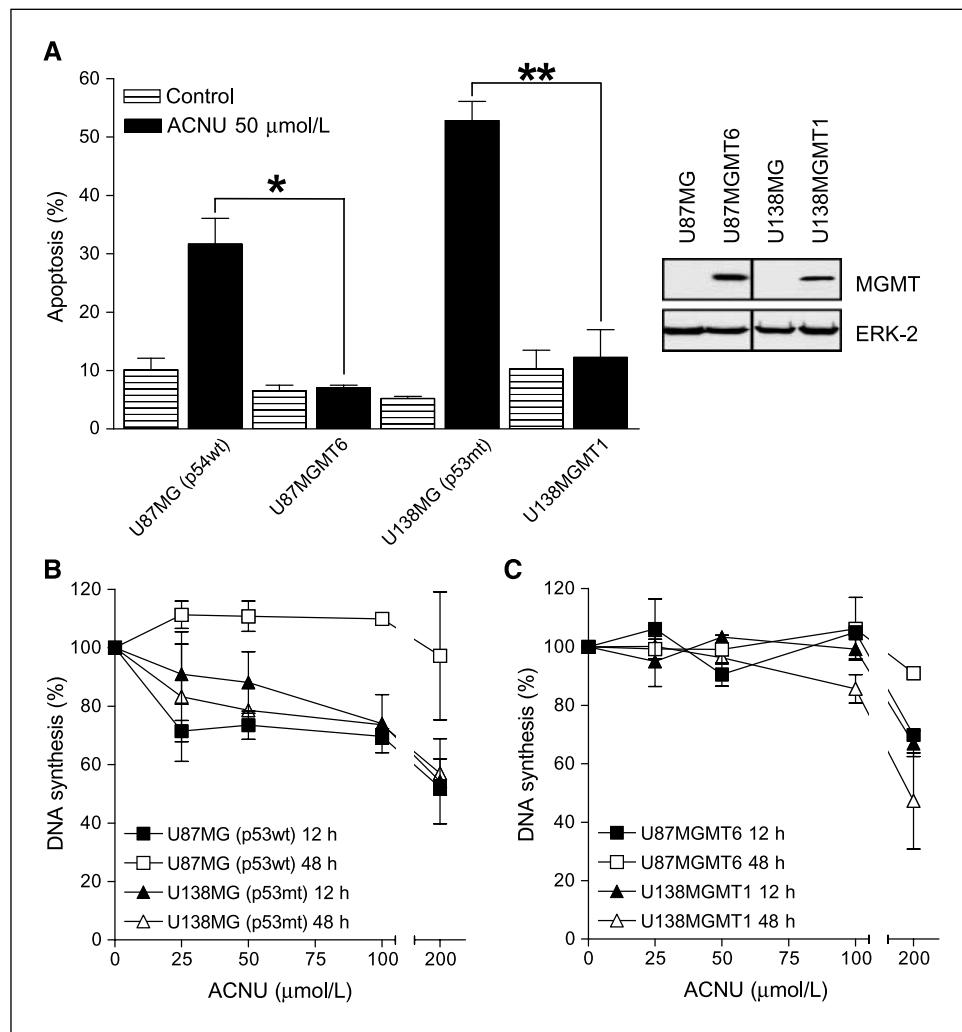
**Expression of DNA repair genes in glioma cells after ACNU treatment.** The effect of ACNU treatment on the expression of

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. The induction of *xpc* and *ddb2* was completely lacking in U87MG (p53wt) cells stably transfected with MGMT cDNA (U87MGMT cells), which shows that the induction of these DNA repair genes originated from  $O^6$ -chloroethylguanine, or the processing of  $O^6$ -chloroethylguanine DNA adducts. XPC and DDB2 are supposed to be involved in interstrand cross-link repair. Therefore, the data further support the hypothesis that p53mt glioma cells are defective in the repair of interstrand cross-links derived from  $O^6$ -chloroethylguanine, which provoke cell death.

**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



**Figure 3.** Influence of p53 on ACNU-induced apoptosis. *A*, p53 nuclear protein levels in U87MG (p53wt) and U138MG (p53mt) cells at different time points (indicated in the figure) after 50  $\mu\text{mol/L}$  ACNU treatment. ERK-2 is shown as a loading control. *B*, apoptosis induction 144 h after 100  $\mu\text{mol/L}$  ACNU in U87MG (p53wt) cells transfected with an empty vector (U87MG siPuro) or with a specific siRNA sequence targeted to p53 (U87sip53). \*, difference is statistically significant ( $P < 0.05$ ). *C*, apoptosis of U87MG (p53wt) and U138MG (p53mt) cells after 50  $\mu\text{mol/L}$  ACNU at 144 h in the presence and absence of the p53 inhibitor pifithrin- $\alpha$ . \*\*,  $P < 0.01$ . *D*, Apoptosis in LN-229 (p53wt) and LN-308 (p53mt) cells after ACNU treatment. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



**Figure 4.** Influence of MGMT on O<sup>6</sup>-chloroethylguanine–induced apoptosis and DNA synthesis. *A*, apoptotic response of U87MG (p53wt), U138MG (p53mt), and their respective MGMT-transfected clones U87MGMT6 and U138MGMT1 after 50 μmol/L ACNU at 144 h after treatment. *Inset*, MGMT expression in nontransfected and transfected clones. ERK-2 was used as loading control. \*, P < 0.05; \*\*, P < 0.01. *B* and *C*, DNA synthesis inhibition by ACNU in 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.

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

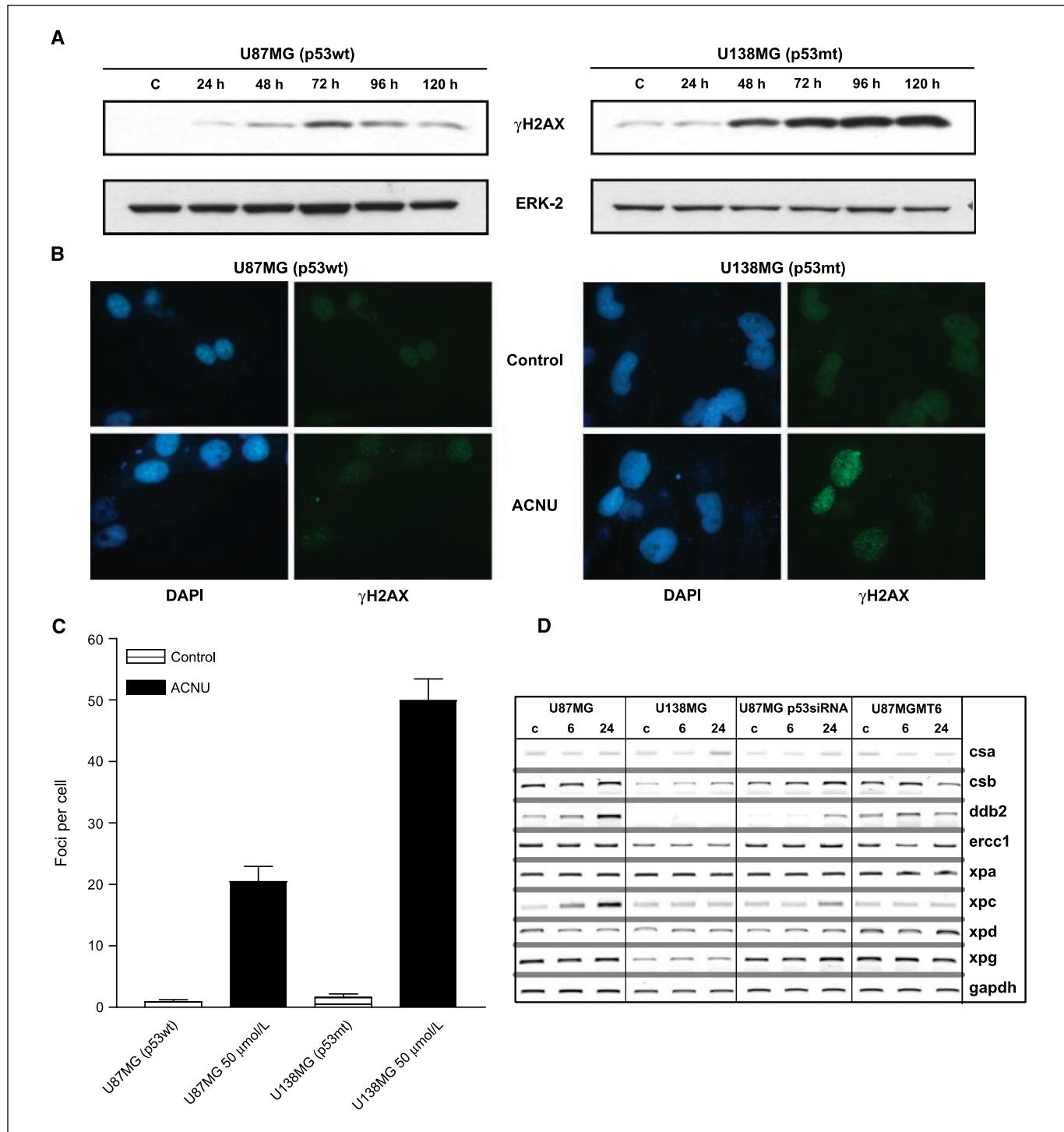
Even after recent advances in therapy, patients suffering from GBM (WHO grade IV) have a very poor prognosis. Treatment consists of surgery followed by radiation and chemotherapy. The most often used chemotherapeutic agents are methylation (such as

temozolamide) and chloroethylating agents (such as ACNU, BCNU, and CCNU). In our previous work, we studied the molecular action of methylation agents in human malignant glioma cells (16). The present work was aimed at elucidating the mechanism of cell death induced by O<sup>6</sup>-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 p53 mutant background. This is important because p53 is often mutated in human gliomas. Thus, secondary GBM that develop from grade II or III astrocytomas exhibit p53 mutations in 57%, whereas primary GBM exhibit p53 mutations in 17% of cases (23). Furthermore, we recently showed that p53 has a strong effect on sensitization of glioma cells to methylation agents such as temozolamide, which is in use for first-line therapy of GBM. The present work shows that glioma cells undergo cell death after ACNU and BCNU treatment by apoptosis. This is a quite late response, occurring no earlier than 3 to 4 days after treatment. Contrary to temozolamide, however, p53 wt glioma cells were more resistant than p53-mutated or p53-inactivated cells. Interestingly, our data also show that although p53 wild-type glioma cells die exclusively by apoptosis, p53 mutant cells treated with ACNU and BCNU exhibit high levels of necrosis (on average 50% of the death fraction). This is unusual and has not been observed for methylation agents, including temozolamide, that almost exclusively induce apoptosis (16). The reason and

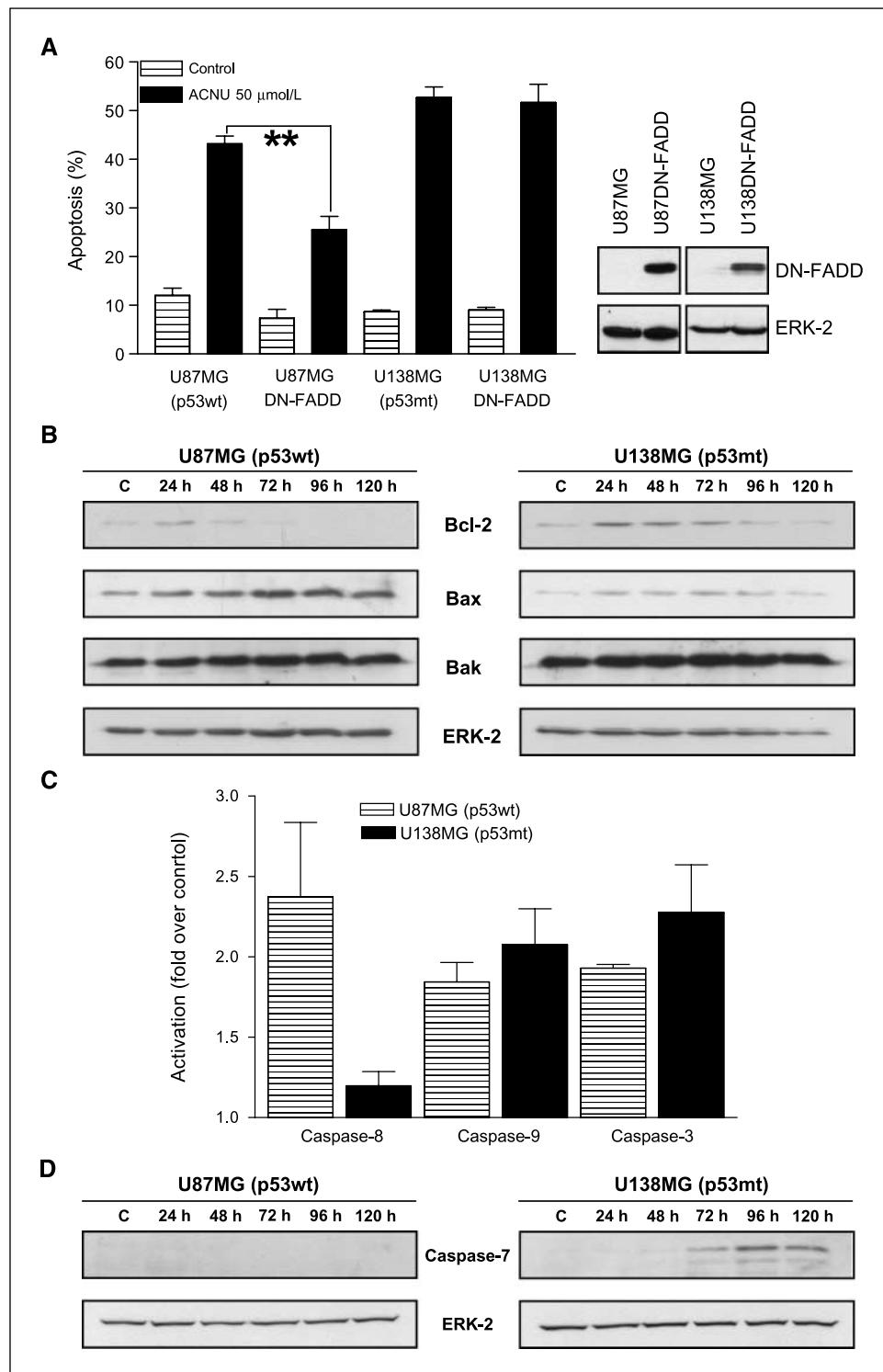
consequences of necrosis induction upon CNU administration will be elucidated in detail in forthcoming studies.

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.** Induction of  $\gamma$ -H2AX and nucleotide excision repair (NER) genes after ACNU treatment. *A*, Western blot analysis of  $\gamma$ -H2AX in U87MG (p53wt) and U138MG (p53mt) cell lines after 50  $\mu$ mol/L ACNU treatment at the indicated time points. ERK-2 was used as loading control. *B*,  $\gamma$ -H2AX foci formation determined by fluorescent microscopy in U87MG (p53wt) and U138MG (p53mt) cells untreated and treated with 50  $\mu$ mol/L ACNU 72 h after treatment. *C*, quantification of the number of  $\gamma$ -H2AX foci by fluorescence microscopy in U87MG (p53wt) and U138MG (p53mt) cells 72 h after 50  $\mu$ mol/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*, *csb*, *ddb2*, *ercc1*, *xpa*, *xpc*, *xpd*, and *xpg*) expression by PCR analysis in U87MG (p53wt), U138MG (p53mt), U87MGSiP53, and U87MMT6 cells 6 and 24 h after 50  $\mu$ mol/L ACNU treatment. *Gapdh* was used as loading control.



**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 μmol/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 μmol/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 μmol/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 μmol/L ACNU treatment. The primary antibody used is specific for the activated form of caspase-7. ERK-2 was used as loading control.

pharmacologic inhibition of p53 by pifithrin-α and by downregulating p53 by siRNA transfection. The data obtained with MGMT-transfected cells further show that the induction of O<sup>6</sup>-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 methylation and chloroethylating agents.

How is cell death executed in response to DNA adducts generated by CNUs? It is well-established that O<sup>6</sup>-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

completely abolished in MGMT-transfected cells, which shows that  $O^6$ -chloroethylguanine 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  $O^6$ -chloroethylguanine 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.<sup>3</sup> 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  $O^6$ -chloroethylguanine 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  $O^6$ -chloroethylguanine adducts, the data support the view that p53 mutant glioma cells are defective in

the repair or processing of  $O^6$ -chloroethylguanine 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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<sup>3</sup> L.F.Z. Batista and B. Kaina, unpublished data.

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