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p53 disruption profoundly alters the response of human glioblastoma cells to DNA topoisomerase I inhibition

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A critical challenge in cancer research is to identify genetic lesions that sensitize patients to chemotherapy. p53, which is mutated in nearly one-third to half of glioblastomas, may be such a lesion. In this paper, we demonstrate that p53 disruption dramatically sensitizes glioblastoma cells to DNA topoisomerase I inhibitor-mediated apoptosis. Using 19 glioblastoma cell lines, including 15 low-passage *ex vivo* cell lines derived from patients, as well as isogenic glioblastoma cells varying in p53 status, we show that clinically relevant levels of SN-38 potently induce cell cycle arrest and temporary senescence in glioblastoma cells with wild-type p53 while causing massive apoptosis in p53-deficient cells ($P < 0.0002$). We demonstrate that glioblastoma cells with wild-type p53 proliferate when recultured in drug-free medium, whereas p53-deficient cells do not. We also show that p16 protein expression is neither necessary nor sufficient for initiation and/or maintenance of SN-38-induced arrest/senescence. These results indicate that p53 disruption has a dramatic effect on how glioblastoma cells process topoisomerase I inhibitor-mediated DNA damage.
Oncogene (2004) **23**, 1283–1290. doi:10.1038/sj.onc.1207244

Introduction

A new approach to cancer therapy focuses on developing inhibitors that are targeted to specific genetic lesions (Druker, 2002; Sawyers, 2002; Shawver et al., 2002). Glioblastoma is the most common malignant brain tumor of adults, and is among the most lethal of all cancers (Kleihues et al., 2002; Mischel and Cloughesy, 2003). Although glioblastomas are highly unresponsive to most standard therapies, they may be very well suited for targeted therapy. Glioblastomas contain a number of clearly defined genetic lesions whose disruption

regulates their biological and clinical behavior (Bachoo et al., 2002). These genetic lesions and the resultant disrupted signaling pathways present an attractive target for therapy (Mischel and Cloughesy, 2003). Our group has focused its efforts on identifying molecular subsets of glioblastomas that may be differentially sensitive to specific targeted therapies (Choe et al., 2002, 2003; Mischel and Cloughesy, 2003; Mischel et al., 2003; Shai et al., 2003).

There appear to be at least two distinct clinical routes to the development of glioblastoma, and each one is associated with a different set of genetic lesions (Kleihues and Ohgaki, 1999). Primary glioblastomas arise as *de novo* grade IV tumors, often in older patients. They frequently harbor amplification/overexpression of the epidermal growth factor receptor (EGFR) (Watanabe et al., 1996; Kleihues and Ohgaki, 1999; Kleihues et al., 2002). In contrast, secondary glioblastomas progress from lower grade gliomas in younger patients, and commonly contain p53 mutations, but not EGFR abnormalities (Kleihues and Ohgaki, 1999; Kleihues et al., 2002). The different clinical and molecular phenotypes of these glioblastomas suggest that they may potentially be susceptible to different therapies. However, to date, p53 status has not been used to guide treatment decisions for glioblastoma patients, although it has been shown to have therapeutic implications for anaplastic oligodendroglioma patients (Ino et al., 2001).

The DNA topoisomerase I inhibitor CPT-11 (a camptothecin derivative) and its active metabolite SN-38 demonstrate impressive activity against glioblastoma cell lines *in vitro* and in animal xenografts (Hare et al., 1997; Coggins et al., 1998). However, early clinical trials indicate that only a subset of glioblastoma patients benefit (Friedman et al., 1999). Determining which glioblastoma patients are most likely to benefit from DNA topoisomerase I inhibition is therefore an important challenge. Previous studies suggest that p53 does not alter the sensitivity of glioblastoma cells (or other types of cancer cells) to topoisomerase I inhibitor-mediated DNA damage using clonogenic assays (Slichenmyer et al., 1993; Weller et al., 1998; Brown and Wouters, 1999; Nieves-Neira and Pommier, 1999). However, there may be molecular differences in how the

DNA damage is processed in p53-deficient cells, which might not be detected by clonogenic assays and could potentially be clinically exploitable. As DNA topoisomerase I inhibitors generate double-stranded DNA breaks (Liu *et al.*, 2000), and because p53 is critical in determining the cellular response to such DNA damage, we hypothesized that loss of functional p53 would profoundly modify the response of tumor cells to DNA topoisomerase I inhibition.

In this paper, we use clinical tumor isolates from 15 glioblastoma patients in *ex vivo* culture of different p53 status, as well as established glioblastoma cell lines and isogenic U87MG differing in p53 status, to demonstrate that there are significant differences in how glioblastoma cells process the damage caused by the inhibition of DNA topoisomerase I in the presence of p53 loss, which may be potentially exploitable in the clinic.

Results

Generation of p53-deficient U87MG glioblastoma cells

As a first step in analysing the role of p53 in determining the response of glioblastoma cells to DNA topoisomerase I inhibition, we introduced HPV16 E6 gene into U87MG cells. The HPV16 E6 protein targets p53 for degradation in a ubiquitin-dependent manner (Scheffner *et al.*, 1993). Immunoblotting demonstrated that three of the E6 transduced clones (clones 4, 12, 13) showed markedly diminished levels of p53 proteins, and lacked p21 induction in response to SN-38 treatment (Figure 1a). Therefore, the E6 transduced clones 4, 12, 13 were deficient in both p53 level and function.

### p53 disruption promotes apoptosis of U87MG glioblastoma in response to SN-38

We performed clonogenic assays to determine the overall sensitivity of U87MG, U87MG-neo and U87MG-E6 cells to SN-38 treatment. As shown in Figure 1b, p53 disruption did not alter the overall sensitivity of glioblastoma cells to DNA topoisomerase I inhibition in a clonogenic assay. We then analysed the effect of p53 disruption on cell cycle distribution in glioblastoma cells treated with SN-38. Glioblastoma cells were incubated for 0–10 days in the presence of the clinical relevant levels of SN-38, 20 ng/ml (Negoro *et al.*, 1991; Friedman *et al.*, 1999). In glioblastoma cells with intact p53 (U87MG-neo), DNA topoisomerase I inhibition caused a decrease in the G1 fraction of cells and an accumulation of cells at G2/M phase ($4n$ DNA content) (Figure 2a). The p53-wild-type glioblastoma cells remained arrested through out the 10-day treatment, and polyploid cells ($8n$ DNA content) became apparent starting 2–3 days post-SN-38 (Figure 2a). On a biochemical level, SN-38 induced transient Cdc2 phosphorylation at 6 h to 1 day (Figure 2b), corresponding to the onset of G2/M arrest, and potent and sustained p21 upregulation (Figure 2c). At 2 days post-SN-38 treatment, the level of total and phosphorylated Cdc2
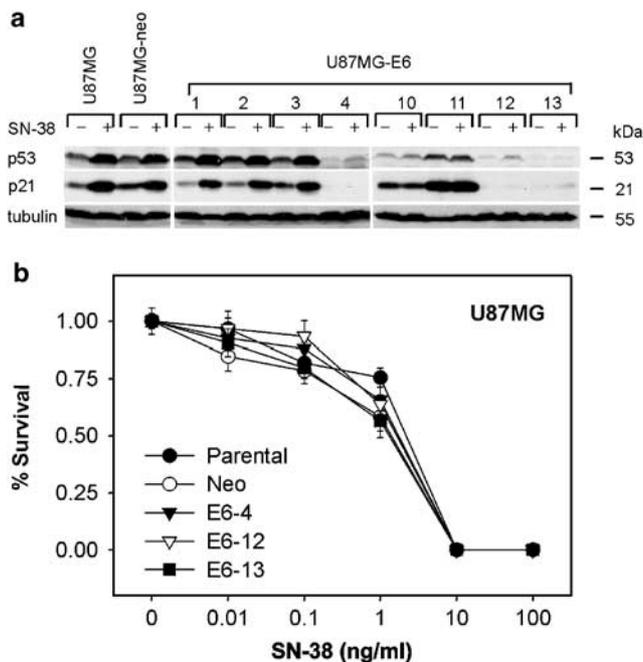
Figure 1 Disruption of p53 by HPV-16 E6 does not alter overall cell sensitivity to SN-38. (a) Western blot analysis confirms the diminished p53 expression and lack of p21 expression in E6 transduced clones 4, 12, 13. −, vehicle treated; +, 5 ng/ml of SN-38 for 24 h. (b) Clonogenic assays demonstrate similar sensitivity to SN-38 in p53-wild-type (parental U87MG and U87MG-neo) and p53-deficient (U87MG-E6, clones 4, 12, 13) glioblastoma cells

diminished in cells with intact p53 (Figure 2b). This is consistent with the previous report that Cdc2 levels are reduced in a p53-dependent manner when DNA is damaged (Azzam *et al.*, 1997; Flatt *et al.*, 2000). No poly(ADP-ribose)polymerase (PARP) cleavage was detected (Figure 2c). Therefore, glioblastoma cells with wild-type p53 (U87MG-neo) underwent cell cycle arrest, but not apoptosis, in response to DNA topoisomerase I inhibition. In line with previous studies, G2/M arrest in cells with intact p53 appeared to involve an initial G2/M arrest triggered by phosphorylation of Cdc2 and prolonged G2/M arrest maintained by further inhibition of Cdc2 activity through upregulation of p21 (Flatt *et al.*, 2000; Magrini *et al.*, 2002).

In striking contrast, SN-38 treatment caused massive apoptosis in p53-deficient U87MG-E6 glioblastoma cells, which was first detected at day 3. By day 10, no viable cells remained. SN-38 caused a decrease in G1 fraction and a transient accumulation of cells in G2/M at 1–2 days (Figure 2d), initiated by increased level of phosphorylated Cdc2 (Figure 2e). However, the G2/M arrest in E6-expressing cells was only transient, probably due to the inability of further inhibition of Cdc2 activity in the absence of p21 (Figure 2f) (Flatt *et al.*, 2000). Noticeably, the total amount of Cdc2 was still abundant at day 7 (Figure 2e), in sharp contrast to the cells with intact p53 where Cdc2 declined to barely detectable level (Figure 2b). By day 3, a prominent sub-G1 peak (cells with a DNA content $<2n$) became apparent (Figure 2d), accompanied by PARP cleavage (Figure 2f). These
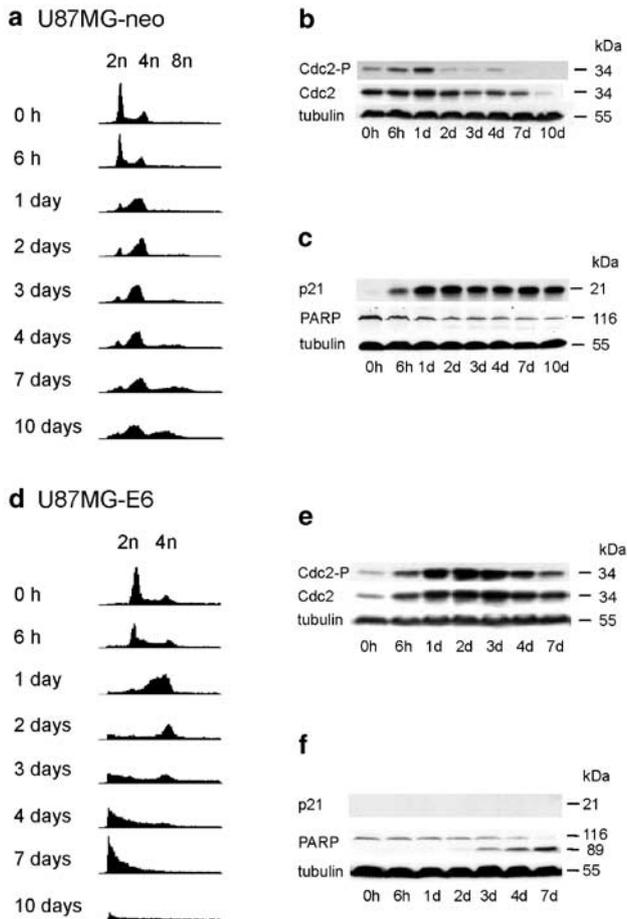
Figure 2 Glioblastoma cells with wild-type p53 (U87MG-neo) undergo cell cycle arrest in response to SN-38 treatment; p53-deficient cells (U87MG-E6) undergo apoptosis. DNA content of U87MG-neo (**a**) and U87MG-E6 (**d**) were analysed by flow cytometry after PI staining. Cells were vehicle treated (0 h) or treated with SN-38 (20 ng/ml), harvested and analysed at the times indicated. Protein lysates were collected from U87MG (**b** and **c**) and U87MG-E6 (**e** and **f**) at the times indicated and analysed for expression of phosphorylated Cdc2 (Cdc2-P), and total Cdc2 (Cdc2), p21 and PARP. β-tubulin level was included to assess protein loading and transfer

results demonstrate that p53-deficient U87MG-E6 glioblastoma cells underwent apoptosis in response to DNA topoisomerase I inhibition.

DNA topoisomerase I inhibition causes cell cycle arrest in patient-derived glioblastoma cells with wild-type p53 and apoptosis in p53-deficient ones

HPV-E6 protein can have effects on cells other than p53 inhibition (Zeng *et al*., 2002). Therefore, we extended our study to the biologically relevant model of 15 low-passage primary glioblastomas in *ex vivo* culture, and two additional established glioblastoma cell lines (T98G and U138MG). We used the Affymetrix GeneChip p53 Assay to analyse the entire coding region of p53 (exons 2–11), and performed automated DNA sequencing on potential mutations suggested by the p53 GeneChip assay (Ahrendt *et al*., 1999; Keshelava *et al*., 2001).

Seven of the 15 primary cell lines were p53 wild type; eight contained p53 mutations (Table 1).

We then analysed the response of this panel of glioblastoma cell lines to DNA topoisomerase I inhibition using flow cytometric and biochemical approaches. Six of the seven p53-wild-type patient cell lines underwent cell cycle arrest in response to SN-38 (Table 1; Figure 3a). SN-38 treatment caused transient Cdc2 phosphorylation corresponding to the onset of cell cycle arrest, followed by prolonged cell cycle arrest associated with increased p21 expression in glioblastoma cells with wild-type p53 (Figure 3b and c). The only p53-intact cell line that did not arrest in response to SN-38 (GM2401) was aneuploid (Table 1), suggesting an alternative disruption in its cell cycle regulation. In contrast, SN-38 treatment caused apoptosis in all eight of the p53-deficient primary cell lines, and two additional p53-deficient established cell lines T98G and U138MG (Table 1; Figure 3d). This was associated with an initial increased Cdc2 phosphorylation (3e), a lack of p21 induction and abundant PARP cleavage (Figure 3f). The total level of Cdc2 was significantly downregulated in p53-intact glioblastoma cells (Figure 3b) but not in p53-mutant ones (Figure 3e). As indicated in Table 1, the percentage of cells with a sub-G1 content of DNA at day 7 in p53-deficient cells was significantly higher than that in p53-wild-type cells ($P < 0.002$; unpaired *t*-test). Thus, in a panel of 19 glioblastoma cell lines tested (Table 1), p53 mutation was highly significantly associated with apoptosis while wild-type p53 was highly significantly associated with cell cycle arrest ($P < 0.0002$; Fisher's exact test).

p16/Ink4a plays an important role in modulating cell cycle arrest in response to chemotherapy, including glioblastoma (Weller *et al*., 1998). Inactivation of p16 is frequent in glioblastomas, mainly due to allelic loss and hypermethylation resulting in the absence of p16 protein, whereas the rate of point mutations is considered to be low (Giani and Finocchiaro, 1994; Jen *et al*., 1994; Merlo *et al*., 1995). Therefore, we analysed p16 protein expression by immunoblotting to determine whether SN-38 response was associated with p16 expression. p16 protein was expressed abundantly in seven of the 19 glioblastoma cell lines (Figure 4), including four cell lines that underwent apoptosis (GM2401, GM97, GM2313 and GM2345) (Table 1). Noticeably, p16 protein was not detectable in two cell lines that arrested in response to SN-38 treatment (U87MG and GM1600; Figure 4 and Table 1). We also did not detect any change in p16 protein level in response to SN-38 treatment (data not shown). These observations suggest that p16 protein expression was neither necessary nor sufficient for cell cycle arrest in response to DNA topoisomerase I inhibition in glioblastoma cells.

SN-38 induced prolonged growth arrest and senescence-like phenotype in p53-wild-type glioblastoma cells

A number of cytotoxic chemotherapies can promote a senescence-like phenotype in vulnerable cancer cells

Table 1 Human glioblastoma cell lines

	Cell lines	p53 status	p16	Response to SN-38	% Sub-G1[a]
ATCC cell lines (N = 4)	U87MG	Wild type	−	Arrest	1.9
	U87MG-E6	Deficient	−	Apoptosis	78
	T98G	Mutant	−	Apoptosis	100
	U138MG	Mutant	−	Apoptosis	40
Primary cell lines (N = 15)	GM2300	Wild type	+	Arrest	0.46
	GM1578	Wild type	+	Arrest	0.38
	GM2455	Wild type	+	Arrest	0.69
	GM1600	Wild type	−	Arrest	1.4
	GM1592	Wild type	±	Arrest	0.46
	GM139	Wild type	±	Arrest	ND
	GM2401[b]	Wild type	+	Apoptosis	100
	GM97	Mutant (exon 5: R175H)	+	Apoptosis	93
	GM2328	Mutant (exon 4: R72P, G76A; exon 5: A161S)	−	Apoptosis	77
	GM2313	Mutant (exon 7: R248W)	+	Apoptosis	66
	GM2217	Mutant (exon 5: Y163S)	ND	Apoptosis	56
	GM2345	Mutant (exon 5: R175H)	+	Apoptosis	100
	GM2493[b]	Mutant (exon 8: R282W)	−	Apoptosis	53
	GM1596[b]	Mutant (exon 4: R72P, G76A; exon 6: Y220H)	−	Apoptosis	100
	GM133[b]	Mutant (exon 5: R175H)	−	Apoptosis	100

[a]Percent of cells with a sub-G1 content of DNA at 7 days after SN-38 treatment; [b]Aneuploid cell line; +, abundant expression; ±, markedly diminished expression; ND, not determined. Association between p53 status and response to SN-38: $P < 0.0002$

(Chang et al., 1999; Roninson, 2003), which results in the expression of senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995). To determine whether the cell cycle arrest seen in glioblastoma cells with wild-type p53 was associated with a senescence-like phenotype, we incubated glioblastoma cells in the presence of 20 ng/ml SN-38 for various time, then fixed and stained for SA-β-gal expression. DNA topoisomerase I inhibition promoted strong SA-β-gal activity in all of the p53-wild-type glioblastoma cells (except for the one aneuploid cell line) (Figure 5). In contrast, none of the p53-deficient glioblastoma cell lines demonstrated evidence of significant SA-β-gal expression, and died through apoptosis (Figure 5).

SN-38-induced arrest/senescence in glioblastoma cells with wild-type p53 is reversible

In order to investigate the reversibility of SN-38-induced growth arrest/senescence-like phenotype in p53-wild-type glioblastoma cells, we treated U87MG-neo and U87MG-E6 cells and low-passage patient-derived glioblastoma cells of varying p53 (and p16) status, and assessed their ability to recover post SN-38 treatment. Cells were treated with 20 ng/ml of SN-38 for 14 days, then were trypsinized and recultured in drug-free medium. The plating efficiency of glioblastoma cells of different p53 status are very similar, ranging from 0.6 to 0.8. As shown in Figure 6a, the viable cells before starting drug treatment were very similar among different cell lines. The cells at the end of the 14-day treatment were fairly enlarged and flattened for p53-wild-type U87MG cells. After being trypsinized and transferred to 100-mm dishes, cells stayed enlarged and flattened (data not shown). Early cell division was noted on enlarged and growth arrested p53-wild-type cells beginning 10 days post-SN-38 treatment, and became

more abundant by day 20 (Figure 6a). Cell colonies were clearly detectable by 20 days post drug withdrawal in glioblastoma cells with wild-type p53 (U87MG-neo, GM1578, GM1600, GM2455) (Figure 6b). In contrast, the p53-deficient cells did not recover their ability to proliferate. The few cells that remained after SN-38 treatment died in subsequent culture in drug-free medium (Figure 6a), and no colony formation was detected (Figure 6b). These data indicate that glioblastoma cells with wild-type p53 can reverse their arrested/senescence-like phenotype and reproliferate after removing the drug.

Discussion

In this paper, we used isogenic U87MG cells varying in p53 status, established glioblastoma cell lines of known p53 status and a large panel of low-passage glioblastoma patient ex vivo cultures, to demonstrate that p53 disruption sensitizes glioblastoma cells to DNA topoisomerase I inhibitor-mediated apoptosis ($P < 0.0002$). We have demonstrated that in the presence of intact p53, diploid glioblastoma cells arrest and develop a senescence-like phenotype. We have also shown that the p53-intact cells retain their ability to reproliferate following withdrawal of SN-38. In contrast, SN-38 effectively induces apoptosis in p53-deficient cells. These findings significantly extend the recent work in HCT116 colon cancer cells (Han et al., 2002; Magrini et al., 2002; te Poele et al., 2002; Roninson, 2003), and demonstrate their validity in patient tumor cell isolates. As p53 is mutated in nearly one-third to half of glioblastoma patients (Kleihues and Ohgaki, 1999; Kleihues et al., 2002), this may have direct clinical importance. Our results suggest the possibility that this molecular/clinical
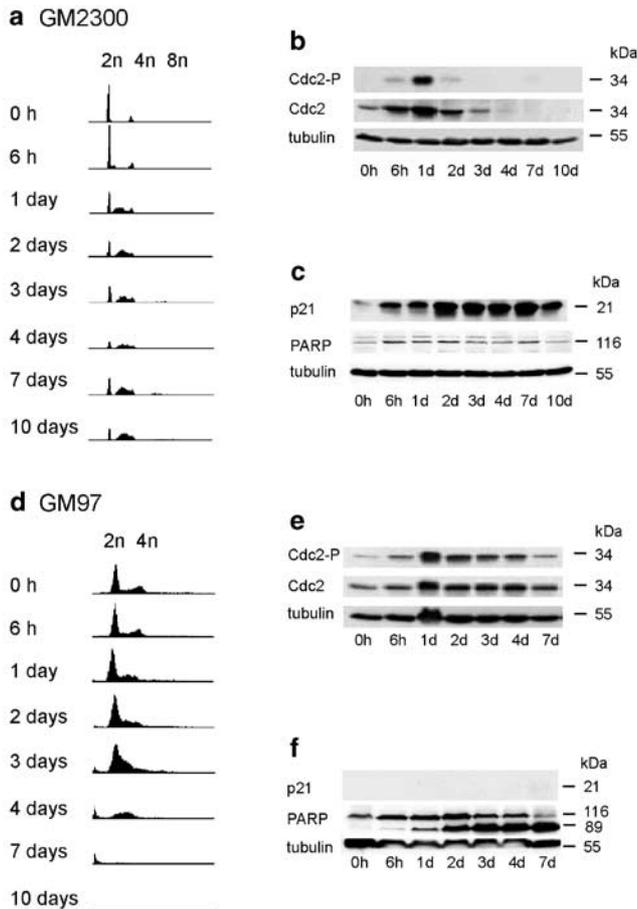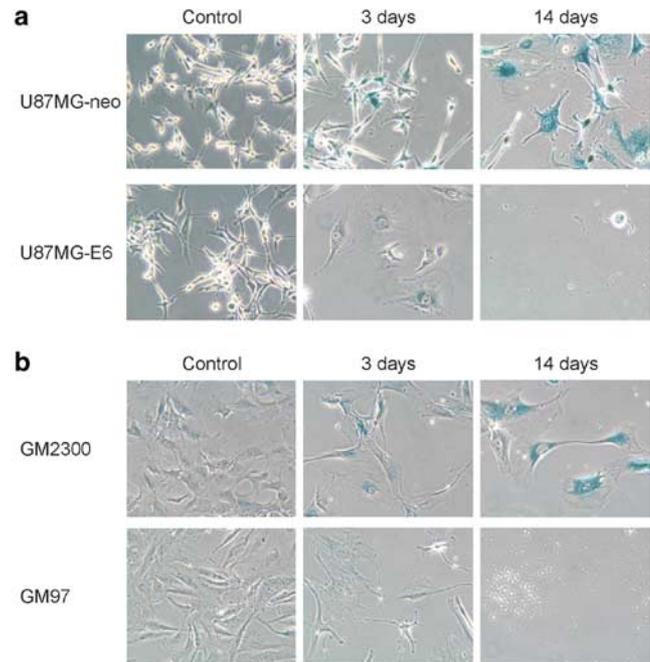
a GM2300

Figure 3 Patient-derived glioblastoma cells with wild-type p53 (GM2300) arrest in response to SN-38 treatment; p53-deficient ones (GM97) undergo apoptosis. GM2300 (**a**) and GM97 (**d**) cells were vehicle treated (0 h) or treated with SN-38 (20 ng/ml), collected at the times indicated and stained with PI for DNA content analysis. Protein lysates were collected from GM2300 (**b** and **c**) and GM97 (**e** and **f**) at the times indicated and analysed for expression level of phosphorylated Cdc2 (Cdc2-P), total Cdc2 (Cdc2), p21, PARP and β-tubulin as indicated
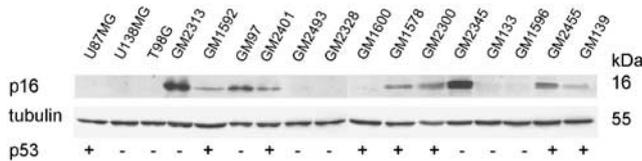

Figure 4 Western blot analysis showing p16 protein expression in glioblastoma cells. The total cell lysates were collected from 17 glioblastoma cell lines and subjected to Western blotting using antibodies to p16/INK4a and β-tubulin as described in Materials and methods. p53 status is shown below the blot as wild-type (+) or deficient (−)

difference can potentially be used to direct DNA topoisomerase I inhibitor therapy to this subset of patients.

The extent of the DNA damage can also determine whether a cancer cell arrests or undergoes apoptosis in response to a genotoxic agent. At low levels of DNA damage caused by the inhibition of DNA topoisomerase I, intact p53 protects HCT116 human colon cancer cells

Figure 5 Glioblastoma cells with wild-type p53 exhibit markers of senescence in response to SN-38 treatment. SA-β-gal expression is detected in p53-wild-type U87MG-neo (**a**, upper panel) and GM2300 (**b**, upper panel), but not in p53-deficient U87MG-E6 (**a**, lower panel) and GM97 (**b**, lower panel) after SN-38 treatment (20 ng/ml). Pictures were taken at the times indicated after SN-38 treatment. All the photographs were taken at $\times 100$ magnification
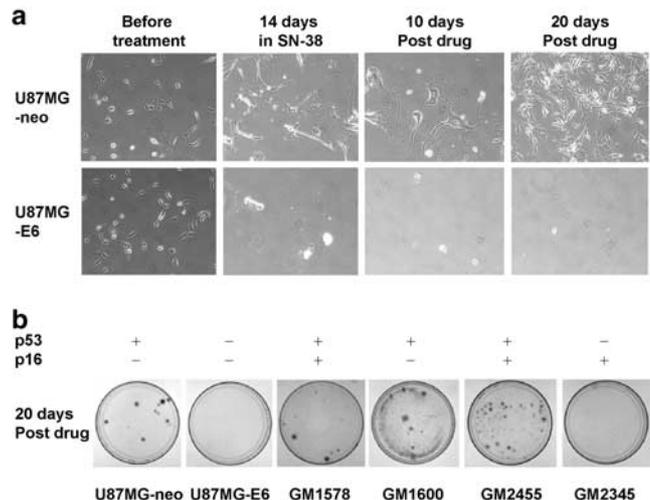
Figure 6 SN-38-induced senescence-like phenotype in glioblastoma cells with wild-type p53 is reversible. Cells were treated with 20 ng/ml of SN-38 for 14 days, then trypsinized and recultured in drug-free medium. (**a**) Glioblastoma cells with wild-type p53 (U87MG-neo) recovered their ability to proliferate in drug-free medium; p53-deficient ones (U87MG-E6) did not recover their ability to proliferate. Pictures were taken at $\times 100$ magnification. (**b**) Glioblastoma cells with wild-type p53 (U87MG-neo, GM1578, GM1600 and GM2455) retained their ability to form colonies following drug withdrawal; p53-deficient ones (U87MG-E6 and GM2345) did not. Cells were fixed and stained in 1% crystal violet in methanol. p53 status is shown as wild-type (+) or deficient (−); p16 status is shown as abundant expression (+) or no expression (−)

from apoptosis whereas at high levels of DNA damage, apoptosis occurs in both p53-wild-type and p53-deficient cells (Han et al., 2002). Consistent with this, we found that at high levels of SN-38 (200 ng/ml, a level that cannot be safely achieved in patients), p53-wild-type glioblastoma cells also begin to undergo apoptosis, but not to the extent seen in p53-deficient cells (data not shown). Thus, p53-independent pathways may have an impact on how the DNA damage is processed by cancer cells, particularly in response to heavy levels of DNA damage. This may account for some of the variability seen between cancer cell types.

Even though it is clear that p53 is an important activator in the apoptotic response to DNA damage (Amundson et al., 1998; Vousden, 2000), there is clear evidence for p53-independent mechanism of apoptosis. For example, c-Abl can contribute to apoptosis via the p53 homolog p73 following DNA damage (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999; Truong et al., 2003), and activation of Jun kinases (JNKs) (Liu et al., 1996; Vivo et al., 2003), Fas pathway (Shao et al., 2001), and increased level of intracellular ceramide (Yang and Duerksen-Hughes, 2001) have all been implicated in modulating apoptosis in a p53-independent fashion. Further studies will be needed to elucidate the mechanisms involved in the apoptotic response to DNA damage in glioblastoma cells with mutant p53.

Senescence-like changes caused by the inhibition of topoisomerase I in HCT116 human colon cancer cells with intact p53 (Han et al., 2002; Magrini et al., 2002; te Poele et al., 2002; Roninson, 2003), likely occur via p21, its transcriptional target. Although we did not specifically test this requirement for p21, our observation that p53-deficient cells failed to upregulate p21 expression in response to SN-38 and underwent apoptosis is in support of this model. The p53/p21 pathway is not the only one involved in chemotherapy-induced senescence. p16/Ink4a also plays a critical role in the senescence response of cancer cells to cytotoxic chemotherapy, including in glioblastoma (Weller et al., 1998; Roninson, 2003), and recent data suggest that p16 may play a role in maintaining senescence following withdrawal of chemotherapy (te Poele et al., 2002; Roninson, 2003). However, we observed that U87MG and GM1600 cells lacking p16 expression developed a senescent-like phenotype with prolonged cell cycle arrest in response to SN-38 (Figures 5 and 6). Further, GM 1578 and GM2455 reproliferated after drug withdrawal despite abundant p16 protein expression (Figure 6). Therefore, p16 protein expression itself was not required to initiate senescence and not sufficient to maintain senescence-like growth arrest in glioblastoma cells treated with SN-38.

The regrowth in p53-wild-type tumor cells after removing the drug does not appear to be due to a few cells that never stopped proliferating and then eventually outgrew the arrested cells, because the early cell division was observed on those enlarged growth-arrested cells (Figure 6a). However, we do not have data to confirm that 100% of these G2/M arrested cells express the senescence marker, so we cannot exclude the possibility that the cells that proliferate after drug withdrawal may derive from a small subpopulation of cells that do not express the SA-β-gal.

In summary, we have shown that p53 disruption sensitizes glioblastoma cells to apoptosis while intact p53 promotes a senescence-like phenotype in response to inhibition of topoisomerase I. Although a myriad of other factors may affect response to CPT-11, such as conversion of drug to active metabolite, in the future, it will be important to determine whether glioblastoma patients whose tumors are p53-wild-type demonstrate worse clinical response to CPT-11 therapy (as well as to a host of new DNA topoisomerase I inhibitors).

Materials and methods

Cell lines and reagents

Human glioblastoma cell lines U87MG (p53--wild-type), T98G (p53-mutant) and U138MG (p53-mutant) were purchased from ATCC (Rockville, MD, USA) and maintained in DMEM (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 2 mM glutamine (Omega Scientific, Tarzana, CA, USA) and 10% FBS (Omega). In total, 15 low-passage primary glioblastoma cultures were established from resections from glioblastoma patients. Briefly, the tumors were diagnosed intraoperatively by a board-certified neuropathologist (PSM), finely minced with scalpels and resuspended in complete Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 20% FBS (Omega), 2 mM glutamine, 5 μg/ml each of insulin and transferrin, 5 ng/ml selenium (ITS Culture Supplement; Collaborative Biosciences, Bedford, MA, USA). The glial nature of the cell lines was confirmed by RT–PCR analysis of glial fibrillary acidic protein, nestin and vimentin. All of the cell lines used here were maintained at 37°C in a humidified 5% CO$_2$ incubator. Primary cell lines used in this study were under passage 15. SN-38 was kindly provided by Pharmacia & Upjohn (Kalamazoo, MI, USA). A measusre of 1 mg/ml SN-38 stock solution was prepared in DMSO and stored at −20°C. All other reagents were from Sigma (St Louis, MO, USA) unless otherwise noted in the text.

Retroviral infection

The PA317 packaging cells carrying the control retroviral vector (LXSN), or HPV type 16-E6 gene (LXSN16E6) were kindly provided by Dr C Patrick Reynolds at Children's Hospital Los Angeles. Viral supernatants were collected from packaging cell lines, filtered through a 0.45-μM filter (Millipore Corp., Bedford, MA, USA) and added to glioblastoma cells with polybrene at a final concentration of 8 μg/ml. After a 4-h incubation, fresh medium was replenished. The infected cells were incubated for 1–2 days and then selected in G418 containing medium (0.4 mg/ml; Omega Scientific, Tarzana, CA, USA) in limiting dilution. Multiple clonal cell lines were obtained by G418 selection and screened for abrogated p53 function by Western blot. U87MG-E6 clone 4 was used in the following experiments whenever encountered; clones 12 and 13 showed essentially the same results.

Clonogenic assays

Cells were seeded (300–350 cells/well) into six-well plates without G418. After 24 h, SN-38 was added to each well at various final concentrations, as indicated in three replicate wells per condition. Control wells received 0.01% DMSO, an

amount equal to the highest concentration of SN-38 treated wells. The plates were incubated for additional 7 days without changing medium. Colonies were then fixed and stained in 1% crystal violet in methanol, and counted only if they numbered more than 50 cells. Data were analysed and graphed using SigmaPlot 4.0 (Jandell Scientific, San Rafael, CA, USA).

Cell cycle analysis

Attached cells were trypsinized, combined with floating cells, and DNA content was analysed by staining with $20 \mu g/ml$ propidium iodide (PI), 0.2 mg/ml RNase A, and 0.5 mM EDTA as described (Larsen, 1994). DNA content analysis was performed with at least 13 000 events captured using a FACScan flow cytometer (Becton Dickinson, Mansfield, MS, USA). Data were analysed using CellQuest software (Becton Dickinson).

GeneChip p53 array and automated DNA sequencing

We used the Affymetrix GeneChip p53 Assay to analyse the entire coding region of p53 (exons 2–11) of all cell lines, and then performed automated DNA sequencing on the potential mutations suggested by the p53 GeneChip assay (Ahrendt et al., 1999; Keshelava et al., 2001).

Western blot analysis

Whole-cell lysates were prepared by standard procedures (Santa Cruz Biotechnology Research Application Protocol). Protein concentration was determined using BCA™ Protein Assay (Pierce Chemical Company, Rockford, IL, USA). In total, $40 \mu g$ of proteins were separated by electrophoresis through 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked overnight in TBS-Tween containing 5% non-fat dried milk, probed for 1 h with primary antibody (see listed below) and for 1 h with secondary antibody (Cell Signaling Technologies, Beverly, MA, USA), and visualized by ECL detection system (Amersham). Primary antibodies to the following antigens were used: p53 (P5813, Sigma, St Louis, MO, USA), p21 (sc-817, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho Cdc2 (Tyr 15, #9111, Cell Signaling Technologies, Beverly, MA, USA); PARP (D214, #9546, Cell Signaling Technologies); p16 (G175-405, BD Biosciences Clontech, CA, USA) and β-tubulin (T4026, Sigma, St Louis, MO, USA).

SA-β-Gal staining

Cells were stained for β-galactosidase (β-Gal) activity using X-Gal Staining Assay Kit (Gene Therapy Systems Inc., San Diego, CA, USA), according to the manufacturer's instructions. Briefly, cells were washed twice in PBS and fixed in a buffer containing 2% formaldehyde/0.5% glutaraldehyde in PBS for 10 min at room temperature. SA-β-gal staining was carried out in X-Gal staining solution (pH 6.0) containing 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) per ml (stock $= 25$ mg/ml in dimethylformamide), 5 mM potassium ferricyanide and 2 mM $MgCl_2$ in PBS at 37°C. Incubation time was typically 16 h. Cells were rinsed in PBS and fixed in 10% formaldehyde for 10 min.

Regrowth assay

Cells were seeded into six-well plates at a density of $0.03 \ E^6$ cells/well. After 24 h, the cells received 20 ng/ml of SN-38. Medium containing fresh SN-38 was replenished every 2 days. After 14 days of drug treatment, the cells were trypsinized and recultured in 100-mm dishes in drug-free medium. The cells were photographed at relevant time points as indicated. At 20 days postdrug withdrawal, the cells were fixed and stained with 1% crystal violet in methanol.

References

Agami R, Blandino G, Oren M and Shaul Y. (1999). *Nature*, **399**, 809–813.

Ahrendt SA, Halachmi S, Chow JT, Wu L, Halachmi N, Yang SC, Wehage S, Jen J and Sidransky D. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 7382–7387.

Amundson SA, Myers TG and Fornace Jr AJ. (1998). *Oncogene*, **17**, 3287–3299.

Azzam EI, de Toledo SM, Pykett MJ, Nagasawa H and Little JB. (1997). *Cell Growth Differ.*, **8**, 1161–1169.

Bachoo RM, Maher EA, Ligon KL, Sharpless NE, Chan SS, You MJ, Tang Y, DeFrances J, Stover E, Weissleder R, Rowitch DH, Louis DN and DePinho RA. (2002). *Cancer Cell*, **1**, 269–277.

Brown JM and Wouters BG. (1999). *Cancer Res.*, **59**, 1391–1399.

Chang BD, Broude EV, Dokmanovic M, Zhu H, Ruth A, Xuan Y, Kandel ES, Lausch E, Christov K and Roninson IB. (1999). *Cancer Res.*, **59**, 3761–3767.

Choe G, Horvath S, Cloughesy TF, Crosby K, Seligson D, Palotie A, Inge L, Smith BL, Sawyers CL and Mischel PS. (2003). *Cancer Res.*, **63**, 2742–2746.

Choe G, Park JK, Jouben-Steele L, Kremen TJ, Liau LM, Vinters HV, Cloughesy TF and Mischel PS. (2002). *Clin. Cancer Res.*, **8**, 2894–2901.

Coggins CA, Elion GB, Houghton PJ, Hare CB, Keir S, Colvin OM, Bigner DD and Friedman HS. (1998). *Cancer Chemother. Pharmacol.*, **41**, 485–490.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I and Pereira-Smith O. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 9363–9367.

Druker BJ. (2002). *Cancer Cell*, **1**, 31–36.

Flatt PM, Tang LJ, Scatena CD, Szak ST and Pietenpol JA. (2000). *Mol. Cell. Biol.*, **20**, 4210–4223.

Friedman HS, Petros WP, Friedman AH, Schaaf LJ, Kerby T, Lawyer J, Parry M, Houghton PJ, Lovell S, Rasheed K,

Cloughsey T, Stewart ES, Colvin OM, Provenzale JM, McLendon RE, Bigner DD, Cokgor I, Haglund M, Rich J, Ashley D, Malczyn J, Elfring GL and Miller LL. (1999). *J. Clin. Oncol.*, **17,** 1516–1525.

Giani C and Finocchiaro G. (1994). *Cancer Res.*, **54,** 6338–6339.

Gong JG, Costanzo A, Yang HQ, Melino G, Kaelin Jr WG, Levrero M and Wang JY. (1999). *Nature*, **399,** 806–809.

Han Z, Wei W, Dunaway S, Darnowski JW, Calabresi P, Sedivy J, Hendrickson EA, Balan KV, Pantazis P and Wyche JH. (2002). *J. Biol. Chem.*, **277,** 17154–17160.

Hare CB, Elion GB, Houghton PJ, Houghton JA, Keir S, Marcelli SL, Bigner DD and Friedman HS. (1997). *Cancer Chemother. Pharmacol.*, **39,** 187–191.

Ino Y, Betensky RA, Zlatescu MC, Sasaki H, Macdonald DR, Stemmer-Rachamimov AO, Ramsay DA, Cairncross JG and Louis DN. (2001). *Clin. Cancer Res.*, **7,** 839–845.

Jen J, Harper JW, Bigner SH, Bigner DD, Papadopoulos N, Markowitz S, Willson JK, Kinzler KW and Vogelstein B. (1994). *Cancer Res.*, **54,** 6353–6358.

Keshelava N, Zuo JJ, Chen P, Waidyaratne SN, Luna MC, Gomer CJ, Triche TJ and Reynolds CP. (2001). *Cancer Res.*, **61,** 6185–6193.

Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC and Cavenee WK. (2002). *J. Neuropathol. Exp. Neurol.*, **61,** 215–225.

Kleihues P and Ohgaki H. (1999). *Neuro-oncol.*, **1,** 44–51.

Larsen JK. (1994). *Methods Cell Biol.*, **41,** 377–388.

Liu LF, Desai SD, Li TK, Mao Y, Sun M and Sim SP. (2000). *Ann. NY Acad. Sci.*, **922,** 1–10.

Liu ZG, Baskaran R, Lea-Chou ET, Wood LD, Chen Y, Karin M and Wang JY. (1996). *Nature*, **384,** 273–276.

Magrini R, Bhonde MR, Hanski ML, Notter M, Scherubl H, Boland CR, Zeitz M and Hanski C. (2002). *Int. J. Cancer*, **101,** 23–31.

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB and Sidransky D. (1995). *Nat. Med.*, **1,** 686–692.

Mischel PS and Cloughesy TF. (2003). *Brain Pathol.*, **13,** 52–61.

Mischel PS, Shai R, Shi T, Horvath S, Lu KV, Choe G, Seligson D, Kremen TJ, Palotie A, Liau LM, Cloughesy TF and Nelson SF. (2003). *Oncogene*, **22,** 2361–2373.

Negoro S, Fukuoka M, Masuda N, Takada M, Kusunoki Y, Matsui K, Takifuji N, Kudoh S, Niitani H and Taguchi T. (1991). *J. Natl. Cancer Inst.*, **83,** 1164–1168.

Nieves-Neira W and Pommier Y. (1999). *Int. J. Cancer*, **82,** 396–404.

Roninson IB. (2003). *Cancer Res.*, **63,** 2705–2715.

Sawyers CL. (2002). *Curr. Opin. Genet. Dev.*, **12,** 111–115.

Scheffner M, Huibregtse JM, Vierstra RD and Howley PM. (1993). *Cell*, **75,** 495–505.

Shai R, Shi T, Kremen TJ, Horvath S, Liau LM, Cloughesy TF, Mischel PS and Nelson SF. (2003). Gene expression profiling identifies molecular subtypes of gliomas. *Oncogene*, **22,** 4918–4923.

Shao RG, Cao CX, Nieves-Neira W, Dimanche-Boitrel MT, Solary E and Pommier Y. (2001). *Oncogene*, **20,** 1852–1859.

Shawver LK, Slamon D and Ullrich A. (2002). *Cancer Cell*, **1,** 117–123.

Slichenmyer WJ, Nelson WG, Slebos RJ and Kastan MB. (1993). *Cancer Res.*, **53,** 4164–4168.

te Poele RH, Okorokov AL, Jardine L, Cummings J and Joel SP. (2002). *Cancer Res.*, **62,** 1876–1883.

Truong T, Sun G, Doorly M, Wang JY and Schwartz MA. (2003). *Proc. Natl. Acad Sci. USA*, **100,** 10281–10286.

Vivo C, Liu W and Broaddus VC. (2003). *J. Biol. Chem.*, **278,** 25461–25467.

Vousden KH. (2000). *Cell*, **103,** 691–694.

Watanabe K, Tachibana O, Sata K, Yonekawa Y, Kleihues P and Ohgaki H. (1996). *Brain Pathol.*, **6,** 217–223.

Weller M, Rieger J, Grimmel C, Van Meir EG, De Tribolet N, Krajewski S, Reed JC, von Deimling A and Dichgans J. (1998). *Int. J. Cancer*, **79,** 640–644.

Yang J and Duerksen-Hughes PJ. (2001). *J. Biol. Chem.*, **276,** 27129–27135.

Yuan ZM, Shioya H, Ishiko T, Sun X, Gu J, Huang YY, Lu H, Kharbanda S, Weichselbaum R and Kufe D. (1999). *Nature*, **399,** 814–817.

Zeng M, Kumar A, Meng G, Gao Q, Dimri G, Wazer D, Band H and Band V. (2002). *J. Biol. Chem.*, **277,** 45611–45618.