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Short communication

Glioma cell sensitivity to topotecan: the role of p53 and topotecan-induced DNA damage

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Abstract

Topotecan is a topoisomerase I inhibitor which is currently evaluated as an adjuvant agent for malignant glioma. Here, we analysed the effects of topotecan on 12 human malignant glioma cell lines in vitro. All cell lines expressed topoisomerase I mRNA. High p53 protein levels, but not genetic or functional p53 status, were associated with increased topotecan-induced DNA/topoisomerase I complex formation. Neither functional p53 status, nor p53 protein levels, nor complex formation predicted topotecan-induced growth inhibition. We thus confirm a possible role for p53 protein in modulating topoisomerase I activity but conclude that the major molecular determinants of topotecan sensitivity in glioma cells await identification.

Keywords: Topotecan; Topoisomerase; Glioma; p53; Chemotherapy

1. Introduction

The median survival for human glioblastoma patients receiving multimodality treatment including radiochemotherapy is in the range of 12 months. One of the candidate drugs for the radiochemotherapy, or adjuvant chemotherapy after radiotherapy, for glioblastoma is topotecan. Topotecan is an inhibitor of topoisomerase I that has shown modest activity against multiple solid tumors (Dancey and Eisenhauer, 1996), including minor responses in recurrent glioma (Macdonald et al., 1996; Friedman et al., 1998). Novel topoisomerase I inhibitors for malignant glioma are currently being developed (Pollack et al., 1999).

Topotecan induces DNA lesions via the formation of stable cleavable complexes of topoisomerase I and DNA in vitro and in vivo (Subramanian et al., 1995). The tumor suppressor gene product, p53, controls the response to genotoxic stress in mammalian cells. Several lines of evidence indicate that p53 modulates topoisomerase I function. Thus, p53 forms complexes with topoisomerase I (Gobert et al., 1996, 1999), and both wild-type and mutant p53 may activate topoisomerase I (Albor et al., 1998; Mao et al., 2000).

Therefore, in the present study, the degree of topotecan-induced complex formation and the p53 status were examined as possible predictors of topotecan sensitivity in a panel of 12 human malignant glioma cell lines.

2. Materials and methods

2.1. Materials and cell lines

Topotecan was kindly provided by SmithKline Beecham (Munich, Germany). The human malignant glioma cell lines, kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland), were characterized extensively in previous studies (Weller et al., 1998; Ishii et al., 1999). D247MG was derived from a gliosarcoma, LN-319 from an anaplastic astrocytoma, all other cell lines were derived from glioblastomas. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal calf serum, 2 mM glutamin and penicillin (100 IU/ml)/streptomycin (100 μg/ml) (Weller et al., 1998).

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Topoisomerase I mRNA expression was assessed by RT-PCR. Total RNA was isolated using the RNeasy Mini

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Kit (Quiagen, Hilden, Germany). cDNA was prepared using random hexamer primers and Superscript II (Gibco, Gaithersburg, MD). Topoisomerase I-specific primers were obtained from MWG Biotech (Ebersberg, Germany). The primer sequences were as follows: topoisomerase I sense (nucleotides 210–229), 5′-ACATGAGTGGGGACCAC-CTC-3′, topoisomerase I antisense (nucleotides 881–898), 5′-GTGGGGCAAATACTGGACC-3′, yielding a PCR product of 688 base-pairs; human β-actin sense (nucleotides 26–46), 5′-TGTTTGAGACCTTCAACACCC-3′, human β-actin antisense (nucleotides 896–877), 5′-AGCA-CTGTGTTGGCGTACAG-3′, yielding a PCR product of 871 base-pairs. The PCR conditions for topoisomerase I were: 5 min 94°C; 35 × 30 s/94°C, 30 s/48°C, 60 s/72°C; 7 min 72°C. The annealing temperature was 55°C, and 30 cycles were run for the β-actin control PCR. The PCR products were run on 2% agarose gels and visualized by ethidium bromide staining.

2.3. p53 reporter assay

The cells were transfected with the PathDetect® p53 *cis*-reporter gene plasmid (#219092, Stratagene, La Jolla, CA), which encodes firefly luciferase in a p53-dependent manner. For each transfection, 0.2 μg DNA and 0.6 μl FuGene (Roche, Mannheim, Germany) were used. The cells were either co-transfected with 0.02 μg of the pFC–p53 positive control plasmid or with the pcDNA-3 plasmid. At 24 h after transfection, the cells were washed with phosphate-buffered saline (PBS) and lysed using 40 μl/well of Reporter Lysis Buffer (Promega, Madison, WI). The lysate was transferred to a LumiNunc™ plate (Nunc, Roskilde, Denmark), 100 μl of Luciferase assay substrate (Promega) were added automatically, and luminescence was measured in a LumimatPlus (EG&G Berthold, Pforzheim, Germany). Background was subtracted from all values and the remaining luciferase activity was expressed in percentage of the respective positive control (wild-type p53 transfection). Thus, the results become independent from transfection efficiency, which varies among the different cell lines. All experiments were performed in triplicate and repeated twice.

2.4. Immunoblot analysis

The levels of p53, p21 or β-actin were examined by immunoblot analysis as described (Weller et al., 1998), in untreated cells or irradiated cells (4 h, 6 Gy), using irradiation as a defined genotoxic stress stimulus. To quantify p53 levels, p53/β-actin ratios were determined by densitometry. These data allowed the classification of the cell lines into two groups of cell lines with either low or high p53 protein levels (see Table 1).

2.5. Determination of cleavable DNA topoisomerase I complexes

Cleavable DNA topoisomerase I complex formation was assessed as previously described (Winter and Weller, 1998). Briefly, the cells were DNA-labeled with 2 μCi/ml [methyl-^{3}H]-thymidine (specific activity: 20–40 Ci/mmol) overnight, adjusted to 100,000 cpm/ml, incubated for further 24 h, treated with topotecan for 30 min, washed with PBS and lysed with 1 ml prewarmed (65°C) lysis solution (1.25% SDS, 5 mM EDTA, pH 8.0, herring sperm DNA, 0.4 mg/ml). After shearing of chromosomal DNA by repeated passing through a 22-gauge needle, the lysates were transferred to a reaction tube containing 250 μl 325 mM KCl, vortexed vigorously for 10 s, incubated for 10 min on ice, and centrifuged for 10 min at 13,000 rpm at

Table 1
p53 status, topotecan-induced cleavable complex formation, and topotecan sensitivity of human malignant glioma cell lines

	Functional p53 status[a]	p53 protein levels[b]	Complex formation (*x*-fold increase)[c]	EC_{50} for growth inhibition [μM][d]
U87MG	wild-type (36%)	low	8	0.08
D247MG	wild-type (77%)	low	6	0.75
LN-229	wild-type (100%)	high	no data	0.06
A172	wild-type (25%)	low	10	0.06
U138MG	wild-type (9%)	high	15	3
Median:			9.0	0.08
LN-18	mutant (1%)	high	14	0.04
LN-428	mutant (0%)	high	6	0.25
T98G	mutant (0%)	high	15	> 4
LN-319	mutant (1%)	high	16	> 4
U251MG	mutant (0%)	high	19	0.025
U373MG	mutant (0%)	high	25	0.02
LN-308	deleted (0%)	absent	12	> 4
Median:			15.0	n.a.

[a] p53 activity in the reporter assay given in percentages of p53 wild-type control.
[b] Determined by immunoblot analysis.
[c] For details, see Materials and methods.
[d] Data derived from concentration response curves such as shown in Fig. 1.

4°C. The pellets were resuspended in 1 ml washing solution (10 mM Tris–HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, 0.1 mg/ml herring sperm DNA) and kept at 65°C for 10 min. The suspensions were cooled on ice for 10 min and recentrifuged. The pellets were washed and resuspended in 200 μl H_2O (65°C). Radioactivity was measured in a Wallac Liquid Scintillation Counter. Cleavable complex formation was expressed as *X*-fold increase over baseline at a fixed concentration of topotecan (10 μM).

2.6. Viability assay

Growth inhibition studies were performed in 96-well plates. The cells were seeded at 5×10^3 cells per well, allowed to attach for 24 h, exposed to topotecan for 120 h, and survival/growth was assessed by crystal violet staining (Weller et al., 1998). This paradigm was chosen in analogy to the clinical application where long-term low level exposure to topotecan, e.g., by daily infusions for days, is considered to be the most effective type of application. EC_{50} values were derived from graphic extrapolation.

2.7. Statistical analysis

The EC_{50} values for topotecan-induced growth inhibition and the levels of cleavable DNA/topoisomerase I complex formation were correlated by Pearson–Product moment correlation. To assess differences of the EC_{50} values and the complex formation data in wild-type vs. mutant p53 status or cell lines with low vs. high p53 protein levels, the Mann–Whitney *U*-test was used because the EC_{50} values within each group cannot be assumed to follow a normal distribution. The results for the subgroups of cell lines are shown as median [25% percentile–75% percentile].

3. Results

3.1. p53 status, topotecan-induced DNA / topoisomerase I complex formation and topotecan-induced inhibition of glioma cell proliferation

The functional p53 status of the 12 cell lines was examined by assessing protein accumulation by immunoblot in response to irradiation (6 Gy, 4 h) and by p53 reporter assay. For the statistical analysis, the basal levels

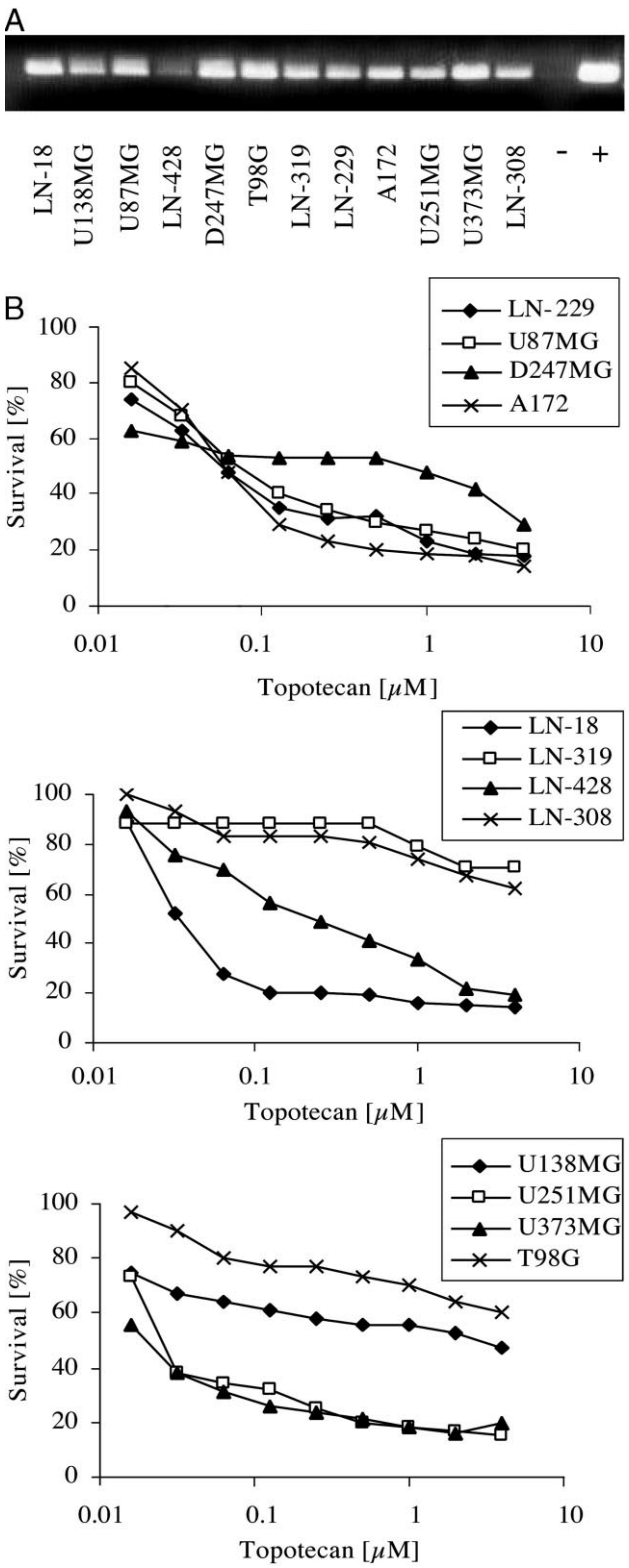

Fig. 1. Topoisomerase I mRNA expression and topotecan sensitivity in human malignant glioma cell lines. (A) Total RNA was reverse-transcribed and examined for topoisomerase I cDNA by PCR. Equal loading was ascertained by β-actin amplification (data not shown). The lane labeled − contains water instead of RNA, the lane labeled + shows amplification from a human topoisomerase I cDNA fragment. (B) The cells were treated with topotecan for 120 h. Survival/growth was asssessed by crystal violet assay. Data are mean values of three separate experiments, where each concentration of drug was assayed in five wells of a 96-well plate, and are expressed as mean percentages of optical density values compared with vehicle-treated cells (range of standard deviations: 4–15%).

of p53 protein were graded as high and low according to densitometric values (Table 1). LN-308 cells do not express p53 protein irrespective of irradiation. Five cell lines, U138MG, U87MG, LN-229, A172 and D247MG, showed p53 protein accumulation in response to irradiation whereas the other seven cell lines did not (data not shown). The same cell lines were also positive in the p53 reporter assay, indicated in brackets in the Table 1.

All human malignant glioma cell lines expressed topoisomerase I mRNA as assessed qualitatively by RT-PCR (Fig. 1A). There was significant variation in the induction of cleavable DNA/topoisomerase I complexes in response to topotecan (Table 1), ranging from a 5- to 25-fold increase from baseline. LN-229 cells did not incorporate sufficient label to assess complex formation.

Concentration response curves for topotecan-induced growth inhibition were obtained for all 12 cell lines (Fig. 1B). These curves were used to obtain EC_{50} values as summarized in the Table 1. These EC_{50} values ranged from 0.02 to more than 4 μM, spanning more than 2 logs. Four of the cell lines (U138MG, T98G, LN-319, LN-308) were rather resistant to topotecan.

3.2. Statistical considerations

As expected, cell lines retaining wild-type p53 function exhibited low baseline p53 levels whereas cell lines harbouring p53 mutations accumulate p53 protein as a result of the prolonged half-life of such mutants. LN-308 lack p53 protein altogether (Table 1). The difference in topotecan-induced cleavable DNA/topoisomerase I complex formation between p53 wild-type and p53 mutant cell lines failed to reach statistical significance ($P = 0.13$). In contrast, cell lines with high p53 protein levels, irrespective of function, formed more complexes than did cell lines with low p53 protein levels (15.2 [14.0–19.2] vs. 9.0 [6.8–10.8], $P = 0.04$). Statistical analysis disclosed that neither functional p53 status, nor p53 protein levels, nor complex formation predicted topotecan-induced growth inhibition.

4. Discussion

Topotecan is a promising candidate for the adjuvant chemotherapy of malignant gliomas, specifically when used as a radiosensitizing agent (Lamond et al., 1996). The molecular mechanisms determining topotecan sensitivity in glioma cells have remained obscure. However, clarification of the nature of such predictors of topotecan sensitivity might help to prospectively identify glioma patients that will respond to adjuvant topotecan chemotherapy.

In the present study, we determined the spectrum of topotecan activity in a panel of 12 human malignant glioma cell lines and examined whether functional p53 status, p53 protein levels or the extent of cleavable DNA/topoisomerase I complex formation after exposure to topotecan predict glioma cell sensitivity to topotecan.

In our previous study (Winter and Weller, 1998), we had noted that the p53 response pathway defined by p53 and p21 protein accumulation was triggered by topotecan in LN-229 cells that retain p53 wild-type function, but not in LN-18, T98G or LN-308 cells, which are mutant for p53. To elucidate the role of the p53 response in determining topotecan sensitivity in LN-229 cells, we introduced a dominant-negative p53 mutant into these cells. Interestingly, forced expression of dominant-negative p53 sensitized LN-229 cells to topotecan in that the EC_{50} was shifted 5-fold. Conversely, topotecan sensitivity of LN-18 cells, which are mutant for p53, was unaffected by expressing the dominant-negative p53 gene. Thus, it is reasonable to assume that p53 plays a role in the topotecan sensitivity of glioma cells retaining wild-type p53 function. However, the present study shows that other factors determining topotecan sensitivity must be present as well since there was overall no change in topotecan sensitivity of cell lines retaining wild-type p53 and those which do not (Table 1). Although wild-type p53 cell lines have been proposed to be more sensitive to topoisomerase I inhibitors than p53 mutant cell lines (O'Connor et al., 1997), no impact of p53 status on topotecan sensitivity became apparent in this panel of glioma cell lines. In fact, apoptosis induced by topoisomerase I inhibitors may involve p53, whereas loss of clonogenicity may not, in an ovarian carcinoma cell line (McDonald and Brown, 1998). Conversely, other authors reported that p53 might even protect colon carcinoma cells from the cytotoxicity of the camptothecin analog and topoisomerase I inhibitor, SN-38 (Te Poele and Joel, 1999).

We also find that differences in the degree of topotecan-induced formation of cleavable DNA/topoisomerase I complexes do not predict overall survival after exposure to topotecan in this panel of glioma cell lines, suggesting that factors acting down-stream of initial complex formation modulate cellular responses to topotecan-induced DNA damage, e.g., DNA repair enzyme systems. Also, we have previously noted that antiapoptotic gene products such as BCL-2 can interfere with cell death induced by the topoisomerase II inhibitor, teniposide, downstream of teniposide-induced cleavable complex formation (Winter et al., 1998).

The correlation of enhanced complex formation, suggestive of enhanced topoisomerase I activity, with the absolute levels of p53 protein levels, irrespective of genetic status, corresponds nicely to the observation that both wild-type and mutant p53 variants may activate topoisomerase I (Albor et al., 1998; Mao et al., 2000). Taken together, our data suggest that there is significant regulation of topotecan sensitivity in glioma cells down-stream of the p53-controlled response to genotoxic stress and the formation of cleavable DNA/topoisomerase I complexes induced by topotecan.

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