ABSTRACT

Topotecan is a novel topoisomerase I inhibitor that may have a role in the adjuvant chemotherapy of several solid tumors, including malignant glioma. Here, we have characterized the time- and concentration-dependent toxicity of topotecan in four human malignant glioma cell lines, LN-18, LN-229, LN-308 and T98G. High micromolar concentrations of topotecan, which are unlikely to be achieved in plasma in human patients in vivo, were cytotoxic within 48 hr, induced DNA fragmentation, did not induce major cell cycle changes, failed to consistently alter BCL-2 or BAX protein levels but inhibited RNA synthesis and induced cleavable DNA/topoisomerase I complex formation. Prolonged exposure for 72 hr to high nanomolar to low micromolar concentrations of topotecan augmented p21 protein levels and induced G2/M arrest but failed to consistently alter BCL-2 and BAX protein levels, did not induce significant DNA/topoisomerase I complex formation and did not inhibit RNA synthesis. Neither short-term nor long-term topotecan toxicity was blocked by ectopic expression of bcl-2 or wild-type p53. Transfer of a mutant p53 gene enhanced topotecan sensitivity in wild-type p53 LN-229 but not mutant p53 LN-18 cells. CD95 ligand (CD95L)-induced apoptosis was synergistically enhanced by short-term/high concentration but not long-term/low concentration exposure to topotecan, suggesting that topotecan sensitizes human malignant glioma cells to CD95L-induced apoptosis via inhibition of RNA synthesis. These data suggest that topotecan needs to be administered in high concentrations, such as an intratumoral polymer, to limit glioma cell growth in synergy with CD95L in vivo.

Human malignant glioma is an inevitably lethal neoplasm that is rather refractory to current approaches of cytoreductive surgery, radiotherapy, chemotherapy and immunotherapy (Fine et al., 1993; Lesser and Grossman, 1994; Weller and Fontana, 1995). Our major focus of interest has been the induction of apoptosis in human glioma cells via activation of the cytokine receptor, CD95 (Weller et al., 1994a, 1995a, 1995b, 1998; Wagenknecht et al., 1997). CD95 is a death-signaling molecule that is activated when bound by its natural ligand, the CD95 ligand (CD95L), which shows homology to the tumor necrosis factors. We previously screened several cancer chemotherapy drugs for an augmentation of CD95L-induced apoptosis of human glioma cells (Roth et al., 1997). These studies showed that none of the drugs tested was comparable to actinomycin D or cycloheximide in its efficacy to sensitize glioma cells to the acute cytotoxic effects of CD95L. These agents inhibit RNA and protein synthesis, respectively, and are thought to permit apoptosis because the synthesis of cytoprotective proteins by tumor cells is repressed. However, all drugs examined synergized with CD95L-induced growth inhibition as assessed by modified colony formation assays. More recently, we noted that the classic topoisomerase I inhibitor camptothecin was unique among cancer chemotherapy drugs in that it potently enhanced even the acute cytotoxic effects of CD95L on human glioma cells (Weller et al., 1997d). Camptothecin, however, has not become a major cancer drug used clinically because of systemic toxicity. A polymer preparation has recently been shown to prolong survival in the 9L rat glioma model (Weingart et al., 1995).

Topotecan (Herben et al., 1996) is a camptothecin-derived compound that has shown activity against numerous human tumor cell lines and xenografts (Houghton et al., 1992; Panchon et al., 1995; Kaufmann et al., 1996a, 1997; Traganos et al., 1996), including glioma cell lines as well as glioma xenografts (Friedmann et al., 1994; Nakatsu et al., 1997). Although topotecan is known to inhibit topoisomerase I, neither mRNA levels of the enzyme nor cleavable DNA complex
formation predict tumor cell responses to topotecan in vitro (Dubreiz et al., 1995). Thus, the precise mechanism of drug action is still under investigation (Danks et al., 1996). In vivo, exposure to topotecan has been confirmed to induce both DNA topoisomerase I complex formation (Subramanian et al., 1995) and cell death by apoptosis (Seiter et al., 1997).

Topotecan has shown clinical activity in small cell and non-small cell bronchogenic carcinoma, ovarian carcinoma and myeloid leukemia (Beran et al., 1996; Broom, 1996; Kudelka et al., 1996; Perez-Soler et al., 1996; Schiller et al., 1996). Its toxicity is largely limited to myelosuppression (Rowinsky et al., 1992). Because topotecan penetrates the blood-brain barrier and achieves significant levels in the cerebrospinal fluid after intravenous administration (Blaney et al., 1993a; Sung et al., 1994; Baker et al., 1996), it is currently evaluated for the chemotherapy of malignant brain tumors as well as leptomeningeal metastases from solid tumors. Topotecan was found to be without clinical effect in pediatric brain tumors when used at 5.5 to 7.5 mg/m² i.v./24 hr in 3-week intervals (Blaney et al., 1996). Modest activity against recurrent malignant gliomas in adults was noted when the drug was administered for 5 days at 1.5 mg/m² in 3-week intervals (Macdonald et al., 1996). In vitro studies suggest that topotecan administration during radiotherapy may result in enhanced antiangioma activity (Lamond et al., 1996; Marchesini et al., 1996). Here, we examine mechanisms of topotecan toxicity in human glioma cells with a special focus on the CD95/CD95L system and a therapeutic value for CD95-based immunotherapy of malignant glioma.

**Materials and Methods**

**Chemicals, cell lines and cell culture.** Topotecan was obtained from SmithKline & Beecham (King of Prussia, PA). The drug was dissolved at 20 mM in dest. H₂O, sterile filtrated and stored in aliquots at −70°C. All other chemicals were purchased from Sigma Chemical (St. Louis, MO). Radiochemicals were from Amersham (Braunschweig, Germany). T98G human glioma cells were kindly provided by Dr. N. de Tribollet (Lausanne, Switzerland). The cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1% L-glutamin and antibiotics. These cell lines have been characterized in previous studies (Van Meir et al., 1994; Weller et al., 1994a, 1995b, 1997a). The p53 status of these cell lines has been determined to be wild-type for LN-229, mutant for LN-18 and T98G and deletion with no protein detectable in LN-308 cells (Van Meir et al., 1994; Weller et al., 1997b). Glioma cells engineered to express high levels of murine bcl-2 (T98G, LN-229) have been described (Weller et al., 1995a). The generation of LN-229 and LN-18 cells engineered to express the murine temperature-sensitive p53 mutant Val135 has also been reported (Trepel et al., 1998). Pooled transfected cells were compared with neo (bcl-2) or hygro (p53) control cells, which harbor the empty vector. Neuro2A murine neuroblastoma cells expressing murine CD95L were generated and maintained as described (Rensing-Ehl et al., 1995) and served as the source for the CD95L-containing supernatants used here.

**Assessment of viability and apoptosis.** Glioma cell proliferation was assessed by crystal violet staining. Apoptotic cell death was measured by quantitative assessment of DNA fragmentation (Weller et al., 1994b, 1997b). Detached and adherent cells were harvested by centrifugation and trypsinization, respectively, pooled and lysed for 10 min on ice in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.2% Triton X-100, pH = 7.5). Intact DNA was pelleted by centrifugation for 10 min at 13,000 rpm, 4°C. The supernatants containing fragmented DNA were transferred to new tubes. The pellets were disrupted by sonication and resuspended in lysis buffer. Disrupted pellets and supernatants were digested with RNase A (100 µg/ml) for 2 hr at 37°C and DNA content separately determined by 1:10 dilution in 5 mM Tris-HCl/0.5 mM EDTA (pH 7.6) containing 0.5 µg/ml ethidium bromide in a Millipore fluorimeter at 530 nm excitation and 620 nm emission wave lengths. In situ DNA end labeling was performed as previously described (Weller et al., 1994a).

**Determination of cleavable DNA topoisomerase I complexes.** Cleavable DNA topoisomerase I complex formation was assessed as previously described (Li et al., 1993). DNA of exponentially growing cells (10⁶ cells/ml) was labeled with 2 µCi/ml [methyl-3H]thymidine (specific activity: 20–40 Ci/mmol) over night. Cells were washed with PBS 3 times and trypsinized, and an aliquot was counted. The cells were adjusted to 100,000 cpm/ml and then incubated in 12-well plates for further 24 hr. Subsequently, the cells were treated with different concentrations of 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 an 22-gauge needle, the lysates were transferred to a reaction tube containing 250 µl of 325 mM KCl, vortexed vigorously for 10 sec, incubated for 10 min on ice and centrifuged for 10 min at 13,000 rpm at 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 then cooled on ice for 10 min and centrifuged. The pellets were washed once again and resuspended in 200 µl of H₂O (65°C). After the addition of 5 ml of liquid scintillation cocktail, radioactivity was measured in a Wallac Liquid Scintillation Counter.

**Determination of CD95 and CD95L protein levels.** CD95 protein levels were measured as described (Weller et al., 1995b). CD95L protein levels were assessed accordingly (Weller et al., 1997c), using anti-human CD95L antibody (N-20, Santa Cruz, CA; rabbit polyclonal IgG) and rabbit IgG as isotype control antibody. For assessment of CD95L levels, the cells were permeabilized in 75% ice-cold ethanol in PBS for 10 min on ice before the labeling procedure. The specific fluorescence index (SFI) was calculated as the ratio of the mean fluorescence values obtained with the specific CD95 or CD95L antibody and the isotype control antibody (Weller et al., 1995b).

**Measurement of RNA and protein synthesis.** Cells were grown in 12 well-plates (10⁴ cells/ml) and incubated with different concentrations of topotecan, actinomycin D or cycloheximide for 8 hr. During the last hour of incubation, the cells were pulse-labeled with 0.5 µCi/ml [5,6-³H]uridine (specific activity: 40 Ci/mmol) to determine RNA synthesis. The cells were washed with ice-cold PBS (2×) and ice-cold 6% trichloroacetic acid (2×) to remove unincorporated, acid-soluble label. After lysis with 0.1 N NaOH (1 ml) overnight at room temperature, 0.5 ml of the lysate was mixed with 4 ml scintillation cocktail and counted in a liquid scintillation counter. For the determination of protein synthesis, the cells were pulse-labeled during the last hour of incubation with 1 µCi/ml L-[4,5-³H]leucine (specific activity: 167 Ci/mmol). After washing with ice-cold PBS (3×), the cells were lysed with 0.1% SDS (0.5 ml/well) for 30 min at 37°C. Proteins were precipitated by addition of ice-cold 15% trichloroacetic acid (0.5 ml/well) and pelleted by centrifugation (13,000 rpm, 10 min, 4°C). The supernatant (trichloroacetic acid-soluble fraction) was counted in a liquid scintillation counter after addition of 5 ml scintillation cocktail. The pellet (trichloroacetic acid-precipitable fraction) was washed with 6% trichloroacetic acid (3×), dissolved in 0.5 ml 0.1 N NaOH, and the radioactivity of the precipitated proteins measured after addition of 5 ml scintillation cocktail.

**Immunoblot analysis.** Immunoblot studies were performed according to standard procedures as previously described (Weller et al., 1994a, 1997b). p53 antibody pAb1801 was from Oncogene Science (Uniondale, NY), human bcl-2 antibody from Dakopatts (Glostrup,
Denmark). p21 and bax antibodies were obtained from Santacruz (Santa Cruz, CA).

Flow cytometric cell cycle analysis. For cell cycle analysis, the glioma cells were exposed to different concentrations of topotecan for 24, 48 or 72 hr, washed and incubated with trypsin for 3 min at 37°C, harvested, washed and fixed with 70% ice-cold ethanol. Then, 10^6 cells were stained with propidium iodide (50 μg/ml in PBS, containing 100 μg/ml RNase A), washed and subjected to flow cytometric analysis of DNA content using a Becton Dickinson FACS caliber cytometer; 10^5 cells were analyzed. Results are presented as histograms.

Statistics. EC_{50} values were determined by linear regression analysis. Effects of simple treatments were compared by Student's t test. Composite treatments were analyzed by analysis of variance (ANOVA).

Results

Topotecan toxicity and cell cycle effects in human malignant glioma cells. Four different human malignant glioma cell lines were exposed to topotecan for 24 to 72 hr. Viability and proliferation were inhibited in a concentration- and time-dependent fashion (fig. 1). The EC_{50} values were above 100 μM at 24 hr, in the low micromolar range at 48 hr except for LN-308 cells, and below 1 μM at 72 hr except for LN-308 cells (table 1). Thus, the cell line with the longest doubling time, LN-308, was most resistant to topotecan. Yet continuous cell cycle progression per se was not required for topotecan toxicity because exposure to topotecan in serum-free medium, which reduces [3H]thymidine incorporation to <10% and accumulation of cells in G0/G1, did not induce resistance to topotecan except for a minor protection in T98G cells. Light microscopic monitoring, in situ DNA end labeling and quantitative DNA fragmentation indicated that the glioma cells killed by topotecan were undergoing apoptosis (see below, data not shown).

Next, we examined concentration- and time-dependent effects of topotecan on the cell cycle distribution in the four cell lines. The cells were treated with 0.01, 0.1, 1, 10 or 100 μM topotecan, and cell cycle analysis performed at 24, 48 or 72 hr. A representative experiment for T98G cells at 24 hr and 72 hr is shown in figure 2. At 24 hr, 0.01 μM topotecan induced a moderate increase in the G2/M fraction with a concomitant reduction in G0/G1 cells in all cell lines. At 0.1 μM, topotecan induced a prominent G2/M arrest in all cell lines as well as an increase in S phase cells for T98G, LN-18 and LN-308. Topotecan at 1 μM had similar effects as 0.1 μM but S accumulation became more prominent than G2/M arrest. Concentrations of 10 and 100 μM had little effects on the cell cycle distribution. Longer exposure times of 48 hr and 72 hr did not result in significantly different findings with regard to cell cycle distribution except for a progressive accumulation of dead cells with higher concentrations of topotecan (see fig. 2, sub-G0/G1 fraction at 72 hr with topotecan at 10 and 100 μM). The cell cycle studies thus indicated that topotecan inhibits glioma cell growth by different mechanisms at different concentrations but failed to identify differential cell cycle changes as a predictor for the higher resistance to topotecan, such as LN-308 cells.

To further examine the significance of the cell cycle arrest in G2/M induced by topotecan for drug-induced apoptosis, we treated the glioma cells with topotecan at 0.1 μM in the presence of increasing concentrations of caffeine which overcomes G2/M arrest by activating p34cdc2 kinase (Yao et al., 1996). Caffeine had strong time-dependent cytotoxic effects on the glioma cells when applied alone at concentrations exceeding 1 mM. As expected, caffeine at 1 mM reduced the number of cells arrested in G2/M by topotecan, without affecting cell cycle distribution when administered alone (fig. 3A). However, topotecan cytotoxicity was essentially unaffected by caffeine since the effects of topotecan and caffeine were additive but not synergistic, as illustrated in figure 3B.

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<th>Topotecan-induced changes of p53, p21 and BCL-2 family protein levels. Drug-induced apoptosis is thought to</th>
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<td>Topotecan-induced inhibition of glioma cell proliferation: EC_{50} values at 24, 48 and 72 hr</td>
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Fig. 1. Topotecan inhibits the proliferation of human malignant glioma cells. LN-LN-229, T98G, LN-18 or LN-308 cells were exposed to increasing concentrations of topotecan for 24 hr ( ), 48 hr ( ) or 72 hr ( ). Survival was assessed by crystal violet assay. Data are expressed as mean and S.E.M. (n = 3).

Fig. 2. Topotecan induces concentration- and time-dependent changes in the cell cycle distribution. LN-18 LN-229 LN-308 T98G. Topotecan at 0.01, 0.1, 1, 10 or 100 μM induces a prominent G2/M arrest in all cell lines. The cells were treated with 0.01, 0.1, 1, 10 or 100 μM topotecan, and cell cycle analysis performed at 24, 48 or 72 hr.
depend on the constitutive and drug-induced levels of various gene products involved in the regulation of cell death, such as p53 and p53 response genes and BCL-2 family proteins. Here, we asked whether constitutive expression of these proteins or topotecan-induced changes in their levels predicted the response to topotecan (fig. 4). Expectedly, the single p53 wild-type cell line, LN-229, showed a concentration-dependent increase in p53 levels. There were no changes in the levels of mutant p53 proteins in LN-18 and T98G cells and no p53 detected in LN-308 cells. Induction of p53 protein expression in LN-229 cells was paralleled by an accumulation of p21 protein. Interestingly, p21 was also moderately up-regulated in T98G, LN18 and LN-308 cells at 0.1 but not at 0.01 or 1 μM topotecan. This increase in p21 levels must be independent of p53 transcriptional activity. There was no major change in the levels of BAX or BCL-2 proteins except for a minor increase of BAX in LN-229 and T98G cells and a minor decrease of BCL-2 in LN-229 cells at 1 μM topotecan. The changes of BAX and BCL-2 in LN-229 cells correspond to those expected for a cell line with wild-type p53 status.

Fig. 2. Topotecan induces concentration-dependent changes in cell cycle distribution in T98G human malignant glioma cells. T98G cells were untreated or exposed to topotecan at 0.01, 0.1, 1, 10 or 100 μM for 24 (left) or 72 (right) hr. Cell cycle analysis was performed by flow cytometry as described in Methods.

Fig. 3. Modulation of topotecan-induced cell cycle changes and cytotoxicity by caffeine. A, LN-229 cells were exposed to 0.1 μM topotecan in the absence (open bars) or presence (black bars) of 1 mM caffeine. Cell cycle analysis was performed at 48 hr as in figure 2. The figure shows changes in the relative fractions in each cell cycle phase (PP, polyploid cells). B, LN-229 cells were exposed to 0.1 μM topotecan ( ), 1 mM caffeine (○) or both (●). Data are expressed as mean percentages of survival (n = 3, S.E.M. <10%). To determine whether the effects of cotreatment with caffeine and topotecan were antagonistic, additive or synergistic, we calculated the predicted effects of cotreatment, based on the assumption of independent (additive) effects. Calculation was done according to the fractional product method (Webb) as described (Roth et al., 1997). The predicted effect of additive, independent actions of topotecan and caffeine is shown ( ). This is very close to what was observed experimentally.

Fig. 4. Topotecan-induced changes in the levels of apoptosis-regulatory gene products. LN-229, T98G, LN-18 or LN-308 glioma cells were untreated (lanes 1, 5, 9, 13) or exposed to topotecan at 0.01 (lanes 2, 6, 10, 14), 0.1 (lanes 3, 7, 11, 15) or 1 (lanes 4, 8, 12, 16) μM for 72 hr. Changes in the levels of p53, p21, BAX and BCL-2 were monitored by immunoblot analysis as described in Methods; 20 μg of soluble protein lysates were loaded per lane.

Modulation of topotecan toxicity by ectopic expression of bcl-2 and p53. To address the role of p53 status and expression of antiapoptotic genes such as bcl-2 more directly, we examined the effects of ectopic bcl-2 and p53 expression on topotecan toxicity of LN-229 and T98G cells. The generation of glioma cell clones expressing murine bcl-2 or the murine temperature-sensitive p53 Val135 mutant has been described (Weller et al., 1995a; Trepel et al., 1998). Ectopic expression of bcl-2 failed to increase glioma cell resistance to topotecan (fig. 5A). The same glioma cell transfectants have...
previously been shown to be more resistant to CD95 antibodies, irradiation, BCNU, cisplatin, teniposide, vincristin, and staurosporine (Weller et al., 1995a, 1997d; Roth et al., 1997).

Forced expression of mutant p53 at 38.5°C sensitized LN-229 cells, which are wild-type for p53, to topotecan. Mutant p53 induced an EC50 shift in LN-229 cells from 0.3 to 0.06 μM at 38.5°C. When the grafted p53 protein was induced to assume wild-type conformation at 32.5°C, there was a significant sensitization to topotecan, compared with hygro control cells, at concentrations up to 0.1 μM, with no difference above that concentration. Topotecan toxicity of LN-18 cells, which are mutant for p53, was unaffected by p53 gene transfer at either temperature.

**Topotecan sensitizes human glioma cells to CD95L-induced apoptosis: requirement for inhibition of RNA synthesis.** Next, we asked whether topotecan enhances glioma cell apoptosis induced by CD95L. The glioma cells were exposed to different concentrations of topotecan and CD95L for 16 hr and survival assessed immediately thereafter. We observed that topotecan at concentrations of 10 to 100 μM sensitized LN-1229, T98G and LN-18 glioma cells, but not LN-308 cells, for CD95L-induced apoptosis (fig. 6, left). While synergy is apparent from the graphs, formal confirmation of synergy between CD95 ligand and topotecan was obtained using the fractional product method (Roth et al., 1997). In contrast, when the glioma cells were coexposed to CD95L and topotecan for 72 hr, no synergy became apparent (fig. 6, right). This is evident from the parallel curves on the right (72 hr) but not on the left (16 hr). Note that much lower concentrations of topotecan were used in the long-term coinubation assays since topotecan toxicity greatly increases with length of exposure (fig. 1). No synergy can be detected if topotecan toxicity alone exceeds, such as 90%.

The next series of experiments was designed to elucidate the mechanism of synergy of topotecan plus CD95L toxicity. The potentiation of CD95L-induced apoptosis was not associated with enhanced formation of cleavable DNA topoisomerase I complexes. While topotecan induced complex formation in a concentration-dependent manner (fig. 7A), coexposure to CD95L did not alter this effect of topotecan (fig. 7B). Quantitative assessment of DNA fragmentation, however, revealed prominent synergy between CD95L and topotecan (fig. 7C), suggesting that synergy operates somewhere between the induction by topotecan of initial DNA damage, detected by measurement of cleavable complex precipitation, and quantitative DNA fragmentation.

CD95-mediated apoptosis of human glioma cells is dramatically enhanced by exposure to CD95 antibodies or CD95L and inhibitors of RNA or protein synthesis such as actinomycin D or cycloheximide (Weller et al., 1994a; Roth et al., 1997). Because topotecan inhibits topoisomerase I and since topoisomerase I activity may play a role in transcription (Horwitz et al., 1971), we examined whether topotecan affected RNA and protein synthesis in the glioma cells (fig. 8). In fact, the
concentrations required to enhance CD95L-induced acute cytotoxicity inhibited RNA synthesis significantly within 8 hr of exposure, suggesting that an actinomycin D-like effect may be responsible for synergy of topotecan and CD95L. In contrast, there were only moderate topotecan-induced changes in protein synthesis. Actinomycin D and cycloheximide served as positive controls for the inhibition of RNA and protein synthesis.

Levels of apoptosis-regulatory gene products during topotecan- and CD95 ligand-induced apoptosis. We also asked whether synergistic killing of glioma cells induced by topotecan and CD95L was associated with changes in the expression of genes that are involved in the regulation of susceptibility to apoptosis (fig. 9). These experiments were performed in LN-229 cells, which have wild-type p53 status, and in T98G cells, as a representative of the three cell lines T98G, LN-18 and LN-308 that are mutant for p53. Note that exposure in these experiments was 16 hr, as opposed to 72 hr in figure 4. As expected, topotecan augmented p53 protein levels in LN-229 but not in T98G cells. The concentrations of topotecan required for p53 activation in LN-229 cells (4 μM) were insufficient to synergize with CD95L in LN-229 cell killing (fig. 6). Moreover, there was no change in the extent of p53 induction whether CD95L was present. p21 was induced by topotecan in LN-229 in parallel with p53. In T98G cells, there was induction of p21 at 20 μM but not at 4 or 100 μM. The changes in p21 were unaffected by CD95L in both cell lines. There was a strong increase in BAX protein in LN-229 in parallel with p53. In T98G cells, there was induction of p21 at 20 μM but not at 4 or 100 μM. The changes in p21 were unaffected by CD95L in both cell lines. There was a strong increase in BAX protein in LN-229 in parallel with p53.
Because changes in CD95 and CD95L protein expression at the cell surface have recently been attributed a major role in drug-induced apoptosis (Friesen et al., 1996; Müller et al., 1997), we examined whether topotecan-induced sensitization to CD95-mediated apoptosis was associated with changes in the levels of CD95 or CD95L at the surface of all four cell lines (fig. 10). LN-229 cells showed an increase of CD95 protein as evidenced by a SFI shift from 1.54 to 2.02 after 16-hr exposure to 1 μM topotecan but not at 100 μM. The other three cell lines that are mutant for p53 exhibited no significant increase in CD95 levels, suggesting that p53 mediates topotecan-induced up-regulation of CD95 protein expression. Interestingly, CD95 protein levels were slightly decreased in all cell lines after exposure to 100 μM topotecan for 16 hr. This is likely to result from the inhibition of new mRNA synthesis at these concentrations (fig. 8). There was no change of CD95L protein expression in either cell line after exposure to topotecan at 1 or 100 μM for 16 hr. The data for LN-229 and T98G are summarized in figure 10.

Fig. 8. Synergy of CD95L and topotecan requires topotecan-mediated inhibition of RNA synthesis. T98G (open bars), LN-18 (gray bars) or LN-308 (black bars) cells were exposed to increasing concentrations of topotecan (A, B) or actinomycin (C) or cycloheximide (D). RNA synthesis (A, C) or protein synthesis (B, D) were measured at 7 to 8 hr as described in Methods. Data are expressed as mean percentages and S.E.M. of RNA or protein synthesis in untreated controls. CPM in untreated cells were in the range of 12,000 (T98G), 8000 (LN-18) and 3000 (LN-308) in the RNA assay and 1700 (T98G), 700 (LN-18) and 450 (LN-308) in the protein assay.

Fig. 9. Topotecan-induced changes in the levels of apoptosis-regulatory gene products: no modulation by CD95L. LN-229 or T98G glioma cells were not drug-treated (lanes 1, 5, 9, 13) or exposed to topotecan at 4 (lanes 2, 6, 10, 14), 20 (lanes 3, 7, 11, 15) or 100 μM (lanes 4, 8, 12, 16) for 16 hr, in the absence (left, 1–4, 9–12) or presence (right, 5–8, 13–16) of CD95L (50 U/ml for LN-229, 10 U/ml for T98G). Changes in the levels of p53, p21, BAX and BCL-2 proteins were monitored by immunoblot analysis as described in Methods; 20 μg of soluble protein lysates were loaded per lane.

Fig. 10. Topotecan does not modulate CD95 or CD95L protein expression at the cell surface. LN-229 or T98G cells were untreated or exposed to topotecan at 1 or 100 μM for 16 hr. CD95 or CD95L protein expression were measured by flow cytometry as previously described (Weller et al., 1995b, 1997c).
**Discussion**

Topotecan belongs to a new generation of drugs that are thought to have a role in the adjuvant chemotherapy of several solid tumors, including malignant glioma. The present study (1) sought to characterize topotecan toxicity of cultured human malignant glioma cells and (2) to examine possible synergy of topotecan toxicity and CD95L-induced apoptosis.

First, we show that cultured human malignant glioma cells show differential time- and concentration-dependent susceptibility to topotecan (fig. 1, table 1). High micromolar concentrations of topotecan do not induce major changes in the cell cycle distribution (fig. 2) and kill glioma cells within 24 to 48 hr. This toxicity involves inhibition of RNA synthesis (fig. 8A), prominent formation of cleavable DNA/topoisomerase I complexes (fig. 7A), is not blocked by bcl-2 (fig. 5A), and does not require wild-type p53 activity. However, this type of topotecan toxicity does not play a role in the antineoplastic actions of topotecan administered systemically to human cancer patients in vivo because plasma concentrations do not exceed 0.45 μM (Blaney et al., 1993b). Prolonged drug exposure induced glioma cell death at significantly lower concentrations which are even achieved in human cerebrospinal fluid (Blaney et al., 1993a). At these concentrations, topotecan augmented p21 levels, presumably by both p53-dependent and p53-independent pathways (fig. 4). Despite induction of p21, the glioma cells arrested in G2/M and not in G0/1 (fig. 2). There was a minor increase in cleavable DNA topoisomerase I complexes (fig. 7A), and these concentrations had little effect on RNA and protein synthesis (fig. 8, A and B). Neither of these parameters, nor the constitutive or induced levels of expression of several apoptosis-regulatory proteins (fig. 4), predicted the differential sensitivity of the 4 cell lines to topotecan (fig. 1, table 1). Although LN-308, the most resistant cell line, had the lowest doubling time, the speed of cell cycle progression alone could not account for the topotecan resistance of LN-308 cells because (1) serum deprivation, which reduces [3H]thymidine incorporation to <10%, did not confer topotecan resistance except for a minor protection in T98G cells (data not shown) and since (2) ectopic expression of wild-type p53, which induced complete growth arrest, failed to abrogate topotecan toxicity (fig. 5B). Topotecan toxicity of malignant glioma cells as characterized here resembled camptothecin toxicity of leukemia cells in that the level of induced cleavable DNA topoisomerase complexes was not predictive of toxicity but differed in that cell cycle arrest predicted resistance in leukemia but not in glioma cells (figs. 1 and 2) (Dubrez et al., 1995).

Second, a principal goal of this project was to identify topotecan as an anti-glioma agent that could act in synergy with CD95L to limit glioma growth in vivo. In contrast to numerous other anticancer drugs (Roth et al., 1997), topotecan synergistically enhanced CD95L-induced apoptosis of human glioma cells in short-term cytotoxicity assays. This synergy was restricted to short-term, high concentration exposure to topotecan and was probably a consequence of inhibition of RNA synthesis by topotecan. Yet, RNA synthesis was also inhibited in LN-308 cells which were refractory to topotecan-mediated sensitization to CD95L-induced apoptosis (figs. 6 and 8). Inhibition of RNA and protein synthesis has long been known to sensitize many cells to the cytotoxicity of cytotoxic cytokines like TNF and CD95L (Yonehara et al., 1989; Weller et al., 1994a). This observation has given rise to the assumption that tumor cells express cytoprotective proteins constitutively or after exposure to the cytokines. These proteins, which await identification, would block the cell death pathway activated by cytotoxic cytokines. Changes in CD95 or CD95L levels (Friesen et al., 1996; Müller et al., 1997) were not involved in topotecan toxicity or topotecan-induced sensitization to CD95L-induced apoptosis of malignant glioma cells. Topotecan induced CD95 protein expression in the single cell line with wild-type p53, LN-229, but not in the other three cell lines (fig. 10). Yet, LN-229 did not stand out in regard to topotecan sensitivity or topotecan-mediated sensitization to CD95L-induced apoptosis (figs. 1 and 6). Further, CD95 protein expression in LN-229 cells was induced only by low micromolar concentrations of topotecan which do not cause prominent inhibition of RNA synthesis (fig. 8) and fail to enhance the cytotoxic effects of CD95L (fig. 6). The lack of a requirement for wild-type p53 activity for induction of apoptosis is a common feature of apoptosis induced by CD95L and by topotecan, an important feature in view of the high frequency of p53 gene alterations in malignant glioma. In contrast to numerous other cancer chemotherapy drugs (Trepel et al., 1998), topotecan toxicity was not attenuated by forced expression of wild-type p53 (fig. 5), a possible consequence of p53-mediated up-regulation of topoisomerase I activity (Gobert et al., 1996).

The present study shows that topotecan has to be administered *via* a locoregionary approach if the synergy of topotecan and CD95L is to be exploited for the clinical management of malignant glioma. Further, preclinical studies suggest that the combination of topotecan with selective other anticancer drug may result in stronger antitumor activity (Jensen et al., 1997; Kaufmann et al., 1996b) as has been shown for CD95L in clonogenicity assays (Roth et al., 1997). Thus, we hope that immunochemotherapy, such as based on the synergy of CD95L and topoisomerase I inhibitors and possibly other cytotoxic drugs, may eventually result in improved outcome for human patients with malignant glioma.

**References**


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