Induction of Apoptosis and Cell Cycle Arrest in Glioma Cells by GL331 (a Topoisomerase II Inhibitor)

YUN CHEN1, YEN-HAO SU2, CHIH-HSIEN WANG2, JIANN-MING WU1, JIN-CHERNG CHEN3 and SHENG-HONG TSENG2

1Department of Surgery, Far Eastern Memorial Hospital, 21, Sec. 2, Nan-Ya South Road, Pan-Chiao, Taipei 220;
2Department of Surgery, National Taiwan University Hospital and National Taiwan University College of Medicine, 7, Chung-Shan S. Road, Taipei 100;
3Department of Surgery, Buddhist Tzu Chi Dalin General Hospital, 2, Ming Shen Road, Dalin, Chia-Yi, Taiwan

Abstract. Background: GL331 is a topoisomerase II inhibitor and, in this study, the effects of GL331 upon rat C6 glioma cells were investigated. Materials and Methods: The glioma cells were treated with GL331, then a cytotoxicity assay was done to evaluate the cytotoxic effects of GL331 and flow-activated cell sorter analysis was performed to analyze the cellular apoptosis and cell cycle distribution of the glioma cells. The expressions of p53, p21 and 14-3-3Û in glioma cells treated with GL331 were investigated by Western blot analysis. Results: GL331 was demonstrated to exert cytotoxic effects upon and induce apoptosis of the C6 glioma cells in a concentration- and time-dependent manner (p<0.05). GL331 also caused the glioma cells to accumulate in the G2/M-phase of the cell cycle and increased the expressions of p53, p21 and 14-3-3a. Conclusion: GL331 exerts cytotoxic effects and induces apoptosis in C6 glioma cells, accompanied by cell accumulation in the G2/M-phase and activation of p53, p21 and 14-3-3a.

Malignant glioma, the most common primary brain tumor, is associated with poor prognosis, although multidisciplinary treatment strategies including surgery, radiotherapy and chemotherapy have been used (1). Topoisomerase, an intracellular enzyme, is important for regulating the mitosis process (2). The enzyme elicits transient single- or double-stranded enzyme-linked deoxyribonucleic acid (DNA) strand breaks, allows DNA relaxation and/or the passage of DNA strands prior to DNA strand resealing (3, 4). Of the two types of topoisomerase, topoisomerase II mainly affects DNA replication, transcription, sister chromatid dysjunction, segregation of newly replicated chromosome pairs, chromosome condensation and alteration of DNA superhelicity during cell division (2, 4, 5).

Since topoisomerase II plays an important role in the regulation of the structure and function of DNA, this enzyme is considered a target for chemotherapeutic agents (6). Further, topoisomerase II inhibitors have been found to interfere with the breakage-reunion process of DNA through stabilizing the formation of topoisomerase II complexes (cleavable complexes), with this stabilization being involved in the induced cytotoxicity (4, 7, 8). Therefore, topoisomerase II inhibitors have been used for the treatment of various kinds of cancers including those of the colon, breast, lung, pancreas, head and neck, and ovarian variants and lymphoma (6). GL331 is a semi-synthetic topoisomerase II inhibitor derived from a plant toxin, podophyllotoxin (Genelabs Inc., CA, USA), which has been used for the treatment of patients with nasopharyngeal or gastric cancers (9-11). GL331 has similar chemical and physical characteristics to etoposide, another kind of topoisomerase II inhibitor; however, the cellular reactions elicited by these two drugs differ (12). The effect of GL331 on the induction of apoptosis for various kinds of cancer cells was three to ten times the effect of etoposide (12-14). Further, the induction of apoptosis by GL331 in adriamycin-resistant murine lymphoma cells was 40 times that of etoposide (10). In recent years, GL331 has also been found to be a potent inhibitor of tumor-induced angiogenesis (15). Thus, the mechanisms by which GL331 influences cancer cells might be different from those of etoposide. Glioma cells have inherent and significant topoisomerase II activity and other topoisomerase II inhibitors, such as etoposide and teniposide, have been found to be effective for the treatment of gliomas (2, 5, 9, 16-18). Thus, in this study, the effects of GL331 upon cultured glioma cells were investigated, including areas such as cytotoxicity effects, the cell cycle, cell cycle regulators and the induction of apoptosis, which have not previously been reported.
**Materials and Methods**

**Tissue culture and cell line.** The cell line used in this study was the rat C6 glioma cell line (19, 20). All the cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, at 37°C in a humidified 5% CO₂ incubator.

**Cytotoxic effects of GL331 on C6 glioma cells.** The GL331 sensitivity of the C6 cells (Genelab Inc.) was determined in vitro using the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide)-based colorimetric assay. The MTT was purchased from the Sigma Chemical Co. (MO, USA). The C6 cells were seeded in triplicate (5x10⁴ cells per well) in a flat-bottomed 24-well microtiter plate, and cultured overnight prior to treatment with various concentrations of GL331 (0, 1, 5, 10, 25, and 100 nM, dissolved in dimethyl sulfoxide [Sigma]) for various time periods (24, 48 and 72 hours). Subsequent to the removal of the drug, the cells were incubated for a total of 5 days after cell seeding. The extent of cell proliferation and viability was then determined using the MTT assay. The GL331 concentrations at which 50% of the C6 cells survived were designated as LC₅₀.

**Analysis of GL331-induced apoptosis of glioma cells by Annexin-V staining.** The apoptotic fraction of cultured C6 glioma cells, subsequent to GL331 treatment, was investigated using flow-activated cell sorter (FACS) analysis. Briefly, following treatment of the glioma cells with various concentrations of GL331 (0, 10, 100 nM) for periods of 24, 48, or 72 hours, 10⁶ C6 glioma cells were trypsinized and washed twice with phosphate-buffered saline (PBS). Then the cells were stained with Annexin-V using a commercial kit (Annexin V: FITC apoptosis detection kit, BD, PharMingen). The apoptotic fraction of the glioma cells was determined by FACS analysis.

**Analysis of the cell cycle distribution of glioma cells following GL331 treatment.** The cell cycle distribution of cultured C6 glioma cells subsequent to GL331 treatment was investigated using flow-activated cell sorter (FACS) analysis. Briefly, following treatment with various concentrations of GL331 (0, 10, 100 nM) for periods of 24, 48, or 72 hours, 10⁶ C6 glioma cells were trypsinized and washed twice with PBS. The cells were then stored in 1 ml of 80% alcohol/PBS at −20°C for subsequent experimentation. For FACS analysis, the cells were centrifuged at 6,000 rpm for 5 minutes, and subsequently washed with PBS, following which they were incubated with 0.5 ml 0.5% Triton X-100/PBS and 5 μg RNase A for 30 minutes. The cells were then stained with 0.5 ml 50 μg/ml propidium iodide/PBS in the dark and analyzed using FACSscan flow cytometry. The cell cycle distribution of the glioma cells following GL331 treatment was then assessed.

**Whole-cell extracts preparation and Western blot analysis.** Glioma cells were lysed in a buffer containing 20 mM HEPES at pH 7.6, 75 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na₂VO₃, 50 mM NaF, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin and 100 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride. The cell lysate was rotated at 4°C for 30 minutes, centrifuged at 10,000 rpm for 10 minutes and the precipitates produced discarded. The concentration of protein in the supernatant was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard. The expressions of p53, p21 and 14-3-3-σ of the glioma cells after GL331 treatment were analyzed by Western blot analysis. Briefly, the glioma cells were treated with 100 nM GL331 for a variety of drug-exposure times (30 minutes, 1, 6, 24, 48 and 72 hours), following which the cellular protein (20-50 μg) was loaded onto 10% SDS-polyacrylamide gels. The protein bands were then transferred electrophoretically to PVDF membranes (Micron Separations Inc., Westborough, MA, USA). The membranes were probed with anti-p53, anti-p21, anti-14-3-3-σ, or anti-α-tubulin (Santa Cruz Biotechnology, CA, USA), followed by a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Detection of the antibody reactions was performed with Western blotting reagent ECL (Santa Cruz Biotechnology), and the resultant chemiluminescence was assessed by exposure to Kodak Medical X-ray films (Eastman Kodak Company, Rochester, NY, USA). Differences in the degree of expression of the proteins were analyzed by ABC-Tiger Gel V2.0 (software from Taigen Bioscience Corp., Taipei, Taiwan).

**Statistical analyses.** One-way analysis of variance (ANOVA) by Scheffe’s post hoc multiple comparisons was used for statistical analyses of the survival curves, cellular apoptosis and cell cycle distribution. Significance was accepted as p<0.05.

**Results**

**GL331 induced time- and concentration-dependent cytotoxicity on C6 glioma cells.** The cytotoxicity effect of GL331 on the C6 glioma cells was studied using an MTT-based colorimetric assay. Figure 1 presents the survival curves for C6 cells treated with various GL331 concentrations and exposure durations; the effect of GL331 on the C6 cells was concentration- and time-dependent. Increasing the concentration and length of GL331 treatment significantly increased the cytotoxicity effect on the C6 glioma cells (p<0.02). The mean LC₅₀ of 24-, 48- and 72-hour GL331 treatment for glioma cells were 68.2, 25.4 and 13.9 nM, respectively, suggesting that C6 glioma cell cytotoxicity was associated with length of GL331 exposure.

**Induction of apoptosis of glioma cells by GL331.** The degree of apoptosis of the glioma cells induced by GL331 was analyzed by FACSscan and the apoptotic fraction was found to be related to the concentration and exposure time of the cells to GL331 (Figure 2). For glioma cells treated with 0, 10 or 100 nM of GL331 for 24 hours, the apoptotic fraction was 4.1±0.4%, 5.3±0.3% and 6.0±0.3% (mean±standard deviation), respectively. For glioma cells treated with GL331 for 48 hours, the apoptotic fraction was 4.1±0.1% and 4.2±0.2% for the cells treated with 0 or 10 nM GL331, respectively. In contrast, the apoptotic fraction of the cells treated with 100 nM GL331 was increased to 19.2±1.4%, which was significantly higher than the former two results (p<0.005). When the drug exposure time was extended to 72 hours, the apoptotic fraction was 5.9±0.3% and 3.4±0.5% for the cells treated with 0 or 10 nM GL331, respectively. In contrast, the apoptotic fraction of the cells treated with 100 nM GL331 was increased to 22.2±1.3%, which was significantly higher than the former two...
results (p<0.005). The apoptotic fraction in the cells treated with 100 nM GL331 for 72 hours was significantly higher than those treated with the same dosage for 48 hours (p<0.05). These data revealed that higher concentration and longer GL331 treatment induced more glioma cells to become apoptotic than lower concentration and shorter GL331 treatment. The effects of GL331 on the glioma cell apoptosis and cytotoxicity were further compared, and the proportion of 100 nM GL331-induced glioma cell apoptosis was found to be less than the proportion of induced cytotoxicity at the same GL331 concentration (24-hour GL331 treatment, 6.0±0.3% vs. 67.9±2.7%; 48-hour GL331 treatment, 19.2±1.4% vs. 93.0±1.7%; 72-hour GL331 treatment, 22.2±1.3% vs. 95.7±0.8%, p<0.00001).

GL331 induced glioma cell transition to the G2/M-phase. The cell cycle distribution of the C6 glioma cells used, but not related
to the drug-exposure time (Figure 3). For the glioma cells treated with 10 nM GL331, the cell cycle distribution showed no or slight change as compared to the control cells, even when the drug exposure time was extended from 24 hours to 48 or 72 hours. In contrast, when the cells were treated with 100 nM GL331 for 24, 48, or 72 hours, the fraction of the cells in the G0/G1-, S-, or G2/M-phases ranged from 43.4±1.7% to 44.8±2.0%, 8.8±0.8% to 10.1±0.4% and 46.4±1.1% to 47.2±2.6%, respectively; while the fraction of the control cells in the G0/G1-, S-, or G2/M-phases ranged from 55.8±2.3% to 65.8±2.8%, 9.1±1.0% to 14.3±0.7% and 25.1±1.9% to 29.9±2.9%, respectively. These data revealed that 100 nM GL331 treatment significantly reduced the proportion of the glioma cells in the G0/G1-phase, while it increased the proportion in the G2/M-phase, as compared with the control groups and the cells treated with low-dose GL331 (10 nM) (p<0.005). The proportion of the cells in the S-phase was only minimally changed. Thus, GL331 seemed to encourage a transition of cells from the G0/G1- to the G2/M-phase; however, the drug-exposure time seemed not to affect the cell cycle distribution significantly.

GL331 elicited increase of the expressions of p53, p21 and 14-3-3σ within glioma cells. The expressions of p53, p21 and 14-3-3σ in glioma cells treated with 100 nM GL331 for a variety of time-intervals was analyzed by Western blot analysis (Figure 4). The expression of p53 was slightly increased for the treated glioma cells from 6 to 48 hours subsequent to the initiation of GL331 treatment, with the peak at 24 hours (1.9-fold of the control). Similarly, the expression of 14-3-3σ was slightly increased from 6 to 72 hours subsequent to the initiation of GL331 treatment, with the peak at 24 hours (1.5-fold of the control). Further, the expression of p21 was markedly enhanced for the treated glioma cells from 6 to 48 hours subsequent to the initiation of GL331 treatment, with corresponding peaks at 6 and 24 hours, respectively (28.3- and 26.5-fold of the control). The data suggest that p53 and 14-3-3σ were activated slightly and p21 was activated markedly within 6 hours following the initiation of GL331 treatment, an activation which was maintained for a period of 48 to 72 hours.

Discussion

In this study, significant concentration- and time-dependent GL331 cytotoxicity was demonstrated for C6 glioma cells. The GL331 LC50 for C6 glioma cells were 68.2, 25.4 and 13.9 nM after 24-, 48- and 72-hour treatments, respectively. The C6 glioma cell LC50 was lower than that reported for other cancer cells such as nasopharyngeal, hepatocellular, gastric, cervical and colon cancers (500-2000 nM) (13). The results of the current study indicate that glioma cells are more sensitive to GL331 than other cancer cells reported in the literature. GL331 induces apoptosis for several types of cancer cell lines (13) and, in this study, we demonstrated this apoptosis induction for C6 glioma cells. The GL331-induced apoptosis of glioma cells appeared to be both a concentration- and time-dependent phenomenon, the greater the concentration of GL331, the more the exposed cells became apoptotic. By comparing the results of cytotoxicity and apoptosis, it was noted that the proportion of GL331-induced glioma cell apoptosis was less significant than the proportion of induced cytotoxicity at the same GL331 concentration. Therefore, it seems reasonable to suggest that other mechanisms, in addition to apoptosis, may play a role in GL331 glioma cell cytotoxicity. Further, we noted that GL331 induced the accumulation of glioma cells in, principally, the G2/M-phase. A previous study has shown that GL331 increases mainly the S-phase fraction with a concomitant decrease in the G1-phase fraction for nasopharyngeal carcinoma cells, and that there is no change to the G2/M-phase fraction under such conditions (21). The results from that study would appear to be inconsistent with those of our study, but the differences might be related to the concentration and exposure time of GL331 used for each of the studies, and to the different cell types tested.

The mechanism of the induction of cellular apoptosis by GL331 remains unclear and may be different from that of other topoisomerase II inhibitors such as etoposide (21). In the course of the induction of apoptosis for cancer cells, both etoposide and GL331 reduce the activity of protein tyrosine kinase, but only GL331 increases the activity of protein tyrosine phosphatase amongst nasopharyngeal,
hepatocellular, gastric, cervical and colon cancer cells (21). In addition, GL331 stimulates the cellular cdc2 kinase activity and enhances the cyclin B-associated cdc2 kinase activity, and subsequently encourages apoptosis for nasopharyngeal carcinoma cells (14). Further, the GL331-induced apoptosis amongst leukemic cells is found to be related to the decreased activity of poly (ADP-ribose) polymerase (PARP) and the extent of apoptosis for these leukemic cells is alleviated by overexpression of bcl-2 (22). All these mechanisms may contribute to GL331-induced cellular apoptosis, however, other pathways such as cell cycle regulation might also play a role, since GL331 exposure essentially elicited G2/M accumulation for the glioma cells. In our study, the expressions of p53 and 14-3-3σ were slightly increased and p21 was markedly increased within 6 hours of GL331 treatment, and the change of these cell cycle regulators was maintained for 48 to 72 hours. Further, the increased proportion of cells present in the G2/M-phase of the cell cycle following GL331 exposure was preceded by GL331-mediated up-regulation of the expressions of p53, p21 and 14-3-3σ. All these data suggest that these cell cycle regulators might be related to the GL331-induced G2/M accumulation of glioma cells.

A previous study has demonstrated GL331 may induce cell death through either a p53-dependent or a p53-independent pathway (22, 23); in addition, activation of p21 may be mediated through either a p53-dependent or a p53-independent pathway (23). Since GL331 only caused mild change of p53, and the activation of p21 through the p53-independent pathway correlates closely with G2/M arrest and cellular apoptosis (23), thus, activation of p21 through the p53-independent pathway was considered more important in the GL331-induced apoptosis of glioma cells than the p53-dependent pathway. However, it is still possible that the activation of p21 is through the p53-dependent pathway, because small changes in the level of p53 protein may stimulate a high expression of p21 (22-24). The function of p21 is closely related to cdc2 kinase, a cyclin-dependent kinase (CDK) (24). Cdc2 is constitutively present throughout the cell cycle and is responsible for the regulation of G2 progression and G2-M transition (25-28). The activation of p21 inhibits the activity of cdc2 by associating with the active Tyr-dephosphorylated form of cdc2, such inhibition correlating with the suppression of cell proliferation and G2/M arrest for colon cancer cells treated with quinones and non-neoplastic human mammary epithelial cells treated with genistein (23, 26). The mildly increased expression of the conserved phosphoserine-binding protein 14-3-3σ may also contribute to the GL331-induced G2/M accumulation of the glioma cells, since 14-3-3σ forms a cytoplasmatic complex with cdc2 during G2 arrest and sequesters cdc2/cyclin B1 in the cytoplasm of the damaged cell resulting in G2/M arrest (25, 29). However, we considered 14-3-3σ only played a minor role because its activation was only mild. As a whole, GL331 might act through a p53-independent pathway to activate the expression of p21, which might then negatively regulate the activity and function of cdc2, such events eventually resulting in G2/M accumulation of the GL331-exposed glioma cells.

Acknowledgements

GL331 was kindly given by Ming-Liang Kuo (Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan, R.O.C.).

References


Received June 6, 2005
Accepted July 25, 2005