GL331, a Topoisomerase II Inhibitor, Induces Radiosensitization of Human Glioma Cells

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Abstract. *Background: GL331 is a topoisomerase II inhibitor and its effects on cultured human T98G glioma cells were investigated in this study. The effects of combined GL331 and radiation treatment on human T98G glioma cells were also examined. Materials and Methods: The glioma cells were treated with GL331 and the clonogenic assay was used to study its cytotoxicity effects. A combination treatment, using either concomitant irradiation at the beginning or end of the GL331 treatment (designated as the RT-GL331 and GL331-RT protocols, respectively), was used to investigate the radiosensitization effects of GL331. The levels of radiosensitization of various protocols were evaluated by the dose-enhancement ratio and Isobologram analysis. The cell cycle distribution of the glioma cells treated with various protocols was explored using flow-activated cell sorter (FACS) analysis. Results: The clonogenic assay demonstrated that GL331 exerts cytotoxic effects on the glioma cells in a concentration- and time-dependent manner ($p<0.001$). The combination treatments, RT-GL331 and GL331-RT, increased the dose-enhancement ratio, with a higher ratio for the RT-GL331 protocol. Isobologram analysis revealed sub-additive glioma cell cytotoxicity for the GL331-RT protocol; in contrast, mainly supra-additive effects were demonstrated for the RT-GL331 protocol. FACS analysis revealed that GL331 and radiation caused the glioma cells to accumulate in the G2/M-phase of the cell cycle and combination of these two treatments as the RT-GL331 protocol further increased the G2/M fraction of the glioma cells. Conclusion: The induction of GL331 radiosensitization was sequence-dependent, with stronger cytotoxic effects on the gliomas noted where radiation was delivered concomitantly with the beginning of GL331 treatment. Such radiosensitization effects might be, at least partly, related to the increased cell accumulation in the G2/M-phase.*

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The diagnosis of malignant glioma, the most common primary brain tumor, is associated with poor prognosis (1). Numerous attempts have been made to develop an effective treatment strategy for malignant glioma. Such strategies include radiotherapy, chemotherapy, or concomitant radiotherapy and chemotherapy, administered alternately or sequentially. Topoisomerase, an intracellular enzyme, is important for regulating mitosis (2). Since topoisomerase II is important for the regulation of the structure and function of DNA, this enzyme is considered to be a target for chemotherapeutic agents (3). Topoisomerase II inhibitors are known to interfere with the cleavage-repair process of DNA *via* stabilization of topoisomerase II complexes (cleavable complexes). This stabilization is believed to be involved in the induced cytotoxicity (4-6). Therefore, in recent years, topoisomerase II inhibitors have been used for the treatment of various kinds of cancer, including of the colon, breast, lung, pancreas, head and neck, ovarian variants and lymphoma (3).

GL331 is a semi-synthetic topoisomerase II inhibitor derived from a plant toxin, podophyllotoxin (Genelabs Inc., CA, USA) (7). GL331 has been found to induce cell death in various kinds of cancer cells *in vitro* (8-13). It has also been used for the treatment of patients with nasopharyngeal and gastric cancers (14, 15). Glioma cells reveal inherent and significant topoisomerase II activity and GL331 has been found to exert cytotoxic effects upon and induce apoptosis of the glioma cells (16, 17). In addition, radiation

therapy is one of the major treatment strategies for glioma (7). The radiosensitization effects of GL331 were studied in this report by comparison to the effects of GL331 and radiation for the treatment of human T98G glioma cells across a range of experimental conditions to determine the optimal combination for maximization of cytotoxicity. To the best of our knowledge, this is the first report of the radiosensitization effects of GL331 on glioma cells.

Materials and Methods

Tissue culture and cell line. The cell line used in this study was the human T98G glioma cell line (purchased from the American Type Culture Collection, Manassas, VA, USA). 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% CO_2 incubator.

Cytotoxic effects of GL331 on T98G glioma cells. The GL331 (Genelab Inc.) sensitivity of the T98G cells was determined *in vitro* using the clonogenic assay (18, 19). Briefly, 12-well trays were incubated with 2 mL of medium containing 750 cells and incubated overnight, The cultures were then incubated with various concentrations of GL331 (0, 1, 10, 25, 50 and 100 nM, dissolved in dimethyl sulfoxide [Sigma-Aldrich, St. Louis, MO, USA]) for various time-periods (24 or 72 h), were washed free of drug with phosphate-buffered saline (PBS), and were incubated in fresh, drug-free medium for 14 days. The colonies were fixed and stained with methanol containing 0.5% methylene blue to aid counting. The colony-forming efficiency (number of colonies formed/number of cells plated) ranged from 20-50%. The extent of cell proliferation and viability was then determined. The GL331 concentrations at which 10, 20, 40, or 50% of the T98G cells survived were designated as LC_{10}, LC_{20}, LC_{40} or LC_{50}. The LC_{50}s for the T98G glioma cells were compared with those reported in the literature for other cancer cells. The LC_{10}s, LC_{20}s, and LC_{40}s were compared across the various combined-treatment conditions as described below.

Radiation therapy. The effects of radiation on T98G glioma cells were studied using ^{137}Cs-irradiation doses of 0, 100, 200, 400, or 600 cGy. The clonogenic assay was carried out as described above. The extent of cell proliferation and viability were then determined. The radiation doses at which 10, 20, 40 or 50% of the T98G cells survived were designated as LC_{10}, LC_{20}, LC_{40} or LC_{50}.

Comparison of combination therapy of GL331 and concomitant radiation at the beginning or end of GL331 treatment (RT-GL331 or GL331-RT protocols, respectively). To investigate the effects of GL331 and radiation treatment on T98G glioma cells, all permutations of GL331 concentration (0, 1, 10, 25, 50 and 100 nM), treatment duration (24 or 72 h) and radiation dosage (0, 100, 200 and 400 cGy) were tested, with radiation delivered at the beginning or end of the GL331 treatment. The cell proliferation and viability were then determined using the clonogenic assay as described above. The LC_{10}, LC_{20} and LC_{40} data, as well as the dose enhancement ratio, were analyzed for various treatment conditions. The additivity of the combined treatment was assessed using isobologram analysis, which may be used when one or more of the therapeutic agents are characterized by a non-linear dose-response curve (20). This method allows for evaluation of an additivity envelope, against which the combined-treatment data-points are compared. Briefly, the locations of the data-points with respect to the envelope indicate the type of interaction, with data-points to the left, right and within the envelope, suggesting positive (enhancement or supra-additivity) and negative effects (inhibition, antagonism or sub-additivity) and independent mechanisms or synergistic effects, respectively (4, 20).

Analysis of the cell cycle distribution of glioma cells following GL331 treatment and radiotherapy. The cell cycle distribution of cultured T98G glioma cells subsequent to GL331 treatment were investigated using flow-activated cell sorter (FACS) analysis. Briefly, following treatment of glioma cells with various concentrations of GL331 (0, 10, 100 nM) for periods of 24 or 72 h, 10^6 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 FACScan analysis, the cells were centrifuged at 6,000 rpm for 5 min, washed with PBS and then incubated with 0.5 ml 0.5% Triton X-100/PBS and 5 mg RNase A for 30 min. The cells were subsequently stained with 0.5 ml 50 mg/ml propidium iodide/PBS in the dark and the stained cells were analyzed using FACScan flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry System, San Jose, CA, USA). The cell cycle distribution of the glioma cells following GL331 treatment was then assessed.

The effects of the RT-GL331 and GL331-RT protocols on the cell cycle distribution of glioma cells were further analyzed. After treatment with 10 nM of GL331 for 24 or 72 h and a radiation dosage of 100 cGy, before or after treatment, the cell cycle distribution of the cultured T98G glioma cells was investigated using FACS analysis as described above.

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

Results

GL331 induced time- and concentration-dependent cytotoxicity for T98G glioma cells. The cytotoxicity effect of GL331 on the T98G glioma cells was studied using the clonogenic assay. The survival curves for the cells treated with various concentrations and exposure durations of GL331 are presented in Figure 1A. The effect of GL331 on the T98G cells was both concentration- and time-dependent ($p<0.001$). Higher concentrations and longer GL331 treatments had significantly greater cytotoxic effects on the T98G glioma cells than lower concentrations and shorter GL331 treatments. The LC_{10} and LC_{20} for glioma cells after 24 h of GL331 treatment could not be calculated because the highest tested GL331 dosage (100 nM) only killed 67% of the cells. The mean LC_{40} and LC_{50} of 24-h GL331 treatment for glioma cells were 68.2 and 45.3 nM, respectively. The mean LC_{10}, LC_{20}, LC_{40} and LC_{50} of 72-h GL331 treatment for glioma cells were 84.0, 53.9, 28.8 and 21.2 nM, respectively.

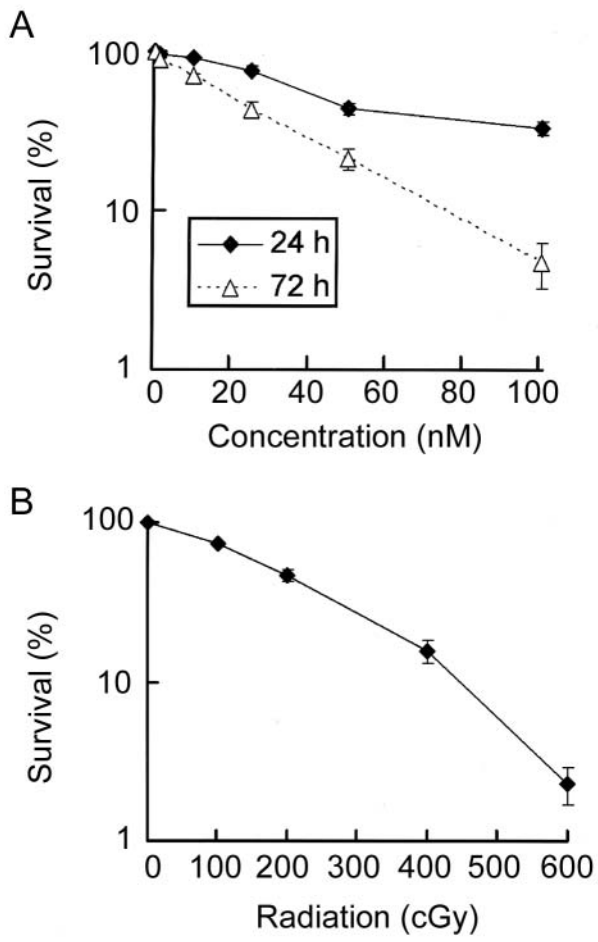

Figure 1. *The cytotoxic effects of GL331 and radiation on T98G glioma cells. A) The survival curves for T98G glioma cells treated with various concentrations of GL331 (0, 1, 10, 25, 50, 100 nM dissolved in dimethyl sulfoxide) for various periods (24 or 72 h). B) The survival curve for T98G glioma cells treated with ^{137}Cs irradiation (0, 100, 200, 400, or 600 cGy). The extent of the cell proliferation and cell viability was determined using the clonogenic assay. Each point is the average of 3 independent trials (9 determinations for each concentration) and are presented as mean ± standard deviation.*

Cell-killing effect of radiation. The effects of various doses of radiation (0, 100, 200, 400, or 600 cGy) on the viability of the T98G glioma cells were studied using the clonogenic assay. The survival curve of the irradiated T98G cells (Figure 1B) indicated that the effect of radiation was dose-dependent ($p<0.0001$). The higher the radiation dosage, the more glioma cells killed. The LC_{10}, LC_{20}, LC_{40} and LC_{50} of the glioma cells receiving radiation were 485.1, 372.5, 244.7 and 188.9 cGy, respectively.

Glioma cell survival with GL331 treatment and early-phase irradiation (RT-GL331 protocol), or GL331 treatment and late-phase irradiation (GL331-RT protocol). The survival curves for GL331-treated glioma cells (0, 1, 10, 25, 50, 100 nM; 24 or 72 h), with concomitant irradiation (0, 100, 200, 400 cGy), by the RT-GL331 and GL331-RT protocols are depicted in Figure 2. Analyses of the survival curves revealed that higher radiation dosages and longer GL331 exposure times killed more glioma cells than lower radiation dosages and shorter GL331 exposure times ($p<0.05$). The combination of 24 h GL331 treatment with 100 cGy irradiation revealed a slightly higher dose-enhancement ratio for the RT-GL331 protocol compared to the GL331-RT analog (2.9 *vs.* 1.5). In contrast, a much higher dose-enhancement ratio was noted for the RT-GL331 protocol in comparison to the GL331-RT analog when the irradiation dosage was increased to 200 cGy (90.9 *vs.* 5.8). The combination of 72-h GL331 treatment with irradiation (Table I) showed a radiation dose-dependent increase for both the RT-GL331 and GL331-RT protocols. The dose-enhancement ratios of the RT-GL331 protocol were slightly higher than those of the GL331-RT analog, using 72-h GL331 treatment and 100 cGy irradiation. However, a much higher dose-enhancement ratio was noted at 200 or 400 cGy for the RT-GL331 protocol in comparison to the GL331-RT analog. These results indicate that the dose-enhancement ratio of either the RT-GL331 or GL331-RT protocols increased with increasing radiation dosage and longer GL331 exposure, with greater T98G glioma cell cytotoxicity when radiation was delivered at the beginning of combination treatment (RT-GL331 protocol).

From isobologram analysis (Figure 3) (20), a mainly supra-additive synergistic response was demonstrated for the RT-GL331 protocol at the 10, 20 and 40% glioma cell survival levels. In contrast, only a sub-additive response was reached at 10, 20 and 40% levels for the GL331-RT analog. These results also indicated greater cytotoxicity for the RT-GL331 protocol in comparison to the GL331-RT analog and that the relative efficacy of combination GL331 and radiation protocols was sequence-dependent for glioma cells.

GL331 and radiotherapy induced glioma cell transition to the G2/M-phase. Following treatment with various concentrations of GL331 (0, 10 and 100 nM) for 24 or 72 h, the cell cycle distribution of the cells was analysed by FACS flow cytometry (Figure 4A and 4B). For the cells treated with GL331 for 24 or 72 h, the cell fractions in the G0/G1-and S-phases decreased and that in the G2/M-phase increased, as compared with the control ($p<0.05$). In addition, the cell cycle distribution of the glioma cells treated with the same concentration of GL331 for 24 or 72 h showed a similar pattern.

The effects of combination therapy with GL331 (10 nM, 24 or 72 h) and concomitant radiation (100 cGy), RT-GL331 or GL331-RT, on the cell cycle distribution of glioma cells were further analyzed (Figure 4C and 4D). After treatment

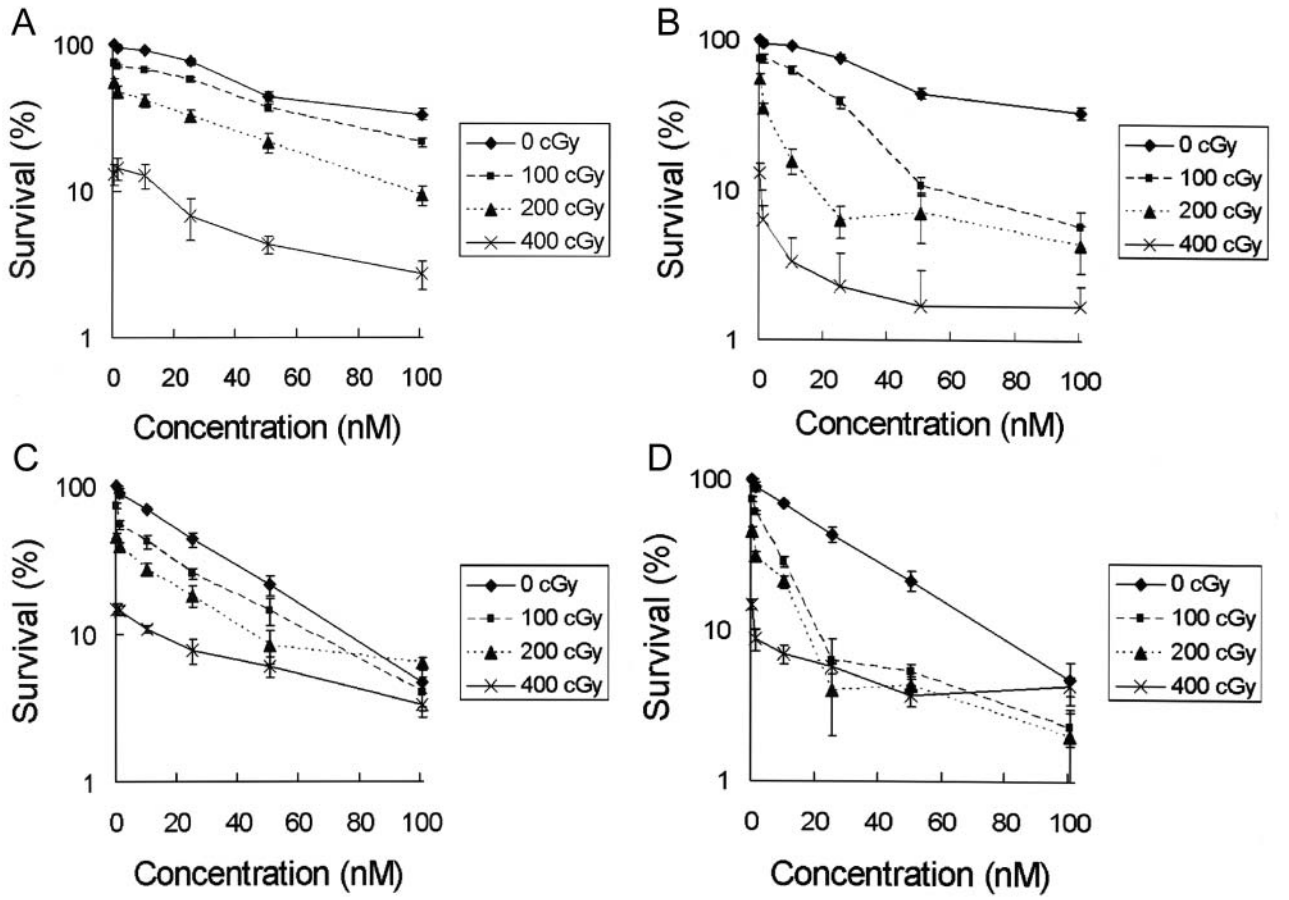

Figure 2. *The effects of GL331 and early- or late-phase irradiation on T98G glioma cells. Survival curves for the cells treated with GL331 and late-phase irradiation (GL331-RT protocol; A and C), or treated with GL331 and early-phase irradiation (RT-GL331 protocol; B and D) (GL331: 0, 1, 10, 25, 50, or 100 nM for 24 or 72 h; irradiation dosage: 0, 100, 200 or 400 cGy) (A & B, 24 h of GL331 treatment; C & D, 72 h of GL331 treatment). Each point on the curve is the average of three independent trials (nine determinations for each concentration) and are presented as mean ± standard deviation.*

Table I. *Enhancement ratio[a] of the LC_{10}, LC_{20}, and LC_{40}[b] of the T98G glioma cells treated with 72-h GL331 and radiation.*

Radiation	GL331-RT[c]			RT-GL331[d]		
	LC_{10}	LC_{20}	LC_{40}	LC_{10}	LC_{20}	LC_{40}
0 cGy	84.0 nM	53.9 nM	28.8 nM	84.0 nM	53.9 nM	28.8 nM
100 cGy	70.9 nM	37.0 nM	11.8 nM	22.5 nM	15.7 nm	6.7 nM
Enhancement ratio	(1.2)	(1.5)	(2.4)	(3.7)	(3.4)	(4.3)
200 cGy	45.6 nM	21.7 nM	0.84 nM	19.7 nM	10.9 nM	0.4 nM
Enhancement ratio	(1.8)	(2.5)	(34.3)	(4.3)	(4.9)	(72.0)
400 cGy	13.5 nM	(-)[e]	(-)	0.78 nM	(-)	(-)
Enhancement ratio	(6.2)	(-)	(-)	(107.7)	(-)	(-)

[a]The dose enhancement ratio is the ratio of the GL331 dose required to kill 90% (LC_{10} level), 80% (LC_{20} level), or 60% (LC_{40} level) of those cells irradiated and treated with GL331 *versus* those treated with GL331 only; [b]LC_{10}, LC_{20}, LC_{40}: the concentration of GL331 at which 90%, 80%, or 60% of the cells were killed; [c]GL331-RT: The glioma cells were treated with GL331 for 72 h and irradiated by various dosage of radiation (0, 100, 200, or 400 cGy) at the end of GL331 treatment; [d]RT-GL331: The glioma cells were treated with GL331 for 72 h and irradiated by various dosage of radiation (0, 100, 200, or 400 cGy) at the beginning of GL331 treatment; [e](-) indicates the LC_{20}, LC_{40} or the enhancement ratio could not be calculated.

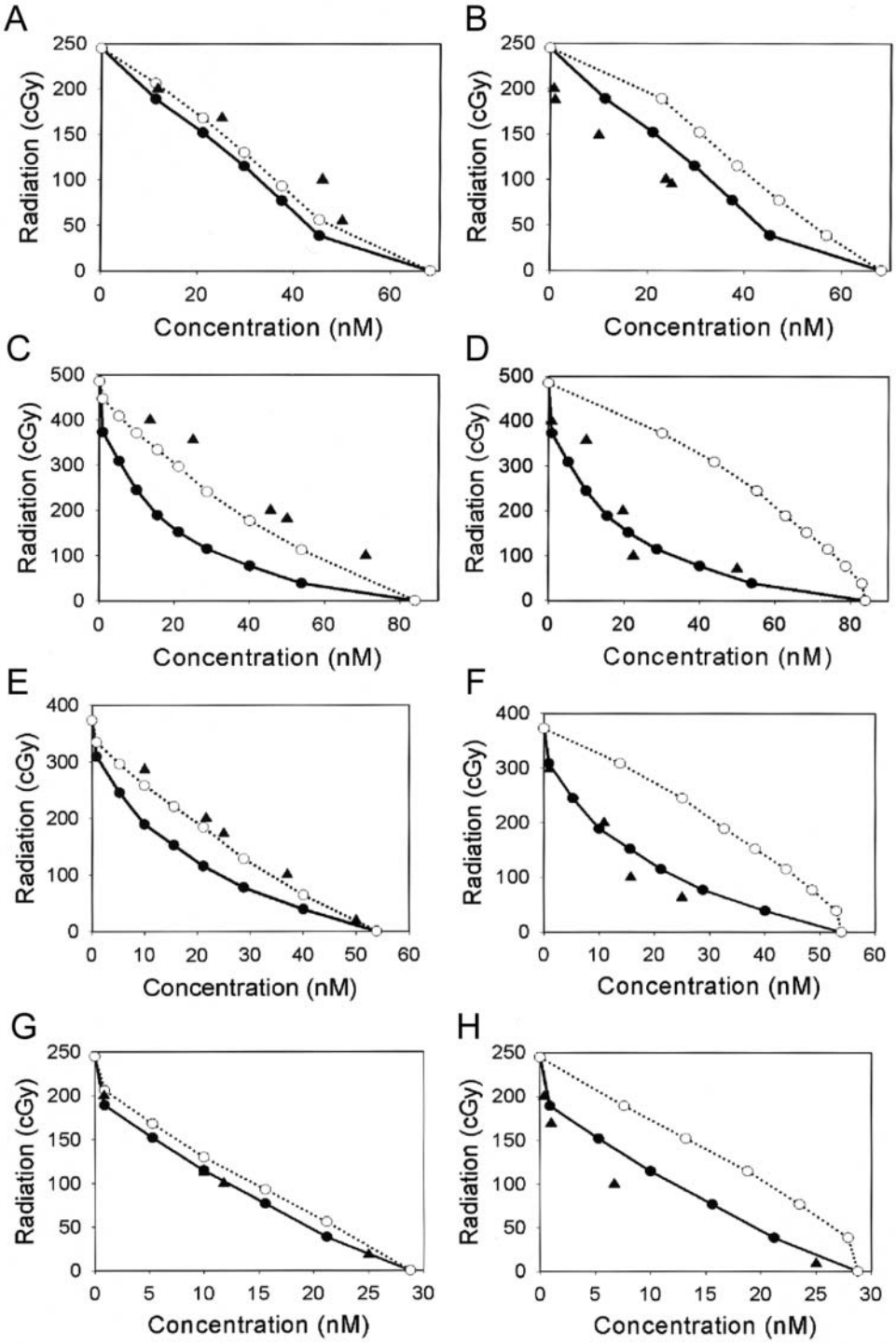

Figure 3. *Isobologram analysis of the effects of GL331 treatment and early-phase irradiation on the T98G glioma cells. A) Isobologram analysis of the outcome (40%) for T98G glioma cells treated with 24-h GL331 and late-phase irradiation (GL331-RT protocol). B) Cells treated with 24-h GL331 and early-phase irradiation (RT-GL331 protocol) (GL331: 0, 1, 10, 25, 50, or 100 nM for 24 h; irradiation dosage: 0, 100, 200 or 400 cGy). C, E and G) Isobologram analysis of the outcome for T98G glioma cells treated with 72-h GL331 and late-phase irradiation, or D, F and H) treated with 72-h GL331 and early-phase irradiation (GL331: 0, 1, 10, 25, 50 or 100 nM for 72 h; irradiation dosage: 0, 100, 200 or 400 cGy). The isobologram analyses took 10% (C, D), 20% (E, F) and 40% (G, H) survival as the end-point for 72-h GL331 treatment. The isoeffect mode I and mode II boundaries delineating the envelope of additivity (hatched line) were calculated from the response to radiation and drug alone (Figure 1). Data points (▲) were determined experimentally from the combination treatment experiments, as revealed in Figure 2.*

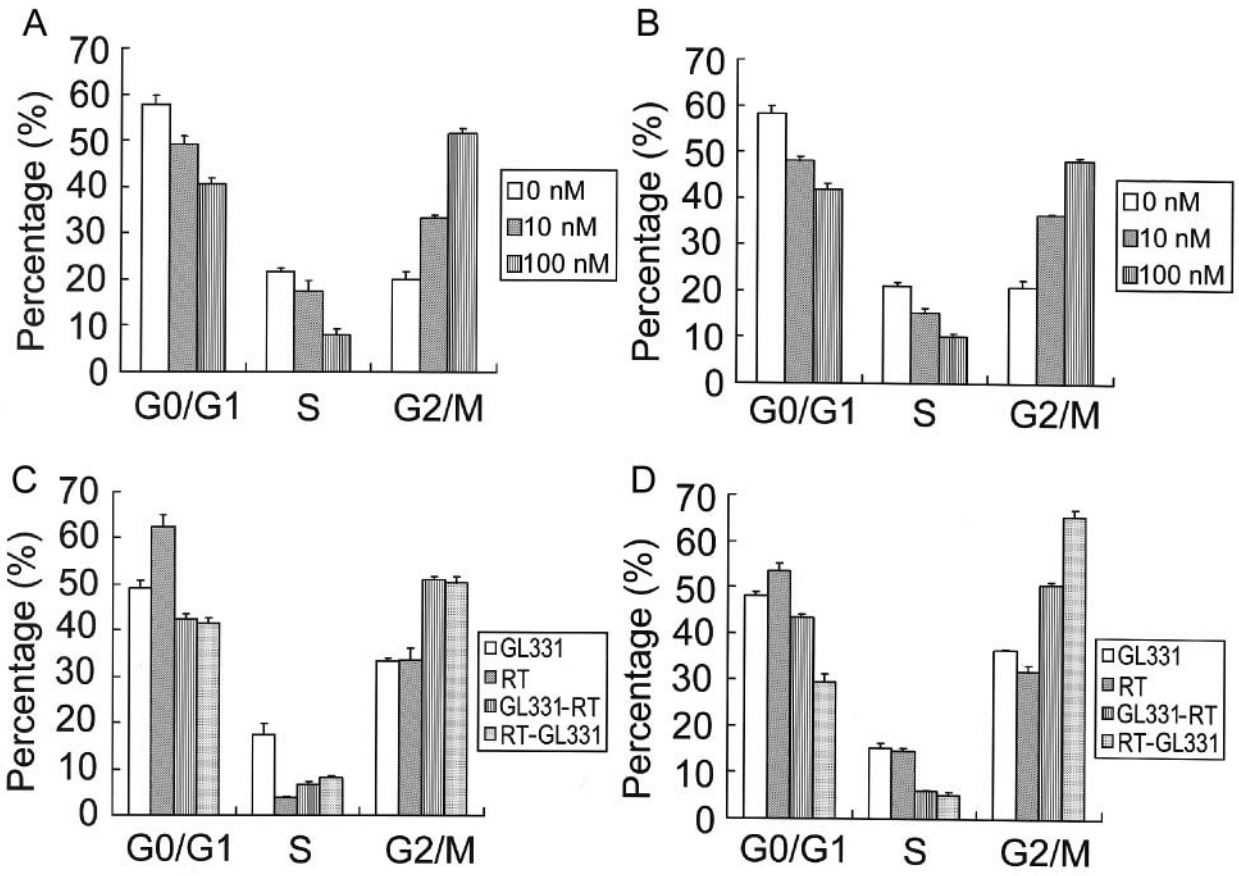

Figure 4. *Cell cycle distribution of the glioma cells treated with GL331 and/or radiation. Cell cycle distribution of the T98G glioma cells following treatment with various concentrations of GL331 (0, 10 and 100 nM) for 24 h (A) or 72 h (B). Cell cycle distribution of the glioma cells following the RT-GL331 or GL331-RT treatments with 10 nM of GL331 for 24 h and 100 cGy radiation delivered at the beginning or end of the GL331 treatment, respectively (C). Cell cycle distribution of the glioma cells following the RT-GL331 or GL331-RT treatments with 10 nM of GL331 for 72 h and 100 cGy radiation delivered at the beginning or end of 72-h 10 nM GL331 treatment), or GL331-RT (100 cGy radiation delivered at the end of 72-h 10 nM GL331 treatment, respectively CD). The cell cycle distribution of the glioma cells was investigated using FACS analysis. Data from three independent experiments are shown as mean ± standard deviation, representing the proportion of cells in the G0/G1- (G0G1), S- or G2/M- (G2M) phase. Bars, standard deviation.*

with RT-24-h GL331 or 24-h GL331-RT protocols, the G0/G1 fraction of the glioma cells was decreased as compared to those of the glioma cells treated with GL331 or radiation alone and the S- and G2/M-fractions were in between those of the cells treated with GL331 or radiation alone ($p<0.05$). In addition, there was no significant difference in the cell cycle distribution between the RT-GL331 and the GL331-RT protocols ($p>0.05$). On the other hand, after treatment with the 72-h RT-GL331 or 72-h GL331-RT protocol, the G0/G1- and S-fractions of the glioma cells decreased and the G2/M-fraction increased, as compared with those of the glioma cells treated with GL331 or radiation alone ($p<0.05$). The change of the cell cycle distribution was more prominent in the glioma cells treated with the 72-h RT-GL331 protocol. These data indicate that combination therapy with GL331 and concomitant irradiation at the beginning or end of GL331 treatment decreased the G0/G1-fraction and increased the G2/M-fraction of the glioma cells. Such change was more prominent after treatment with the 72-h early-phase protocol than with the other treatment protocols.

Discussion

In this study, significant concentration- and time-dependent GL331 cytotoxicity was demonstrated for T98G glioma cells. The GL331 LC_{50}s for the T98G glioma cells were 45.3 and 21.2 nM after 24 and 72 h of treatment, respectively, being similar to those obtained for the rat C6 glioma cells in our previous study (17). Postoperative radiotherapy has been the standard treatment strategy for glioma. As irradiation induces cells to arrest in the G2/M-phase (21) and since topoisomerase II functions as a repair enzyme during this phase, combining radiotherapy and GL331 treatment was

considered a promising approach for the enhancement of glioma cell radiosensitivity. Although establishing the ideal sequence is considered important, a review of the literature revealed that the optimal schedule has yet to be determined (21-23). In this study, we found mainly a supra-additive effect on T98G glioma cells for the RT-GL331 protocol, while only a sub-additive effect was demonstrated for the GL331-RT analog. Notably, a higher dose-enhancement ratio was revealed for the RT-GL331 protocol. Furthermore, the increased dose-enhancement ratio was more significant with longer GL331 exposure (72 h) and higher radiation dosage (200 or 400 cGy). These results suggest that T98G glioma cell cytotoxicity is greater for the RT-GL331 protocol in comparison to the GL331-RT variant. Our finding of sequence-dependent glioma cell radiosensitization with GL331 exposure suggests that optimal effects occur where radiation is delivered concomitantly at the beginning of topoisomerase II treatment. Therefore, the combination of irradiation and GL331 in an optimized treatment schedule may be clinically efficacious for the treatment of malignant gliomas.

The precise mechanisms underlying the sequence dependence of GL331 radiosensitization remain elusive. We found that GL331 caused the glioma cells to accumulate in the G2/M-phase. In addition, radiation also arrests cells in the G2-phase (19). Prolonged G2/M arrest may result where glioma cells are subjected to both GL331 and irradiation (21). The G2/M-phase represents the radiosensitive phase of the cell cycle and radiation-induced G2 arrest has been associated with the degree of cell radiosensitization induced by cytotoxic drugs (21, 22). Therefore, GL331 cytotoxicity, accompanied by G2/M accumulation, may further exacerbate radiation-induced cellular damage (21). In addition, the capability for DNA repair after radiation may also play a role in GL331-induced radiosensitization (23). Since achieving radiosensitization requires that cytotoxic quantities of a drug reach the target cells shortly before irradiation (4), as well as persistent post-irradiation drug treatment which effectively impairs DNA repair (24), the combination drug therapy and concomitant radiation at the beginning of drug treatment is a reasonable treatment schedule. The superior results achieved with the RT-GL331 protocol in comparison to the GL331-RT analog support the proposition described above. GL331 treatment and early-phase irradiation (RT-GL331 protocol) caused a persistent inhibition of topoisomerase II function after radiation, such that the normally rapidly repairable radiation-induced DNA damage would be exacerbated by subsequent GL331 treatment and fixed into lethal lesions, resulting in supra-additive or synergistic cytotoxic effects (4). In contrast, glioma cells treated with GL331 and late-phase irradiation (GL331-RT protocol) regained the ability to repair the radiation-induced injury because the suppression of topoisomerase II was reversed when GL331 was removed from the culture medium. Thus, the stronger cytotoxic effect on the cells induced by the RT-GL331 protocol as compared to the GL331-RT protocol was, at least partly, related to increased cell cycle accumulation in the G2/M-phase. Certainly, other mechanisms, such as the influence of cell-cycle regulators, apoptosis induction, doubling-time of tumor cells, ability to repair DNA damage after irradiation and alteration of cellular radiosensitivity through the modification of topoisomerase II activity, might also play a role in the radiosensitization effects of GL331 (24). Further studies are necessary to elucidate the underlying pathophysiology.

Acknowledgements

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