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Induction of apoptosis in multi-drug resistant (MDR) human glioblastoma cells by SN-38, a metabolite of the camptothecin derivative CPT-11

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Abstract The overexpression of the multidrug resistance (*mdr*1) gene and its product, P-glycoprotein (P-gp), is thought to limit the successful chemotherapy of human tumors. Recent studies demonstrate that SN-38, a metabolite of the camptothecin (CPT) derivative CPT-11, has antitumor effects on several tumors, but the mechanisms responsible for its cytotoxicity remain unclear. We therefore determined whether SN-38 has cytotoxic effects on MDR human glioblastoma GB-1 cells and non-MDR human glioblastoma U87-MG cells. Furthermore, we determined what role SN-38 plays in the induction of cytotoxicity in these tumor cells. In this study, we demonstrated that SN-38 had significantly stronger antitumor effects on GB-1 and U-87MG cells than did CPT ($P < 0.01$ and $P < 0.05$, respectively). In addition, findings obtained using a DNA fragmentation assay, Hoechst 33258 staining, in situ end-labeling and cell cycle analysis demonstrated that SN-38 induced apoptosis in these tumors. Our results suggest that SN-38 has a stronger antitumor effect on malignant glioma cells regardless of MDR expression than does CPT, and therefore can be considered a new chemotherapeutic agent potentially effective in the treatment of human primary or recurrent malignant gliomas resistant to chemotherapy.

Keywords Apoptosis · Chemotherapy · Camptothecin · SN-38 · Multidrug resistance · Glioma

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Introduction

The development of the multidrug-resistance (MDR) phenotype in human tumors is thought to be a major obstacle to successful chemotherapy. The MDR phenotype is associated with increased expression of the *mdr*1 gene [13, 34, 41]. This gene codes for a high molecule weight membrane glycoprotein of 170 kDa: P-gp [19]. Many studies have shown that calcium channel blockers are able to enhance the cytotoxic effect of chemotherapeutic agents in the treatment of MDR tumors [17, 32]. However, the main problem with this chemosensitization is the inability to attain plasma levels in patient corresponding with in vitro effective concentrations because of cardiovascular toxicity [33]. Therefore, there is a considerable need to find new chemotherapy able to circumvent the MDR phenotype.

Camptothecin (CPT) is an MDR phenotype antitumor drug isolated from *Camptotheca acuminata* [43]. CPT potently inhibits topoisomerase I through the formation of stable topoisomerase I-DNA cleavable complexes [15] and has shown significant antitumor activity against mouse L1210 leukemia [43], rat Walker carcinosarcoma [43], and several types of experimental tumors [8]. However, it has been found to show severe toxicity in both animal experiments [37] and clinical studies [12, 31], and has therefore not been used clinically as an antitumor drug. CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-pideridino]carbonyloxy-CPT), a semisynthetic derivative of CPT, has recently been

demonstrated to manifest potent cytotoxicity against several murine tumors after intravenous, oral and intraperitoneal administration [28, 30, 40]. Furthermore, 7-ethyl-10-hydroxy-CPT (SN-38), a metabolite of CPT-11, plays an essential role in mediating the antitumor effects of CPT-11 [21]. However, the mechanism responsible for the cytotoxicity of SN-38 is not yet clear. In this study, we attempted to determine whether SN-38 has antitumor effects on both MDR human glioblastoma GB-1 cells and non-MDR human glioblastoma U87-MG cells, and found that SN-38 inhibits the viability of these two types of cells significantly more than does CPT by induction of apoptosis regardless of MDR expression.

Materials and methods

Reagents

CPT was purchased from Sigma Chemical Co. (St. Louis, Mo.). SN-38 was a generous gift from Daiichi Pharmaceutical Co. (Tokyo, Japan). These agents were obtained in powder form, from which 10 m*M* stock solutions were prepared in dimethyl sulfoxide.

Cell lines

Human glioblastoma GB-1 cells show overexpression of the *mdr*1 gene transcript in Northern blot analysis and a high rate of expression of P-gp in Western blot analysis [29]. Human glioblastoma U87-MG cells obtained from RIKEN Cell Bank (Wako, Japan) demonstrate no expression of P-gp [29]. Tumor cells were cultured in Dulbecco's modified minimal essential medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS, GIBCO Laboratories, Grand Island, N.Y.), 4 m*M* glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

Cell viability

The cytotoxic effects of SN-38 and CPT on tumor cells were quantified using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra-zolium bromide (MTT, CHEMICON, Temecula, Calif.) colorimetric assay [25]. Target cells were seeded at 10^4 cells/well (0.1 ml) in 96-well flat-bottomed plates (Corning, N.Y.) and incubated overnight at 37 °C. SN-38 and CPT were then added (0.01 ml/well) at concentrations ranging between 0.01 μ*M* and 50 μ*M*. Following a 48-h incubation at 37 °C, 0.01 ml MTT reagent was added to each well. Following another 4-h incubation at 37 °C, 0.1 ml isopropanol with 0.04 *N* HCl was added to each well to dissolve the precipitates, and the absorbance was then measured at 570 nm with an autoreader (ER-8000, Sanko Junyaku Co., Tokyo, Japan) within 30 min of dissolution. In addition, a time-course assay of SN-38/CPT-mediated cytotoxicity in glioma cells was performed. Tumor cells were treated with CPT or SN-38 (10 μ*M*) for 6, 12, 24 or 48 h. After treatment, the MTT assay was performed as described above. The statistical significance of the findings was assessed using the paired Student's *t*-test.

Analysis of DNA fragmentation in agarose gel

This assay was performed using methods described previously [25]. Briefly, harvested cells (1×10^7) were centrifuged and washed twice with cold phosphate-buffered saline (PBS). The cell pellet was lysed in 1.0 ml of a buffer consisting of 10 m*M* Tris-HCl, 10 m*M* EDTA and 0.2% Triton X-100 (pH 7.5). After 10 min on ice, the lysate was centrifuged (13 000*g*) for 10 min at 4 °C in an Eppendorf microtube. The supernatant (containing RNA and fragmented DNA, but not intact chromatin) was then extracted first with phenol and then with phenol-chloroform/isoamyl alcohol (24:1). The aqueous phase was brought to 300 m*M* NaCl and nucleic acids were precipitated with two volumes of ethanol. The pellet was rinsed with 70% ethanol, air-dried and then dissolved in 20 μl of a solution including 10 m*M* Tris-HCl and 1 m*M* EDTA (pH 7.5). After digesting RNA with RNase A (0.6 mg/ml, at 37 °C for 30 min), the sample was electrophoresed in a 2% agarose gel with Boyer's buffer (50 m*M* Tris-HCl, 120 m*M* sodium acetate, 2 m*M* EDTA and 18 m*M* NaCl, pH 8.05). DNA was then visualized by ethidium bromide staining. The time-course of percentage DNA fragmentation was assessed by precipitation of high molecular weight DNA with 12.5% trichloroacetic acid and fragmented DNA with 25% trichloroacetic acid using methods described previously [27].

Hoechst 33258 staining of tumor cells treated with SN-38

To determine whether GB-1 and U87-MG cells treated with SN-38 display an apoptotic morphology, tumor cells were stained with the DNA-binding fluorochrome bis(benzimide)trihydrochloride (Hoechst 33258) as previously described [27]. Briefly, treated tumor cells were fixed in 1.0% formaldehyde and 0.2% glutaraldehyde for 5 min, washed with PBS twice and incubated with Hoechst 33258 (8 μg/ml) for 15 min at room temperature. The incidence of apoptotic chromatin changes was determined by counting and scoring 500 cells under UV-fluorescence microscopy.

In situ end labeling

Free 3′-OH ends generated by endonuclease cleavage of genomic DNA during apoptosis were labeled with a commercial kit (ApopTag; Oncor, Gaithersburg, Md.) based on a method similar to that of Gavrieli et al. [9], but which utilized digoxigenin-11-dUTP as label.

Flow cytometry

Tumor cells were treated with SN-38 for 48 h. Then 2×10^6 cells were fixed with 2 ml 70% ethanol on ice for 15 min, and pelleted and stained with propidium iodide (50 μg/ml in PBS) containing 0.5 mg/ml RNase A for an additional 30 min on ice prior to analysis of DNA content by flow cytometry [26]. Cells were tested for cell cycle position using a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) equipped with CellFIT version 2.0 software. The SOBR (Sum of Broadened Rectangles) model provided by this software was used to estimate the percentage of cells in each phase of the cell cycle. This model uses a complex repetitive calculation to produce approximations to the actual histogram, fitting G_0/G_1 and G_2/M populations with single Gaussian curves.

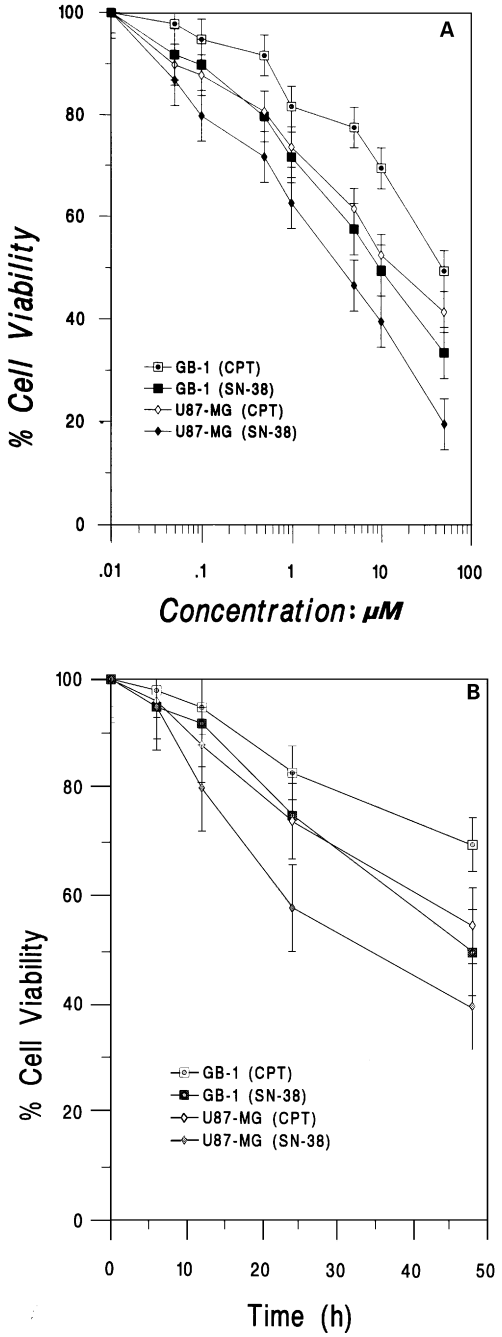

Fig. 1A, B The effects of SN-38 and CPT on GB-1 and U87-MG cells, as determined using a modified MTT assay. Tumor cells were seeded at a density of 10^4 cells/well (0.1 ml) in 96-well flat-bottomed plates and incubated at 37 °C. Values are means ± SD from three separate experiments. **A** Tumor cells were treated with various doses of SN-38 and CPT for 48 h; **B** Tumor cells were treated with SN-38 and CPT (10 μ*M*) for 6, 12, 24 or 48 h

Results

Cell viability

Exponentially growing GB-1 and U87-MG cells were treated with CPT and SN-38. Using a modified MTT assay, as shown in Fig. 1A, SN-38 inhibited the viability of GB-1 and U87-MG cells significantly more than did CPT ($P < 0.01$ and $P < 0.05$, respectively). The $IC_{50\,CPT}/IC_{50\,SN-38}$ (the concentration of CPT which caused 50% inhibition of viability of cells treated for 48 h divided by the corresponding concentration of SN-38) for GB-1 and U87-MG cells was 6.4 and 5.0, respectively. As shown in Fig. 1B, SN-38 inhibited the viability of both GB-1 and U87-MG cells significantly more than did CPT in a time-dependent manner ($P < 0.01$ and $P < 0.05$, respectively). We therefore suggest that SN-38 has a stronger cytotoxicity against malignant glioma cells than does CPT, regardless of their MDR expression.

Induction of apoptosis in tumor cells by SN-38

To investigate whether SN-38-induced cell death occurs by apoptosis, nuclease activity was assessed by extraction and gel electrophoresis of DNA. Tumor cells treated with 10 μ*M* SN-38 for 48 h were found to contain fragmented DNA in multiples of approximately 185 base pairs, giving rise to a characteristic DNA "ladder" pattern of apoptosis (Fig. 2A) [45]. As shown in Fig. 2B, the degree of DNA fragmentation in GB-1 and U87-MG cells treated with SN-38 was increased in a time-dependent manner significantly more than in those treated with CPT ($P < 0.01$ and $P < 0.01$, respectively). GB-1 and U87-MG cells treated with SN-38 (10 μ*M*) for 48 h showed about 38% and 62% DNA fragmentation, respectively. To establish an assay for apoptosis in individual cells, chromatin condensation and nuclear fragmentation, hallmarks of apoptosis, were assessed in treated tumor cells using the nucleic acid stain Hoechst 33258. When assayed 48 h after adding 10 μ*M* SN-38, about 50% of GB-1 cells displayed typical apoptotic morphology (Fig. 3). Furthermore, to evaluate the structural integrity of the DNA in treated tumor cells, an in situ end-labeling technique was used. About 50% of GB-1 cells treated with 10 μ*M* SN-38 for 48 h stained positive for DNA breaks (Fig. 4). Treated U87-MG cells also showed similar results (data not shown). Taken together, these results clearly revealed that SN-38 induce apoptosis in GB-1 and U87-MG cells.

Cell cycle analysis

We studied changes in the intensity of fluorescence of DNA using flow cytometry. As shown in Fig. 5, treatment of GB-1 cells with 10 μ*M* SN-38 for 48 h resulted in a decrease in the percentage of cells in G_0/G_1 phase and an increase in the percentage of cells in S and G_2/M phases, compared with the

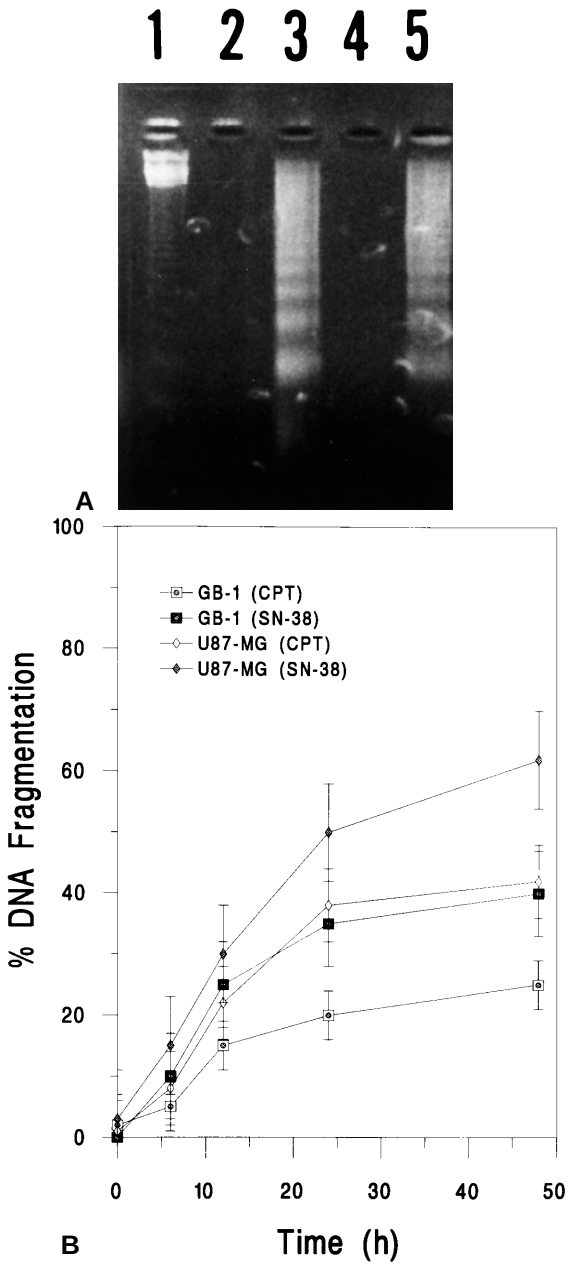

Fig. 2A, B DNA fragmentation assay in agarose gel. **A** Induction of DNA fragmentation by SN-38. Fragmented DNA was isolated 48 h after adding 10 μ*M* SN-38 to GB-1 and U87-MG cells and was electrophoresed in a 2.0% agarose gel containing 0.5 μg/ml ethidium bromide. GB-1 and U87-MG cells were treated without (*lanes* 2 *and* 4, control) or with SN-38 (*lanes* 3 *and* 5, 10 μ*M*). Molecular weight standards of multiples of 123 bp DNA Ladder (GIBCO BRL, Tokyo) are shown in *lane* 1. **B** Percent DNA fragmentation in GB-1 and U87-MG cells treated with SN-38 or CPT (10 μ*M*) for 6, 12, 24 or 48 h, respectively. Values are means ± SD from three separate experiments

corresponding percentages for the control. Moreover, SN-38 induced the accumulation of a discrete subpopulation of signals in the G_0/G_1 cell cycle region (A_0 peak).

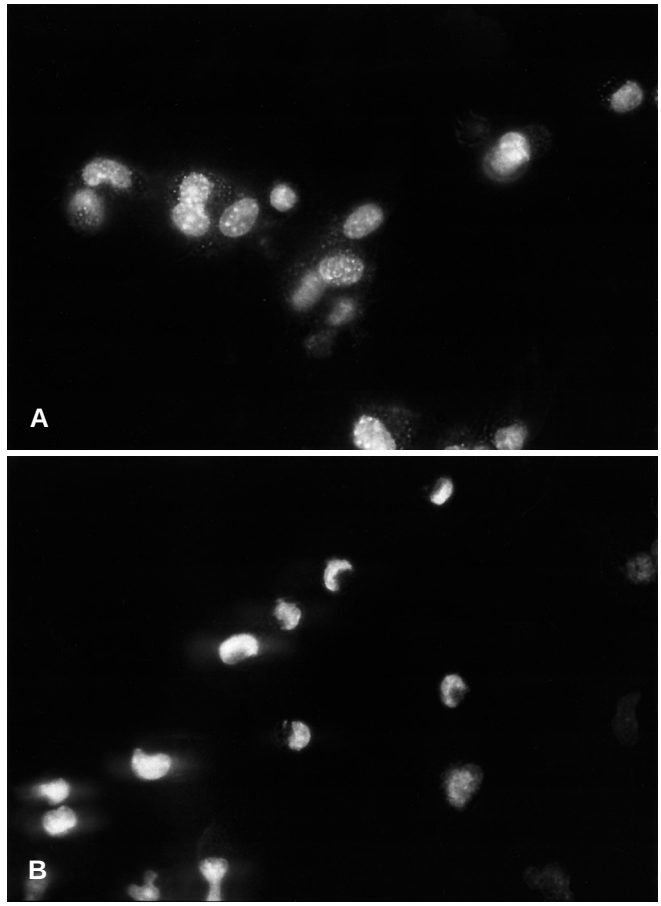

Fig. 3A, B Morphological changes associated with apoptosis in GB-1 cells treated with 10 μ*M* SN-38 for 48 h. After treatment without (**A**) or with SN-38 (**B**), GB-1 cells were fixed and stained with Hoechst 33258 (×400)

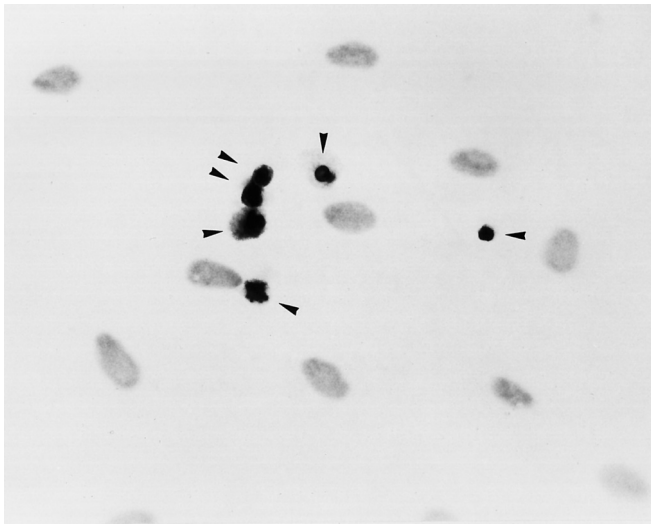

Fig. 4 In situ end labeling of DNA was detected in GB-1 cells treated with 10 μ*M* SN-38 for 48 h. *Arrows* show GB-1 cells positive for DNA breaks (×400)

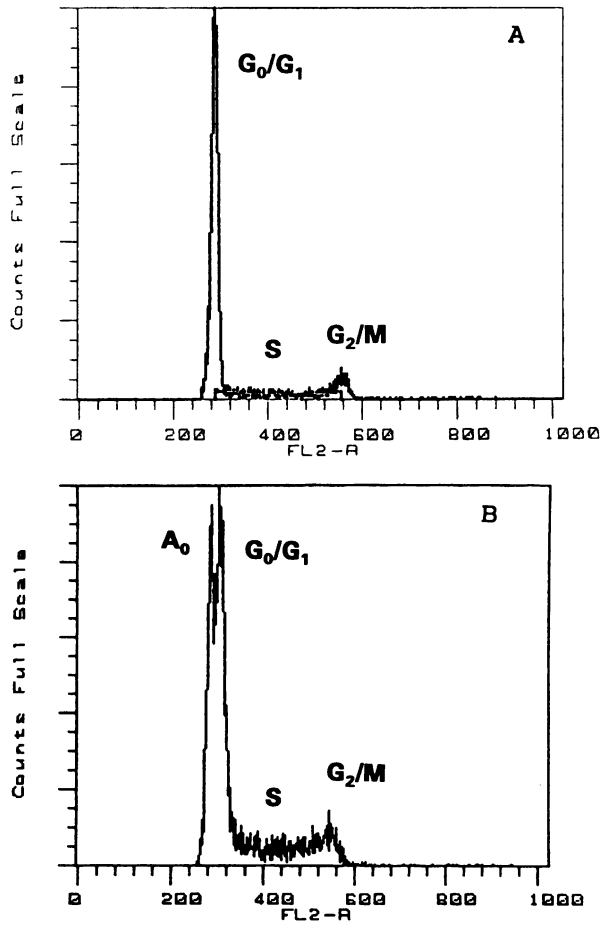

Fig. 5A, B Flow cytometric analysis of GB-1 cells treated without (control, **A**) or with 10 μ*M* SN-38 (**B**) for 48 h. GB-1 cells were subsequently fixed and stained with propidium iodide prior to DNA histogram analysis. In each case cell number (*ordinate*) was plotted against relative fluorescence (*abscissa*). The percentages of cells in the A_0 (a subpopulation of signals under the G_0/G_1 cell cycle region), G_0/G_1, S, and G_2/M phases of the cell cycle were: **A** 0, 82, 12, and 6%, respectively, and **B** 10, 54, 23, and 13%, respectively

Discussion

In this study, we present data showing that SN-38 inhibited the viability of MDR GB-1 and non-MDR U87-MG cells significantly more than did CPT, and subsequently induced apoptosis in these tumor cells. We suggest that treatment with SN-38 may be effective for primary or recurrent malignant gliomas resistant to chemotherapy.

MDR is caused by overexpression of P-gp that binds analogs of ATP and cytotoxic drugs [35], exhibits ATPase activity [36], and serves as an ATP-conducting channel [1]. P-gp therefore appears to function as an energy-dependent transport pump capable of effluxing cytotoxic agents and thereby decreasing their intracellular concentration. Recent studies have demonstrated that the expression level of P-gp may not only predict the response of individual tumors to specific cytotoxic agents but may also provide important criteria for determining a successful chemotherapeutic protocol [3, 10]. Our results showing that SN-38 had a strong cytotoxic effect on human malignant glioma cells regardless of MDR phenotype, may indicate one way in which the MDR phenotype can be circumvented.

Apoptosis (programmed cell death) [11] can be described as a process in which cells actively participate in their own death. The term apoptosis has been used to describe the death of cells killed during normal development or by growth factor deprivation [4, 23, 42, 44]. Apoptotic cells exhibit characteristic changes, including chromatin condensation, membrane blebbing and fragmentation of the DNA into nucleosome-sized pieces [5, 45]. Recently, apoptosis has also been a focus of reseach interest since it can result from the administration of antitumor chemotherapeutic drugs such as CPT [46], cisplatin [7, 26, 27] and etoposide [20, 24, 25]. However, the molecular mechanisms regulating apoptosis induced by chemotherapeutic agents remain unclear.

CPT-11 is converted to SN-38 in mouse serum and tissue homogenate [21]. SN-38 possesses much higher growth-inhibitory activity against several tumor cells than does CPT-11 in vitro, and is thought to play an important role in mediating the antitumor effect of CPT-11 in vivo [21]. The antitumor activity of CPT has been found to be correlated with the drug-induced accumulation of topoisomerase I-DNA cleavable complexes [16] and with inhibitory activity against DNA relaxation by topoisomerase I [18]. Moreover, topoisomerase I prepared from CPT-resistant mammalian cells, such as leukemia cells, is markedly resistant to CPT and CPT-11, and both the amount and the total activity of this enzyme are less in CPT-resistant cells than in wildtype cells [2, 38]. These observations indicate that inhibition of topoisomerase I plays a principal role in mediating the cytotoxicity of CPT and CPT-11. However, other experimental findings call into question the existence of a direct relationship between inhibition of topoisomerase I and CPT or CPT-11 cytotoxicity. For example, CPT has been shown to be especially toxic to cells in S phase, although the levels of topoisomerase I and drug-induced DNA single-strand breaks appear to be relatively constant throughout the cell cycle [14, 22]. In addition, CPT- or SN-38-induced DNA single-strand breaks are rapidly repaired following drug removal, while the cytotoxic effects of these agents are sustained [44]. Thus, the mechanism responsible for the cytotoxicity of CPT and CPT derivatives remains unclear.

The results of cell cycle analysis show that SN-38 induced a decrease in the percentage of cells in G_0/G_1 phase and an increase in the percentage of cells in S and G_2/M phases, compared with the corresponding percentage for the control. In addition, SN-38

accumulated A_0 peak, which has been shown to indicate the presence of apoptotic cells [6, 24, 26, 39]. Our findings suggest two possibilities: (1) tumor cells blocked in G_2/M phase continue to cycle and die at a later stage in the cell cycle; or (2) tumor cell death occurs directly out of G_0/G_1 phase.

In conclusion, our studies demonstrated that SN-38 has a stronger antitumor effect on both MDR and non-MDR glioma cells than does CPT and induces apoptosis in tumor cells. Although further clinical and toxicological studies are needed, SN-38 can nevertheless be considered a new chemotherapeutic agent potentially effective in the treatment of human primary or recurrent malignant gliomas resistant to chemotherapy.

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