CKD-602, a camptothecin derivative, inhibits proliferation and induces apoptosis in glioma cell lines

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Abstract. CKD-602 7-[2-(*N*-isopropylamino)ethyl]-(20S)-camptothecin, belotecan) is a synthetic water-soluble camptothecin derivative and topoisomerase inhibitor that has been shown to have clinical anticancer effect against ovarian and lung cancer. We studied its anticancer effects on four human glioma cell lines, U87 MG, U343 MG, U251 MG and LN229. Cell viability was quantified by a modified 2-(2-methoxy-4-nitropheyl)-3-(4-nitropheyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt and significant time- and dose-dependent cytotoxicity was observed in all cell lines. Susceptibility to CKD-602 at 48 h after treatment varied among the four cell lines and their IC_{50} value was as follows: 9.07 nM (95% CI 0.18-37.42) for LN229, 14.57 nM (95% CI 0.86-47.33) for U251 MG, 29.13 nM (95% CI 0.35-101.23) for U343 MG, and 84.66 nM (95% CI 34.63-148.25) for U87 MG. CKD-602 induced cell cycle arrest at G2 phase and produced antiproliferative activity and apoptosis in all cell lines. Thus, CKD-602 showed a significant anticancer effect on glioma cells *in vitro* and is a promising candidate for further studies on malignant gliomas.

Introduction

Patients with high-grade gliomas have a disappointing survival rate of one year, despite recent advances in best standard therapies, including surgical resection, radiation and new chemotherapeutic agents (1). High-grade gliomas comprise a biologically heterogeneous set of tumors with diverse therapeutic responses to different kinds of anticancer agents. Moreover, these tumors tend to have resistance to prolonged use of the agents, so other drugs need to be evaluated; the development of effective chemotherapeutic agents in various aspects of drug resistance mechanisms and the investigation of effective targeting agents are important and challenging medical problems.

Topoisomerase inhibitors are a class of agents that interfere with DNA 'unwinding' during DNA replication and RNA transcription. They stabilize DNA-topoisomerase complexes through non-covalent interactions to yield enzyme-linked DNA breaks (2). The prolonged exposure of replicating cells to these agents produces lethal DNA breaks that can trigger apoptosis (3). Various kinds of topoisomerase I inhibitors, most of them camptothecin derivatives, have been developed and show significant anticancer effects *in vitro* (4,5) and *in vivo* (6,7). Camptothecin is a plant alkaloid isolated from *Camptotheca acuminate* that shows high anticancer activity (8). Despite its highly potent anticancer effects, the clinical use of camptothecin sodium is hampered by poor solubility and unpredictable toxicities such as hemorrhagic cystitis, myelosuppression and diarrhea (9-11). With the aim of overcoming these limiting factors, a search for structural analogues of camptothecin has been conducted. This resulted in the discovery of a number of camptothecin analogues, such as CPT-11 and topotecan, with better aqueous solubility and bioavailability (12). These show high clinical efficacy in treating human neoplasms such as colorectal cancer, ovarian cancer and leukemia (13,14). CPT-11 and topotecan also showed efficacy in treating patients with brain tumors, especially when combined with other agents, although the study results are not decisive (15,16). However, poor efficacy as a single agent and he reported major toxicities such as CPT-11-induced colitis or interstitial pneumonia are potential limitations for their further clinical use (17,18). CKD-602, 7-[2-(*N*-isopropylamino)ethyl]-(20S)-camptothecin (belotecan, Chong Kun Dang Pharmaceutical Corporation, Seoul, Korea), is a new synthetic water-soluble camptothecin analogue (19). Clinical trials showed improved toxicity profiles and broad antitumor activity against various human tumor cells, with greater potency than CPT-11 and topotecan (20,21).

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In this study, we evaluated the potential anticancer activity of CKD-602 *in vitro* using human glioma cell lines and evaluated its feasibility as an anticancer agent for treating patients with malignant gliomas.

Materials and methods

Reagents. CKD-602 is a semisynthetic analogue of camptothecin containing a 2-(*N*-isopropylamino) ethyl group linkage at position C-7 of the camptothecin ring (22,23). It stabilizes the complex formed between topoisomerase I and DNA, thereby preventing the religation of DNA breaks. This leads to an inhibition of DNA replication and triggers apoptotic cell death (24). For this study, we used 10 mM stock solutions of CKD-602 dissolved in distilled water. CKD-602 was a gift from Chong Kun Dang Pharmaceutical Corporation.

Cell culture. Human glioblastoma cell lines were obtained from the Korea Cell Line Bank (U87 MG and U251 MG, Seoul, Korea) and purchased from ATCC (U343 MG, LN229, American Type Culture Collection, Manassas, VA, USA). U87 MG and U343 MG cells express wild-type *p53*, whereas U251 MG and LN229 cells harbor a *p53* mutation (25). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; welGene, Daegu, Korea) supplemented with 10% inactivated fetal bovine serum (Invitrogen, Grand Island, NY, USA) and 10 mg/ml of an antibiotic-antimycotic agent (Invitrogen) at 37˚C in air with 5% CO_2.

Cell viability. The cytotoxic effect of CKD-602 was quantified using a modified 2-(2-methoxy-4-nitropheyl)-3-(4-nitropheyl) -5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt (CCK-8; Dojindo Molecular Technologies Inc., Gaithersburg, MD, USA). Target cells were plated in 96-well plates (Techno Plastic Products) at a density of $2x10^3$ cells/well (100 μl) and incubated overnight for attachment. In each experimental set, cells were plated in quadruplicate and were treated with different concentrations (0.05, 0.1, 0.2, 0.5, 1, 2, 5 μM) of CKD-602 for 24, 48 and 72 h. CCK-8 reagent was added for colorimetric reactions up to a final concentration of 10% (v/v). Plates were incubated another 3 h at 37˚C. Extinction was measured on an ELISA reader at 450 nm (VERSAmax microplate reader, Molecular Devices, CA, USA). Cell survival was calculated according to the following equation: viability (%) = ($OD_{450[sample]}$/$OD_{450[control]}$) x 100. The percentage of viable cells was calculated and plotted against drug concentrations. The half maximal (50%) inhibitory concentration (IC_{50}) value was calculated from the dose-response curve.

BrdU cell proliferation assay. Cells were seeded at $2x10^3$ cells/well in 96-well plates and incubated overnight. They were treated with different concentrations (0.05, 0.1, 0.2, 0.5, 1, 2, 5 μM) of CKD-602 for 48 h. Cellular proliferations were measured by colorimetric immunoassay based on BrdU incorporation into the cellular DNA following the instructions of the manufacturers (Cell Proliferation ELISA, BrdU kit; Roche Molecular Biochemical, Indianapolis, IN, USA). Briefly, cells were pulsed with BrdU labeling reagent for 3 h, followed by fixation in FixDenat solution for 30 min at room temperature. Thereafter, cells were incubated with 1:100 dilution of antiBrdU-POD for 1 h at room temperature. Finally, the immunoreaction was detected by adding the substrate solution and the color developed was read at 370 nm.

Cell cycle analysis. Cells were plated at $2x10^5$/well in 6-well plates for 24 h before treatment. Cells were treated with different concentrations of CKD-602 for 48 h based on ~2-fold of their IC_{50} values, harvested by centrifugation at 1,200 rpm for 5 min, washed twice in ice-cold PBS, fixed in 70% ethanol, stored at 4˚C for a minimum of 1 h, washed with ice-cold PBS and resuspended in a mixture of 200 μl PBS, 20 μl RNase (10 mg/ml, Qiagen, Hilden, Germany) and 2 μl propidium iodide (PI, 25 mg/ml, Promega, Madison, WI, USA). The cell suspension was passed through a 40 μm pore filter to remove undesirable particles and aggregates. Cell cycle position was evaluated by Becton-Dickinson Fluorescence-Activated Cell Sorting (FACScan) using an excitation laser set at 480 nm and a detection wavelength of 575 nm (Becton-Dickinson, Mountain View, CA, USA). A minimum of 10,000 events/sample was analyzed.

Annexin assay. Apoptosis was quantified using Annexin V-FITC apoptosis kit (Pharmigen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cells were plated at $2x10^5$/well in two wells of 6-well plates. Cells were treated with different concentrations of CKD-602 for 48 h based on ~2-fold of their IC_{50} values and harvested by centrifugation at 700 rpm for 5 min. Cell pellets were resuspended in annexin V binding buffer (150 mmol/l NaCl, 18 mmol/l $CaCl_2$, 10 mmol/l HEPES, 5 mmol/l KCl, 1 mmol/l $MgCl_2$). FITC-conjugated annexin V (1 μM/ml) and PI (50 μM/ml) were added to cells and incubated for 30 min at room temperature in the dark. Analyses were done using a FACScan (Becton-Dickinson). Annexin V- and PI-positive cells were regarded to be apoptotic.

Apoptosis assay. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for apoptosis detection was performed using the Apoptag peroxidase *in situ* apoptosis detection kit (Intergen Company, Purchase, NY, USA). Briefly, cells were plated at $2x10^5$/well on coverslips in 6-well plates. Cells were treated with different concentrations of CKD-602 for 48 h based on ~2-fold of their IC_{50} values. The cells were washed with PBS and fixed in 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 10 min at 4˚C. The fixed cells were then incubated with 3% H_2O_2 in buffer, followed by 10 min proteolytic digestion in prediluted proteinase K. The cells were incubated in equilibration buffer for 2 min, then with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase (TdT)-catalyzed reaction for 60 min at 37˚C in a humidified atmosphere and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were incubated with antidigoxigenin antibody conjugated with peroxidase for 30 min. Color development was done using DAB. The cells were then counterstained with hematoxylin, dehydrated, cleared and coverslipped using a xylene-based mounting medium. TUNEL-positive cells were counted in 10 randomly selected fields.

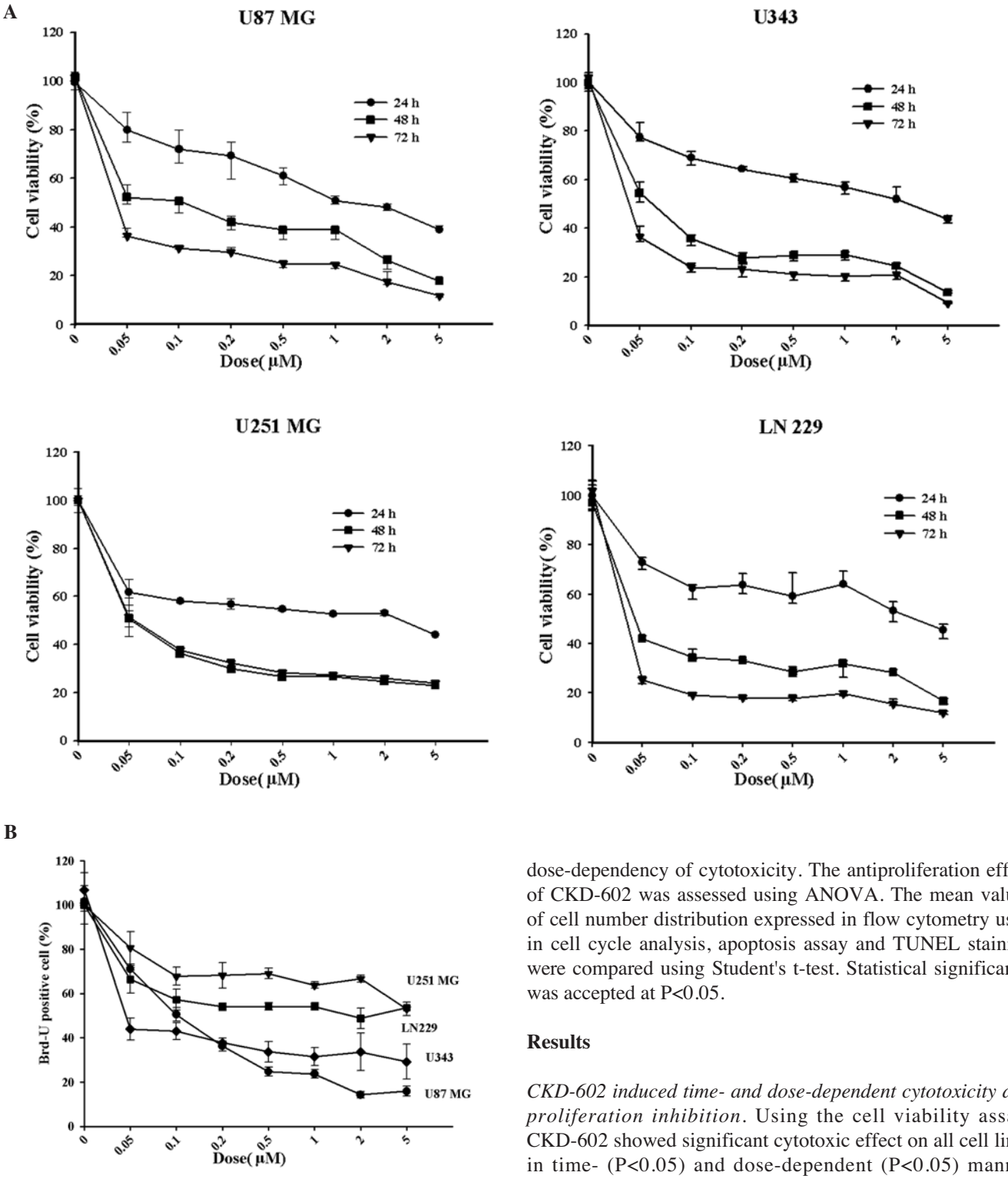


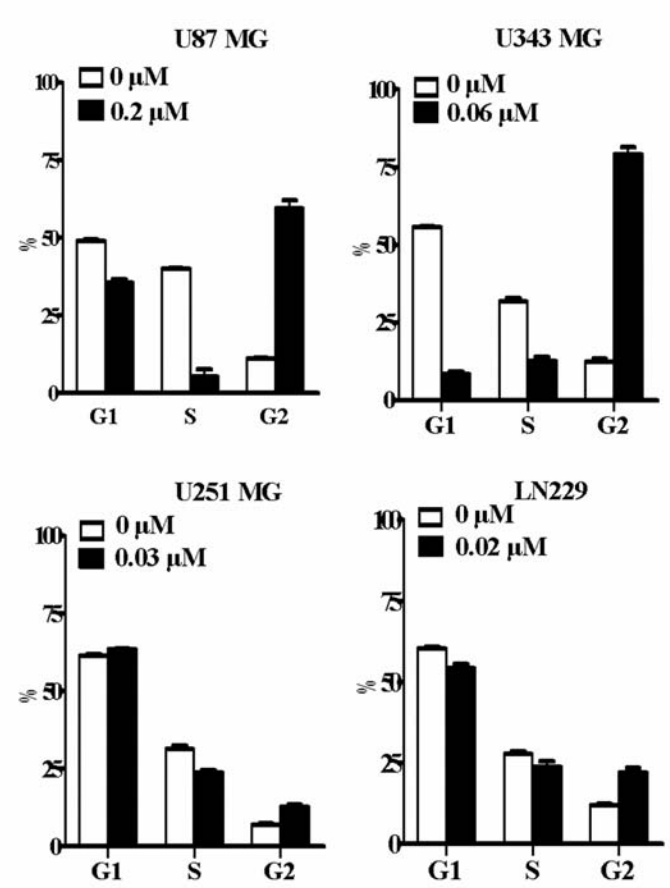
Figure 1. (A) Cell viability after CKD-602 treatment. All cell lines showed significant reduction of viability with time (P<0.05, ANOVA) and dose (P<0.05, ANOVA). Data are expressed as the mean percentage of viable cells ± SD. (B) CKD-602 treatment inhibited cell proliferation in a dose-dependent manner. Mean percentages of proliferative cells observed after BrdU staining are shown (P<0.05, ANOVA).

Statistics. The data are expressed as the mean ± SD and as 95% confidence intervals. Probit regression analysis was performed to determine the IC_{50} for cell viability analysis and repeated measures ANOVA was used to test for time- or dose-dependency of cytotoxicity. The antiproliferation effect of CKD-602 was assessed using ANOVA. The mean values of cell number distribution expressed in flow cytometry used in cell cycle analysis, apoptosis assay and TUNEL staining were compared using Student's t-test. Statistical significance was accepted at P<0.05.

Results

CKD-602 induced time- and dose-dependent cytotoxicity and proliferation inhibition. Using the cell viability assay, CKD-602 showed significant cytotoxic effect on all cell lines in time- (P<0.05) and dose-dependent (P<0.05) manner (Fig. 1A). The IC_{50} (50% inhibition concentration of cell viability) values were 84.66 nM (95% CI 34.63-148.25) for U87 MG cells, 29.13 nM (95% CI 0.35-101.23) for U343 MG, 14.57 nM (95% CI 0.86-47.33) for U251 MG cells and 9.07 nM (95% CI 0.18-37.42) for LN229 cells at 48 h after treatment.

The antiproliferative effect of CKD-602 was calculated from BrdU staining after 48 h exposure to CKD-602 at various concentrations (Fig. 1B). CKD-602 inhibited the proliferation of cells in a dose-dependent manner in all cell lines. U87 MG and U343 MG cells were more sensitive than U251 MG and LN229 cells after CKD-602 treatment with regard to proliferation inhibition (P<0.05).



U87 MG

	0 μM	0.2 μM	P value
G1	48.98 ± 1.02	35.77 ± 1.72	0.00
S	39.98 ± 0.66	2.70 ± 3.79	0.00
G2	11.05 ± 0.51	61.68 ± 3.85	0.00

U343 MG

	0 μM	0.06 μM	P value
G1	55.71 ± 0.32	8.40 ± 1.58	0.00
S	31.84 ± 1.65	12.50 ± 2.48	0.00
G2	12.44 ± 1.42	79.10 ± 4.05	0.00

U251 MG

	0 μM	0.03 μM	P value
G1	61.41 ± 0.75	63.37 ± 0.56	0.02
S	31.56 ± 1.41	23.85 ± 1.21	0.00
G2	7.03 ± 0.76	12.78 ± 1.25	0.00

LN229

	0 μM	0.02 μM	P value
G1	60.34 ± 0.87	54.21 ± 2.36	0.01
S	27.74 ± 1.51	23.80 ± 3.20	0.13
G2	11.92 ± 0.87	21.98 ± 2.69	0.00

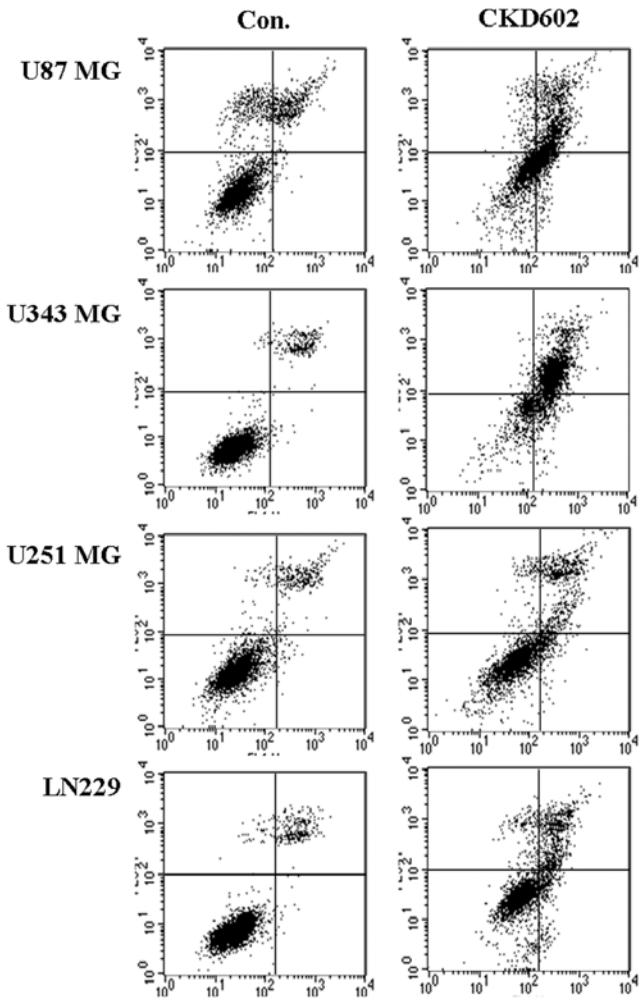
Figure 2. Cell cycle analysis using flow cytometry analysis of glioma cells treated without or with CKD-602 treatment. All cell lines showed G2 phase arrest. Cells were harvested at 48 h and analyzed for DNA content by propidium iodide staining.

CKD-602 induces cell cycle arrest at G2 phase and apoptosis. Cell cycle analysis was performed after treatment with different concentrations (U87 MG; 0.2 μM, U343 MG; 0.06 μM, U251 MG; 0.03 μM, LN229; 0.02 μM, respectively) of CKD-602 for 48 h in each cell line. CKD-602 induced G2 cell accumulation in all cell lines, and the block of G2 phase was higher in U87 MG and U343 MG than in U251 MG and LN229 (Fig. 2). We also observed apoptotic death in all cell lines in different concentrations (U87 MG; 0.2 μM, U343 MG; 0.06 μM, U251 MG; 0.03 μM, LN229; 0.02 μM respectively) of CKD-602 for 48 h by Annexin V and TUNEL staining. FITC-conjugated Annexin V signals demonstrated a significant increase in apoptotic cells after CKD-602 treatment ($P<0.05$, Fig. 3). TUNEL staining also showed that the numbers of apoptotic nuclei increased after CKD-602 treatment. After CKD-602 treatment, 44.25±1.31% in the U87 MG cells, 40.87±5.30% in the U343 MG cells, 9.93±1.59% in the U251 MG cells and 17.62±4.73% in the LN229 cells were positive at TUNEL staining (Fig. 4).

Discussion

CKD-602 is a potent topoisomerase I inhibitor that successfully overcomes the poor water solubility and toxicity of its parent drug, camptothecin (28). It showed potent anticancer activity against various tumor cells *in vitro* and *in vivo* in animal models (19-21). In phase I and II studies for solid cancers, CKD-602 showed significant anticancer activity against ovarian cancer (overall response 45%) and small cell lung cancer (overall response 45%) and dose-limiting toxicity was demonstrated by neutropenia and thrombocytopenia, but not by diarrhea, which is the prototype toxicity of CPT-11 (26-28). Clinical trials including CPT-11 or topotecan for malignant glioma are ongoing and have shown promising results (29,30). Therefore, CKD-602 also appears to have promise in the treatment of patients with gliomas based on its potent anticancer effect, with improved tolerability compared with previous camptothecin derivatives.

In the present study, CKD-602 showed cytotoxicity, causing antiproliferative activity and apoptosis in glioma cell lines in a dose- and time-dependent manner. The cytotoxic effect of CKD-602 was more prominent in the U251 MG and LN229 cell lines with *p53* mutation than in the U87 MG and U343 MG cell lines without *p53* mutation. The U87 MG and U343 MG cell lines showed more prominent inhibition of proliferation, cell cycle arrest at G2 phase and apoptosis than the U251 MG and LN229 cell lines after CKD-602 treatment. The influence of the *p53* gene on the efficacy of topoisomerase inhibitors has been reported with controversy. Wang *et al* demonstrated that *p53* disruption dramatically sensitizes glioblastoma cells to DNA topoisomerase I inhibitor-mediated apoptosis while wild-type *p53* cell lines arrest and develop a senescence-like phenotype after SN-38 treatment (31). In the screening study correlating the effect of anti-



U87 MG

%	0 μM	0.2μM	P value
Viable	73.84 ± 2.34	33.00 ± 5.40	0.00
apoptotic	16.61 ± 0.69	62.51 ± 6.68	0.00
necrotic	9.55 ±2.40	4.48 ± 1.28	0.01

U343 MG

%	0 μM	0.06 μM	P value
Viable	89.97 ± 1.15	14.79 ± 1.52	0.00
apoptotic	9.52 ± 0.92	85.30 ± 1.68	0.00
necrotic	0.52 ± 0.23	1.41 ± 0.26	0.00

U251 MG

%	0 μM	0.03 μM	P value
Viable	85.32 ±1.63	68.85 ± 1.70	0.00
apoptotic	11.60 ± 0.77	27.34 ± 1.72	0.00
necrotic	9.11 ± 4.41	3.82 ± 0.12	0.50

LN229

%	0 μM	0.02 μM	P value
Viable	91.76 ± 0.53	59.32 ± 9.29	0.00
apoptotic	7.35 ± 0.29	33.99 ± 12.98	0.01
necrotic	0.89 ± 0.34	3.82 ± 0.83	0.00

Figure 3. Induction of apoptosis and necrosis in glioma cells after CKD-602 treatment for 48 h demonstrated by FITC-conjugated Annexin V and propidium iodide staining. Significant increases in apoptotic cells after CKD-602 treatment are shown.

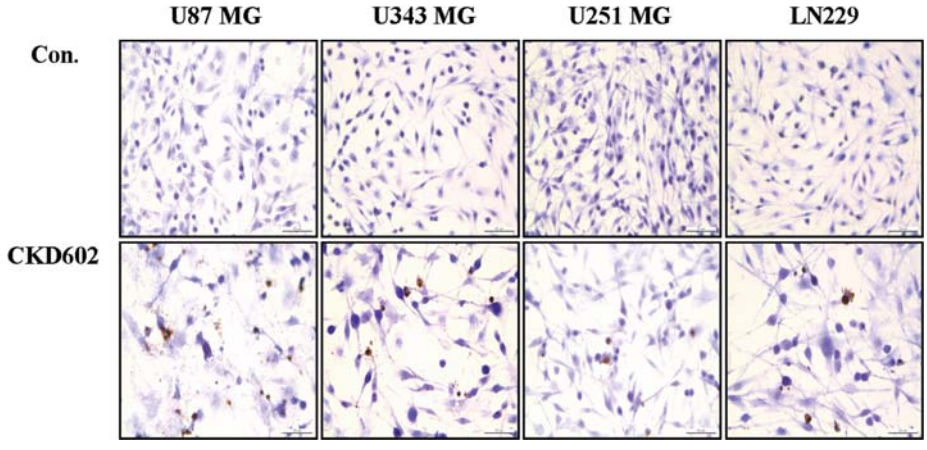


Figure 4. Apoptotic cells were observed after CKD-602 treatment for 48 h following terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (x400 original magnification).

cancer drugs in cell lines with their *p53* status, mutant *p53* sequence tended to exhibit less growth inhibition than the wild-type *p53* cell lines when treated with the majority of clinically used anticancer agents including topoisomerase I inhibitor (32). However, there are recent studies denying the role of *p53* in differential susceptibility to topoisomerase I inhibitor (33,34). Based on this evidence, the relationship between *p53* status and the effect of topoisomerase I inhibitors, including CKD-602, requires further evaluation and study.

Our result suggest that CKD-602 blocks the progression of cell cycles in G2 phase cells, leading to apoptosis and that CKD-602 induced more increase in the percentage of cells in G2 phases in U87 MG and U343 MG than in U252 MG and LN229. This findings coincide with other studies with CPT-11. Nakatsu *et al* (35) reported a decrease in the percentage of cells in G1 phase and an increase in the percentage of cells in S and G2 phases in human glioblastoma GB-1 cells and U87 MG cells after treatment with SN-38. Moreover, CPT induced cytotoxicity to both U251 MG and DAOY cells and produced prolonged G2 delay in these cells (36). This evidence implies that the CKD-602 shares its mechanism of action with its prototype, CPT-11.

In conclusion, CKD-602 showed *in vitro* anticancer efficacy against human malignant glioma cell lines. This potent anticancer activity suggests that CKD-602 is a promising candidate for treating patients with malignant gliomas. Our results provide a rationale for further evaluation of CKD-602 for glioblastomas by *in vivo* and clinical studies.

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