A Novel Camptothecin Analog with Enhanced Antitumor Activity

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Abstract. *Background: The use of camptothecin (CPT) derivatives is limited by severe toxicity associated with the instability of their chemical structure. We have developed a stable CPT derivative (CPT417) and have investigated its biological activity in comparison to the currently used CPT analogs. Materials and Methods: The anticancer effects of CPT417 were assessed in vitro, with glioblastoma and colon cancer cell lines, and in vivo with mice bearing mammary adenocarcinoma tumors implanted subcutaneously in the flank. Cytotoxicity was assessed using vital dye exclusion, timelapse microscopy and colony formation. Results: CPT417 and topotecan inhibited glioblastoma cell growth at comparable levels and both compounds inhibited clonogenicity of colon cancer cells more effectively than irinotecan. CPT417 showed a much greater inhibition of mammary tumor growth compared to topotecan, both by intraperitoneal and oral administration. Conclusion: CPT417 shows dramatically reduced toxicity and an enhanced antitumor activity compared to topotecan.*

Drugs that target deoxyribonucleic acid (DNA) are among the most effective chemotherapeutic agents used clinically but treatment related toxicity and the development of drug resistance limit their effectiveness (1, 2). The cellular responses to DNA damage include damage recognition, repair and induction of signaling cascades leading to cell cycle checkpoint activation, apoptosis and stress related responses (3). Cells typically are arrested at multiple cell cycle checkpoints, at which cells attempt to repair damaged DNA before progressing into mitosis, and numerous key molecules have been identified including damage sensor proteins, transducer kinases and effectors. One of the most important pathways of such checkpoints in mammalian cells is that of double-strand break repair (4).

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Camptothecin (CPT) derivatives emerged as a promising group of chemotherapeutic agents in the 1960's because of their inhibition of topoisomerase I leading to DNA damage (5). Topoisomerase I is a ubiquitous enzyme involved in the regulation of DNA topology during replication, recombination and transcription. Topoisomerase I forms a phosphotyrosine bond with DNA, catalyzing a forward reaction in which DNA is cleaved to allow unwinding, and a reverse reaction, in which DNA is re-ligated (6). CPT interferes with the re-ligation step of this process by reversibly binding to and stabilizing the enzyme/DNA complex. Once the CPT molecule has intercalated with the topoisomerase I-DNA complex, the collision between the complex and the replication fork during the S-phase is thought to result in DNA double-strand breaks (7). Additionally, anticancer properties of CPT that are S-phase-independent have been elucidated, including rapid cessation of RNA synthesis (8) and interference with the cell cycle checkpoint network by inhibiting checkpoint kinase 1 (9). With respect to antitumor activity, the lactone E ring in the CPT molecular structure has been identified as the most critical structural feature (10, 11).

Clinical trials proved CPT to have potent antitumor properties but it was found to be too toxic for practical use. Since then, modifications to the parent molecule have been made to increase efficacy and decrease toxicity (12). In this regard, topotecan and irinotecan are two of the most widely evaluated camptothecin derivatives. However, a phaseI/II clinical trial evaluating combined treatment with topotecan and irinotecan in patients with metastatic colon cancer found no complete or partial responses at doses where toxicity was low (13). Trials evaluating higher doses of these camptothecins against multiple cancer types found some partial responses but hematological toxicities were severe, with neutropenia and thrombocytopenia being the most common (14-16).

The intrinsic instability of the lactone E ring is a major contributing factor to the unwanted toxicity associated with the CPT derivatives used currently. The instability results in an inactive ring-opened carboxylate at pH greater than 4 and in plasma this causes reduced potency (17, 18). The lactone ring of topotecan, for example, is partially

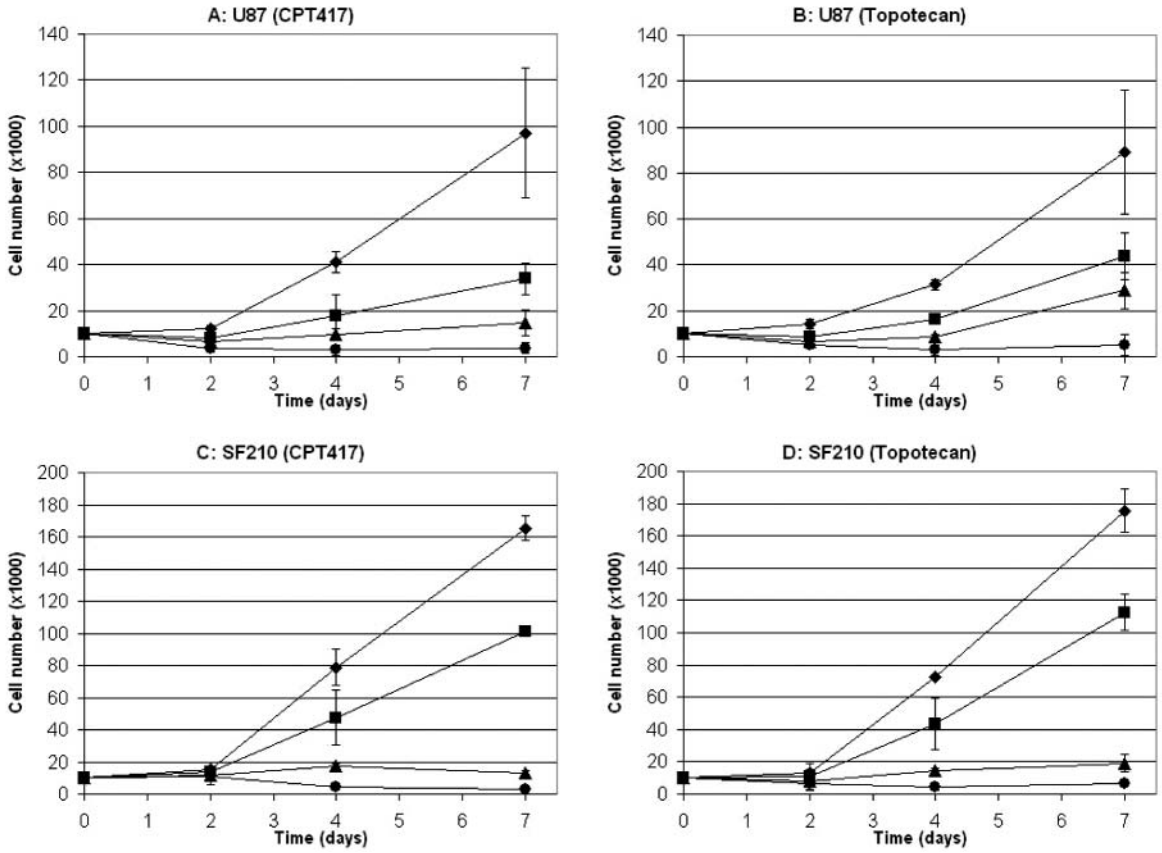

Figure 1. *Growth curves for glioblastoma cells. U87 MG (wild-type p53) and SF210 (mutant p53) glioblastoma cell cultures were analysed for cell viability by trypan blue exclusion for seven days following treatment with CPT417 (A & C) or topotecan (B & D). The concentrations of drugs used were 0 nM (diamonds), 10 nM (squares), 20 nM (triangles) and 40 nM (circles). Shown are the mean and standard deviation of three independent experiments.*

hydrolyzed even prior to clinical administration in parental solution and only a small percentage of lactone rings remain intact 30 minutes after administration (19). In addition to reduced potency, the inactive carboxylate is generally toxic and causes a host of negative side-effects.

We previously characterized a series of novel camptothecin derivatives designed for enhanced stability through increased steric hindrance at the C20(S) position of the lactone E ring (20). The camptothecin-20-(S)-4-fluorophenoxy-acetic acid ester derivative designated CPT417 was characterized as being highly stable and relatively less toxic. Here we evaluate its anticancer potential in both *in vitro* and *in vivo* cancer model systems.

Materials and Methods

Cell culture and analysis of cell fate. Four human cell lines were evaluated: the U87 MG glioblastoma cell line and the VM46 human colon cancer cell line obtained from the American Type Culture Collection (Manassas, VA, USA) and the glioblastoma cell lines SF210 and SF188 from the University of California San Francisco Tumor Bank. Cells were maintained as exponentially growing monolayer cultures at 37˚C and 5% CO_2 in RPMI media supplemented with 50 ng/ml gentamicin, 50 ng/ml amphotericin, penicillin, streptomycin, and 10% fetal bovine serum. Growth serum concentration was reduced to 5.0% prior to drug treatments. Cell viability was quantified in six-well plates with 100,000 cells seeded in each well 24 hours prior to experimental treatment. At specified times after treatment, adherent cells were harvested by trypsinization, pooled with nonadherent cells, stained with Trypan blue dye, and counted on a hemacytometer.

Computerized timelapse microscopy allowed direct observation of morphological changes associated with apoptosis in cell populations across multiple microscopic fields. The fate of approximately 400 cells over a 30-hour observation period was documented using an inverted phase contrast microscope (Axiovert 200; Zeiss, Gottingen, Germany) equipped with an onstage environmental chamber. A software automation program (Openlab; Improvision, Lexington, MA, USA) drove the camera and stage movements and compiled the acquired phase images. Images were subsequently processed as QuickTime movies using Openlab.

Clonogenic survival analysis was performed using six-well plates prepared with a feeder layer of lethally irradiated cells and experimental cells seeded at three different dilutions (50, 100, and

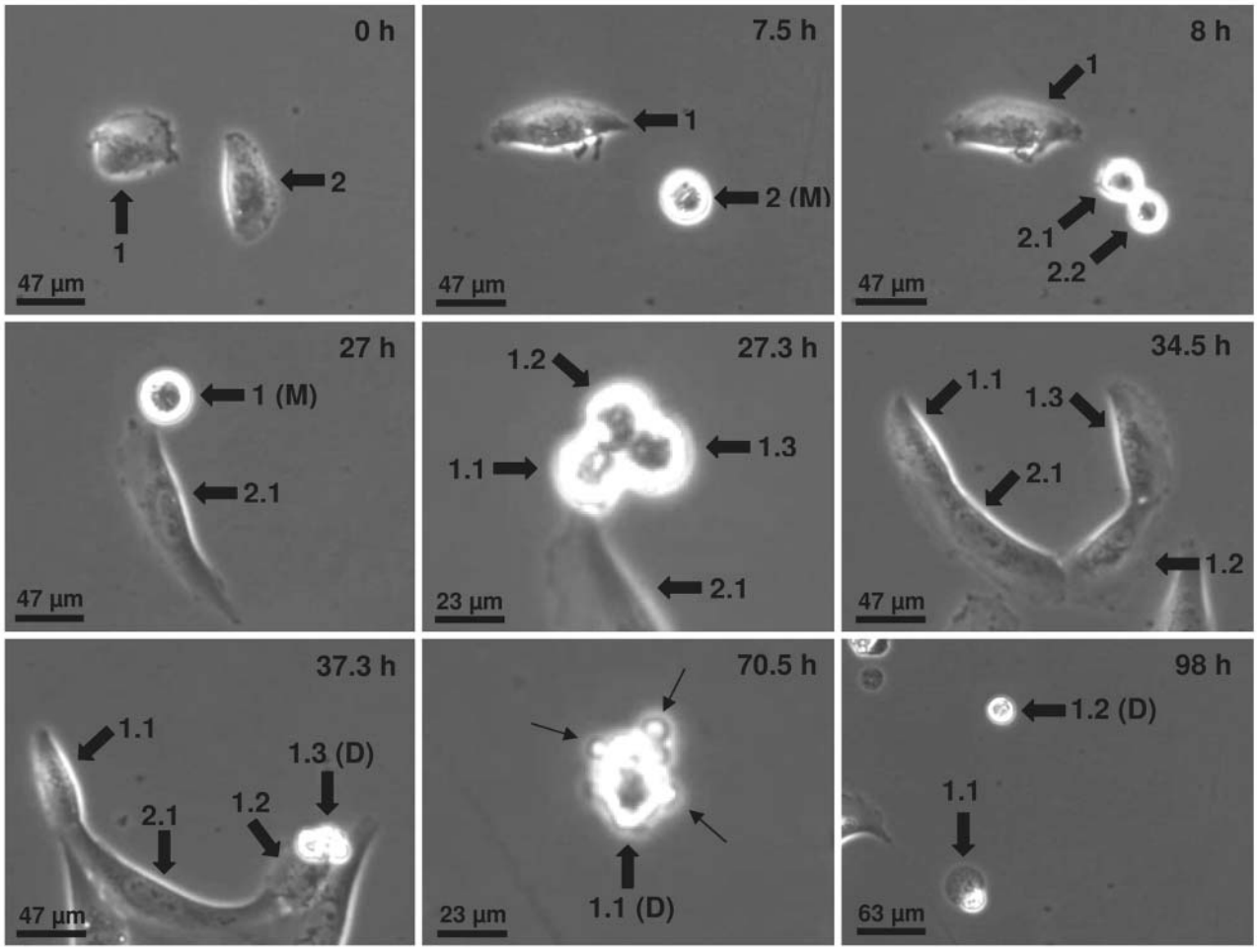

Figure 2. *Cell cycle disturbances in glioblastoma cells. Phase contrast images from timelapse videos follow human SF188 GBM cells (numbered 1 and 2) and their progeny (1.1, 1.2, 1.3 and 2.1, 2.2) treated with 20 nM CPT417. Elapsed time in hours is indicated in upper right corner of each panel with the start of drug treatment designated at time equals 0 hours. Cells are indicated by thick arrows and approximate times of mitosis (M) and cell death (D) are indicated. The 70.5 hour panel is shown at higher magnification to highlight the membrane blebbing characteristic of apoptosis (thin arrows).*

150 cells per well; each dilution plated in multiples of six). These cultures were then incubated for 21 days; colonies of >50 cells were then counted from equivalent dilutions. Cell survival was calculated as the percentage of values obtained from vehicle-treated (control) cultures using the following formula: cell survival (%)=number of colonies in treated group/mean number of colonies in untreated (control) group X 100%.

In vivo tumor model. Maximum tolerated doses at 40 days (MTD_{40}) for healthy male C3H/HeN mice (Simonsen Laboratories Inc., Gilroy, CA, USA) were determined following published methods (21). Mice (20-22 g) were allowed one week for acclimatization prior to MTD_{40} determination. MTG-B mammary adenocarcinoma tumors were established subcutaneously in the flank. After tumors grew to 8 mm diameter (268.08 mm^3), the mice were randomly divided into four groups of ten. CPT417, topotecan or vehicle were administered by intraperitoneal injection to three groups of mice with a single dose (MTD_{40}; injection volume=0.3 ml/20 g animal weight) and a fourth group of mice was treated with CPT417 by oral administration. Drugs were delivered in a solution of 5% ethanol, 5% cremophor EL, 95% saline. Tumors diameters were measured daily using calipers and the time required for tumors to grow to twice their initial size was calculated as the tumor doubling time, with the treatment day defined as day one for all calculation and plots.

Results

CPT417 and topotecan inhibit glioblastoma cell proliferation. We evaluated the cytotoxicities of CPT417 and topotecan using two human cell lines derived from glioblastoma tumors: U87 MG and SF210. U87 MG cells are highly resistant to anticancer therapies (22, 23), possibly contributed to by expression of membrane Met receptors and secretion of significant levels of hepatic growth factor (24). SF210 cells were chosen because they are typical of the

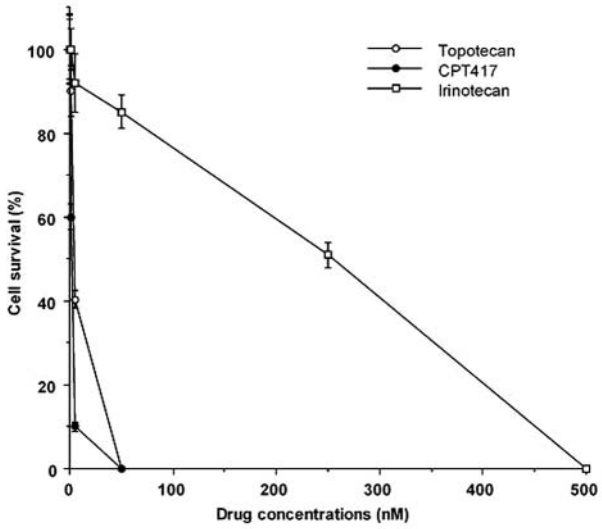

Figure 3. *Clonogenic survival curves for colon cancer cells. VM46 human colon cancer cells were exposed to increasing doses of CPT417, topotecan or irinotecan for 72 hours. Colonies of >50 cells were counted after 21 days of incubation and the percent survival relative to the mean number of colonies in untreated (control) group is plotted with standard deviations.*

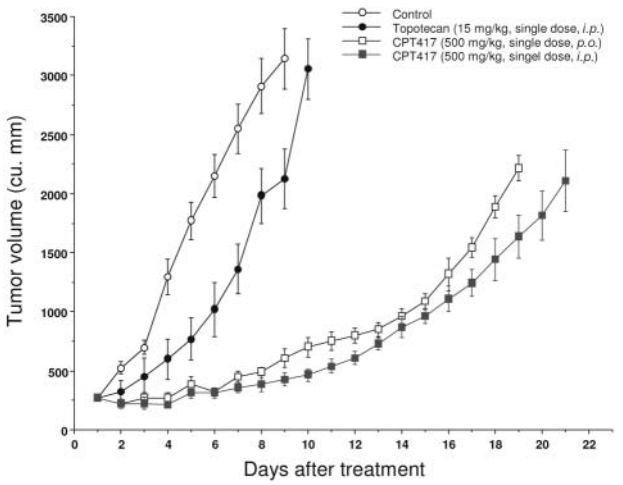

Figure 4. *In vivo efficacies of CPT417 and topotecan against mammary adenocarcinoma tumors. MTG-B mammary adenocarcinoma tumors were established subcutaneously in the flank of C3H/HeN mice. CPT417, topotecan or vehicle with no drug were administered by a single intraperitoneal injection (i.p.) to three groups of mice. Another group of mice was treated by oral administration (p.o.) with CPT417. Average tumor volumes are plotted (n=10).*

majority of glioblastoma tumors in that they express a mutant form of the p53 tumor suppressor gene (25). Both drugs exhibited similarly potent antiproliferative properties against both of the glioblastoma cell lines (Figure 1); no statistical differences were noted.

CPT417 causes aberrant cell division and prolonged cell cycle arrest. Computerized timelapse microscopy allowed the documentation of cell cycle disturbances in human glioblastoma cells treated with CPT417 that are consistent with findings reported for other cell types treated with camptothecins (26-28). Figure 2 shows selected images from a four-day timelapse video following two representative SF188 glioblastoma cells after treatment with CPT417. The average cell cycle time of untreated SF188 cells was 23 hours (SD=4.5 hours). The first cell underwent an aberrant division (tri-pole) 27 hours after drug exposure and all three of its daughter cells eventually exhibited morphological characteristics typical of apoptotic cell death. The second cell underwent mitosis 7.5 hours after treatment (indicating drug exposure during the G2-phase) and both daughter cells were arrested in the cell cycle for the duration of the experiment (91 hours), probably in S-phase due to the mutant status of p53 in these cells (25).

CPT417 inhibits colon cancer cell colony formation. We compared the influences of CPT417, topotecan and irinotecan on clonogenic survival of human colon cancer cells. We used the VM46 colon cancer cell line that expresses a multidrug resistance phenotype (29). Clonogenicity was determined by colony forming efficiency. CPT417 and topotecan performed similarly in this long-term viability assay but irinotecan was less effective (Figure 3).

CPT417 inhibits tumor growth in mice more effectively than topotecan. To evaluate the potential for CPT417 to inhibit the growth of solid tumors, we studied mice bearing mammary adenocarcinoma tumors subcutaneously in the flank. The MTG-B human adenocarcinoma cell line was used because it expresses a multidrug resistance phenotype (30). The effect on the growth of tumors was assessed after a single administration of CPT417 or topotecan at the MTD_{40} determined for each agent (500 mg/kg and 12 mg/kg, respectively). Tumor growth in control mice was rapid with a tumor doubling time (TDT) of 1 day (Figure 4). Topotecan inhibited tumor growth slightly when comparing drug treatments and the control group (TDT=4.5 days) but CPT417 delivered by the same route was significantly more effective in causing growth delay (TDT=26 days; $p<0.001$ by *t*-test), as was CPT417 delivered orally (TDT=9 days; $p<0.001$ by *t*-test).

Discussion

The unique pharmacokinetic parameters of the highly stable lactone ring E of CPT417 likely account for its relative lack of toxicity and enhanced efficacy *in vivo*.

No toxicity was noted in mice treated with CPT417 at doses as high as 500 mg/kg and the TDT value for CPT417

by intraperitoneal injection was 7.1 times higher than that of topotecan. When CPT417 was administered orally, its TDT value was 2.3 times higher than that of topotecan. These differences in therapeutic effect could be explained by CPT417's bioavailability in mice.

CPT417 maintains potent anticancer properties against multiple cancer cell types, including cells expressing the multidrug resistance phenotype. Importantly, CPT417's cytotoxic effects appear to be independent of p53 function as evidenced by its activity against glioblastoma cells harboring mutant p53. Timelapse video stills presented here suggest that cells treated with CPT417 may overcome cell cycle checkpoints, leading to mitotic catastrophe (31) and eventually apoptosis. These data are consistent with the ability of CPT to inhibit checkpoint kinase 1 (32) and suggest that CPT417 could potentiate the efficacy of DNA damaging agents against p53-deficient cancer cells.

Synthesis of CPT417 involves only a one step reaction and does not require rigorous synthesis conditions (20). Moreover, all the raw materials for synthesis of CPT417 are inexpensive. This is in contrast to the synthesis of other CPT derivatives that include multiple-step reactions and expensive raw materials (33). The yields of CPT417 synthesis are also much higher than those for topotecan or irinotecan (20, 33). These practical advantages, combined with the relative lack of toxicity and improved efficacy, suggest a great potential for CPT417 as an antitumor agent.

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