Potent Topoisomerase I Inhibition by Novel Silatecans Eliminates Glioma Proliferation in Vitro and in Vivo

Ian F. Pollack, Melanie Erff, David Bom, Thomas G. Burke, J. Thompson Strode, and Dennis P. Curran

ABSTRACT

Although topoisomerase inhibitors, such as camptothecin and topotecan, have been widely used in the treatment of nonglial tumors, their application for gliomas has been limited by poor efficacy relative to toxicity that may in part reflect limited bioavailability and blood stability of these agents. However, the potential promise of this class of agents has fostered efforts to develop new, more potent, and less toxic inhibitors that may be clinically relevant. Using a cascade radical annulation route to the camptothecin family, we developed a series of novel camptothecin analogues, 7-silylcamptothecins (“silatecans”), that exhibited potent inhibition of topoisomerase I, dramatically improved blood stability, and sufficient lipophilicity to favor blood-brain barrier transit. We explored the efficacy of a series of these agents against a panel of five high-grade glioma cell lines to identify a promising compound for further preclinical testing. One of the most active agents in our systems (DB67) inhibited tumor growth in vitro with an ED50 ranging between 2 and 40 ng/ml, at least 10-fold more potent than the effects observed with topotecan, and at least comparable with those of SN-38, the active metabolite of CPT-11. Because DB67 also exhibited the highest human blood stability of any of the agents examined, this agent was then selected for in vivo studies. A dose-escalation study of this agent in a nude mouse U87 glioma model system demonstrated a concentration-dependent effect, with tumor growth inhibition at day 28 postimplantation (the day control animals began to require sacrifice because of large tumor size) of 61 ± 7% and 73 ± 3% after administration of DB67 doses of 3 and 10 mg/kg/day, respectively, for 5 days beginning on postimplantation day 7. Animals that continued treatment with 10 mg/kg/day in three additional 21-day cycles all remained progression free after >90 days of follow-up but later developed enlarging tumors after treatment was stopped. However, a slightly higher dose (30 mg/kg/day) induced complete tumor regression after only two cycles in all study animals and was effective even if treatment was delayed until large, bulky tumors had developed. Application of the 30 mg/kg/day dose to treated established intracranial glioma xenografts led to long-term (>90 day) survival in six of six animals, whereas all controls died of progressive disease (P < 0.00001). No apparent toxicity was encountered in any of the treated animals. In summary, the present studies indicate that silatecans may hold significant promise for the treatment of high-grade gliomas and provide a rationale for proceeding with further preclinical evaluation of their efficacy and safety versus commercially available camptothecin derivatives.

INTRODUCTION

High-grade gliomas present a major therapeutic challenge (1–4) because these poorly circumscribed, biologically aggressive lesions typically are refractory to surgery, radiotherapy, and conventional chemotherapy. Accordingly, there is a strong rationale for examining the efficacy of novel chemotherapeutic strategies as a means for enhancing disease control. Topo1 inhibitors are a class of agents that interfere with DNA “unwinding” during DNA replication and RNA transcription and stabilize DNA-topo I complexes through noncovalent interactions to yield enzyme-linked DNA single-strand breaks. Prolonged exposure to these agents in replicating cells produces lethal double-strand DNA breaks that can trigger the induction of programmed cell death (5). In this way, these agents function by subverting the normal topo I enzyme to “poison,” and ultimately induce cytotoxicity in, rapidly dividing tumor cells (6), which typically have high levels of topo I activity (7–9). Topo I inhibitors have shown significant promise as antineoplastic agents in in vitro studies. Unfortunately, early topo I inhibitors, such as camptothecin (10, 11), suffered from poor solubility, limited bioavailability secondary to albumin binding, and rapid hydrolysis at physiological pH of the lactone ring to an inactive open form (6, 11).

Subsequently, a number of camptothecin derivatives, such as topotecan and irinotecan (CPT-11), were developed that exhibited somewhat improved aqueous solubility and bioavailability (12, 13). Topotecan has shown substantial promise in preclinical and initial clinical studies with several tumor types (6, 11, 14, 15), but this activity has yet to be duplicated in patients with brain tumors (16–19), despite the apparent cerebrospinal fluid penetration of this agent (20). In one study that used a 24-h infusion of 5.5–7.5 mg/m2 every 21 days, no activity was observed in nine high-grade, nonbrainstem gliomas or in 14 brainstem gliomas (16). Other studies of topotecan in recurrent central nervous system tumors have also observed little if any activity, even using high doses (3.5 mg/m2/day administered in 5-day courses; Refs. 16–19). One potential shortcoming of topotecan that may account for its limited clinical activity against brain tumors is its rapid hydrolysis to the inactive open-ring form (13, 21, 22), which may limit the ability of therapeutically relevant concentrations of the active drug to persist within the tumor for a sufficient duration to induce cytotoxicity. CPT-11, which is also undergoing early clinical testing (12, 23–26), is actually a largely inactive prodrug that is metabolized to form SN-38 (27); significant interpatient differences in metabolism may account for wide variability in the accumulation of active and inactive drug metabolites and hence both toxicity and efficacy (23, 28, 29).

Recognizing the potential promise but apparent limitations of the available topo I inhibitors, we undertook synthesis of a series of novel camptothecin derivatives, using a cascade radical annulation route to the camptothecin family. To enhance lipophilicity (and the potential for transfer across the blood-brain barrier) as well as potentially inhibit hydrolysis and albumin binding and increase blood stability, a series of 7-silyl-modified camptothecins (silatecans) were developed. These agents exhibit potent inhibition of topo I (30, 31), dramatically improved blood stability, and profound inhibition of glioma growth in vitro, with some derivatives exhibiting more than 10-fold greater...
potency than topotecan. More importantly, one of the most active compounds, DB67, which also exhibited the greatest blood stability, demonstrated striking antiproliferative activity in vivo in a series of nude mouse glioma models, with no overt toxicity.

MATERIALS AND METHODS

Cell Culture. The human malignant glioma cell lines U87 and A172, which contain wild-type p53, and T98G, which harbors a homozygous p53 mutation, were obtained from the American Type Culture Collection. LNZ308, a p53-mutated glioma cell line, was kindly provided by Dr. Nicolas de Tribolet (University of Lausanne, Lausanne, Switzerland). Each of these cell lines was maintained in growth medium consisting of α-minimal essential medium supplemented with L-glutamine, ribonucleosides, deoxyribonucleosides, 10% FCS (Life Technologies, Inc., Grand Island, NY), and the following antimicrobial agents: 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cultures were established in 75-cm² flasks (Costar, Cambridge, MA), maintained at 37°C in a humidified atmosphere with 5% CO₂ in air, and subcultured every 4–7 days with 0.25% trypsin in HBSS (Life Technologies).

The low-passage, p53-wild-type malignant glioma cell line (SG388) was established at our institution from a tumor specimen that had been transported directly from the operating room to the laboratory in α-MEM. The histopathological diagnosis was confirmed by a neuropathologist. The specimen was finely dissected to remove excess blood and necrotic material, cut into pieces 1–1 mm³ in diameter, and dissociated by incubation for 1 h at 37°C in 0.25% trypsin and 0.02% DNase. Dissociated cells were filtered through 100-μm nylon mesh and then cultured for 48 h in growth medium. Medium containing nonadherent cells was removed, and fresh medium was applied. Cells were subcultured after reaching confluence and studied in approximately the tenth passage in vitro.

Topo I Inhibitors. A series of 7-silyl camptothecin derivatives were developed using a cascade radical annulation route as described by Curran and co-workers (30–33). The structures of several of these agents are summarized in Fig. 1 and Table 1. These compounds were selected for their potential lipophilicity and topo I inhibitory activity. Many of these agents had been previously reported to have excellent potency in inhibiting topo I-mediated DNA relaxation and enhancing topo I-mediated DNA cleavage (30). Topotecan was obtained from SmithKline Beecham Pharmaceuticals (Philadelphia, PA). Samples of 10,4-methylenedioxycamptothecin were obtained from the NCI, and 7-ethyl-10,11-methylenedeoxycamptothecin was provided by Dr. Mansukh Wani (Research Triangle Institute, NC). All compounds were prepared as 1 mg/ml stock solutions in DMSO and diluted in nutrient medium.

MTS Assay of Cell Proliferation. A colorimetric cell proliferation assay was used in each of the aforementioned cell lines to assess the effect on cell proliferation of a series of 7-silylcamptothecin derivatives versus commercially available derivatives, such as topotecan, and CPT-11, and other promising agents, such as 9-aminocamptothecin, SN-38 (the active metabolite of CPT-11), and 11,11-methylenedioxy-camptothecin derivatives. For these studies, 2 × 10⁴ cells were plated and grown for 12 h in 100 μl of growth medium in 96-well microtiter plates (Costar, Cambridge, MA) and then treated for 4 days with various concentrations of each agent, prepared from a 1-mg/ml stock solution dissolved in DMSO. Control cells were treated with equivalent concentrations of DMSO alone. In all cases, final concentrations of DMSO were ≤0.1%, well below the concentrations that interfere with proliferation in the above cell lines. All studies were performed in triplicate.

After a 4-day incubation period, the number of viable cells was determined by measuring the bioreduction by intracellular dehydrogenases of the tetrazolium compound MTS in the presence of the electron coupling reagent PMS (34). To perform the assay, 20 μl of combined MTS/PMS solution containing 2 mg/ml MTS and 150 μM PMS in buffer (0.2 g/l KCl, 8.0 g/l NaCl, 0.2 g/l KH₂PO₄, 1.15 g/l Na₂HPO₄, 133 mg/l CaCl₂·2H₂O, and 100 mg/l MgCl₂·6H₂O, pH 7.35; Promega Corp., Madison, WI) was added to each well, and the mixture was incubated for 3 h at 37°C in a humidified 5% CO₂ atmosphere. Absorbance at 490 nm was measured using an ELISA microplate reader. Background absorbance of the medium was measured in a triplicate set of control wells that contained medium and the MTS/PMS solution without added cells and was subtracted from the absorbance measured in each of the sample wells to provide a corrected absorbance for each of the wells. Triplicate wells with predetermined cell numbers were subjected to the above assay in parallel with the test samples; this also provided internal confirmation that the assay was linear over the range of absorbances and, hence, cell numbers measured.

Assessment of Blood Stability. The stabilities in human blood of several of the most active camptothecins were assessed using HPLC assays. Whole human blood was obtained from a healthy male donor by drawing into sterile vacutainers containing sodium heparin. The pH of the blood was checked before and after an experiment to confirm that the pH remained at 7.4 ± 0.1. Drug stock solutions were prepared in DMSO at drug concentrations of 2 mM. Aliquots of 1 μl of the DMSO stock drugs were added to 2 ml of blood incubated at 37°C to make a final drug concentration of 1 μM. At different time points, 150-μl aliquots of the blood samples were removed and added to 600 μl of chilled methanol (−20°C) in an HPLC autosampler vial. The vial was vortexed and then centrifuged at 8000 rpm for 45 s. The supernatant of each sample was immediately placed in the autosampler, and HPLC analysis was carried out.

All HPLC analyses were carried out on a Waters Alliance 2690 Separations Module equipped with a Waters Model 474 variable wavelength fluorescence detector. Baseline separations of lactone and carboxylic acids of each of the agents allowed for simultaneous quantification of each species. Lactone and carboxylic acid levels were determined by measuring the peak areas corrected for subtle changes in fluorescence intensity caused by the opening of the lactone ring. Separations were carried out on a Waters Symmetry C-18 column. Conditions for quantitation were optimized for individual camptothecin derivatives. For DB-67, the mobile phase consisted of 40% acetonitrile and 60% of an aqueous buffer containing TEA and acetate. The TEA/acetate buffer (pH 5.5) contained 2% TEA added to distilled, deionized water with pH adjustment to 5.5 made with concentrated acetic acid. Fluorescence excitation for DB-67 was set at 380 nm and emission at 560 nm. Retention times for the lactone and carboxylic species of DB-67 were approximately 6 min and 2 min, respectively. Conditions for the quantitation of SN-38 in blood have been described previously (35–37). For 10,11-methylenedioxyacamptothecin, the mobile phase consisted of 29% acetonitrile and 71% TEA/acetate buffer. Excitation and emission detector settings of 370 and 440 nm, respectively, were used. For 7-ethyl-10,11-methylenedioxyacamptothecin, the mobile phase consisted of 35% acetonitrile and 65% TEA/acetate buffer (excitation and emission detector settings of 370 nm and 440 nm, respectively). Flow rates of 1 ml/min were used in all experiments. The percentage of lactone at equilibrium values representing relative drug stability was determined from the decay profiles by the method of nonlinear least squares.

Assessment of Cell Viability. Among the agents examined in our initial studies, DB67 exhibited the greatest human blood stability and among the most potent effects on cell proliferation. The effect of this agent on cell viability was then examined using a clonogenic assay. For these studies, 250 U87 and SG388 cells were plated and, after an overnight attachment period, grown as described above with selected concentrations of DB67 and with appropriate vehicle controls. To evaluate the effect of exposure time on toxicity, cells were treated with DB67 (or vehicle) for periods of 6 and 96 h. After the treatment period, the cells were washed with inhibitor-free medium and grown in serum-supplemented medium for an additional 2-week period. The plates were then stained with crystal violet, and colonies consisting of groups of 50 or more cells were counted.

Fig. 1. Molecular structure of the parent compound, camptothecin, indicating the locations of the structural modifications noted in Table 1.
Table 1  Median effective concentrations for inhibition of the proliferation of five glioma cell lines by a variety of camptothecin derivatives

<table>
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<tr>
<th>Name</th>
<th>$R^7$</th>
<th>$R^9$</th>
<th>$R^{10}$</th>
<th>$R^{11}$</th>
<th>ED50 U87 ng/ml</th>
<th>ED50 A172 ng/ml</th>
<th>ED50 SG388 ng/ml</th>
<th>ED50 LN-Z308 ng/ml</th>
<th>ED50 T98G ng/ml</th>
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<td>H</td>
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<td>H</td>
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<td>3</td>
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<td>60</td>
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<td>6</td>
<td>3</td>
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Fig. 2. Relationship between topo 1 inhibitor concentration and cell numbers, as assessed semi-quantitatively by a spectrophotometric measurement of MTS bioreduction, is illustrated in the U87 established malignant glioma cell line. Points represent the mean of three measurements; bars, SD. No significant inhibition was detected in control cells treated with equivalent concentrations of vehicle.
Assessment of Antiproliferative Activity in a Nude Mouse s.c. Model.

In vivo assessment of the effect of DB67 on glioma proliferation was performed using a nude mouse s.c. model of the U87 glioma cell line. This model was chosen so that tumor size could easily be followed serially in each animal. For these studies, 4-week-old nu/nu mice (obtained from the National Cancer Institute) were injected in the right flank with $2 \times 10^5$ tumor cells in a volume of 100 μl, suspended in a 1:1 mixture of PBS:Matrigel (Collaborative Research, Bedford, MA). This dose of cells invariably produces palpable tumors within 7 days. Animals were examined daily for evidence of tumor growth, and treatment was initiated when tumors of $\geq 0.5$ cm in diameter were identified. Animal weights were also recorded twice weekly. Animals were sacrificed when maximum tumor diameter in any plane was $\geq 1.5$ cm, in accordance with Animal Research and Care Committee guidelines. Animals that survived to 28 days without requiring sacrifice were retreated with up to three additional 21-day cycles of therapy.

Assessment of Tumor Regression in a Nude Mouse Model. To determine whether DB67 was capable of inducing regression of large, established tumors, cells were implanted as described above and allowed to grow until the maximal tumor diameter exceeded 1 cm. In the absence of treatment, such animals invariably require sacrifice within 1–5 days. Animals then began treatment with 30 mg/kg/day of DB67 for 5 days in two 21-day cycles. Tumor volumes and animal weights were recorded as noted above.

Assessment of Tumor Growth Inhibition in an Intracranial Model.

Animals were anesthetized with ketamine (100 mg/kg) and acepromazine (0.02 mg/kg) and immobilized in a stereotactic frame. Through a small right parietal scalp incision, an opening was made in the bone using a 20-gauge needle tip. U87 cells ($2 \times 10^5$ in 10 μl of PBS) were injected stereotactically into the right frontal lobe to a depth of 2.5 mm using a Hamilton syringe. This dose of cells typically induces tumors that manifest with contralateral hemiparesis within 3–5 weeks.

Seven days after implantation, animals were randomly chosen to receive either DB67 at a dose of 30 mg/kg/day for 5 days in three 21-day cycles, or vehicle, with six animals in each group. The mice were examined daily for postimplantation day 7; control animals received vehicle alone. Groups of four animals each were treated with DB67 doses of 0.3, 3, or 10 mg/kg/day, and seven animals were treated with vehicle alone. Tumor dimensions were measured every other day using vernier calipers. Tumor volume was calculated as $L \times W^2/2$ (where $L$ is the longest diameter and $W$ is the diameter perpendicular to $L$). Comparisons between volumes at various time points were made using Student’s t test. Animal weights were also recorded twice weekly. Animals were sacrificed when maximum tumor diameter in any plane was $\geq 1.5$ cm, in accordance with Animal Research and Care Committee guidelines.

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other commercially relevant camptothecins, such as CPT-11, SN-38, and 7-ethyl-10,11-methylene-dioxycamptothecin. No significant inhibition was seen in con-

ection as assessed by the MTS assay. The results for several of the agents tested in the U87 cell line are shown in Fig. 2. A more extensive summary of the median effective concentrations in this cell line and in the other four glioma cell lines is presented in Table 1. Not unexpectedly, there was some variability between cell lines in their sensitivity to the individual inhibitors; the p53-mutated LN-Z308 cell line generally exhibited the least sensitivity among the five glioma lines examined. Median effective concentrations for the most active lines examined were generally at least 10-fold higher in each of the cell lines (Table 1 and Fig. 3). The effects of DB67 also compared favorably to other commercially relevant camptothecins, such as topotecan and 9-aminocamp-

tothecin (21, 36), as well as all of the other silatecans evaluated.

**RESULTS**

**Effect of Novel topo I Inhibitors on Cell Proliferation.** In each of the malignant glioma cell lines tested, all of the topo inhibitors examined produced concentration-dependent inhibition of cell proliferation as assessed by the MTS assay. The results for several of the agents tested in the U87 cell line are shown in Fig. 2. A more extensive summary of the median effective concentrations in this cell line and in the other four glioma cell lines is presented in Table 1. Not unexpectedly, there was some variability between cell lines in their sensitivity to the individual inhibitors; the p53-mutated LN-Z308 cell line generally exhibited the least sensitivity among the five glioma lines examined. Median effective concentrations for the most active agents (DB67, CHJ439, CHJ792A, DB205A, and 10,11-methylenedioxy-

camptothecin) were as low as 2–3 ng/ml in several of the more sensitive cell lines, such as U87 and SG388. Of the agents examined, DB67 exhibited the best activity profile across the five cell lines, with median effective concentrations ranging from 2 to 40 ng/ml. In comparison, the median effective concentrations for topotecan were generally at least 10-fold higher in each of the cell lines (Table 1 and Fig. 3). The effects of DB67 also compared favorably to other commercially relevant camptothecins, such as CPT-11, SN-38, and 9-aminocamptothecin. No significant inhibition was seen in control cells treated with equivalent concentrations of vehicle (DMSO) in the absence of these inhibitors.

**Assessment of Blood Stability.** DB67 exhibited the greatest stability in human blood of the camptothecins tested and had substantially better stability than SN-38, 10,11-methylenedioxyacamptothecin, and 7-ethyl-10,11-methylene-dioxycamptothecin as illustrated in Fig. 4. The percentage of lactone exceeded 50% for >3 h with DB67 versus <1 h for each of the other agents. In addition, the percentage of lactate at equilibrium value for DB-67 was >30%, compared with only 20% for SN-38 and 7-ethyl-10,11-methylenedioxyacamptothecin, and only 2% for 10,11-methylenedioxyacamptothecin. In addition, the human blood stability of DB-67 compared favorably with other clinically relevant camptothecins, such as topotecan and 9-aminocamptothecin (21, 36), as well as all of the other silatecans evaluated.

**Effect of DB67 on Cell Viability.** The cytotoxic effect of DB67 was confirmed using a clonogenic assay in the U87 and SG388 malignant glioma cell lines. Cells exposed to a range of concentrations of this agent exhibited a steep concentration-dependent decrease in clonogenic activity (Fig. 5). A comparatively modest effect of drug exposure duration on efficacy was observed over the range of exposure times examined. In the SG388 cell line, median effective concentrations were 3–10 ng/ml after a 6-h exposure versus <1 ng/ml after a 96-h exposure, whereas median effective concentrations were 1–3 ng/ml at both exposure intervals in the U87 glioma cell line.

**Assessment of Antiproliferative Activity in a Nude Mouse s.c. Model.** Growth curves for DB67-treated and control animals are shown in Fig. 6. DB67 produced a concentration-dependent delay in tumor growth. Control animals invariably required sacrifice for ex-

cessive tumor growth within 35 days of tumor implantation. Although animals treated with DB67 concentrations of 0.3 mg/kg/day exhibited no discernible tumor growth delay (data not shown), those treated with 3 and 10 mg/kg/day exhibited significant delays in tumor growth. Tumor volumes were 61 ± 7% and 73 ± 3%, respectively, smaller than in control animals at 28 days (the time at which these animals

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**Fig. 5. Relationship between colony counts and DB67 concentration in the U87 (top) and SG388 (bottom) cell lines. Cells were treated with various concentrations of DB67 for 6 and 96 h and then grown for an additional 14 days in the absence of the inhibitor. Colonies were then counted.**

**Fig. 6. Effect of DB67 on tumor growth in a nude mouse s.c. model using the U87 malignant glioma cell line. After tumors of ~0.5 cm in diameter were detected, animals began treatment with DB67, which was administered s.c. on a daily basis for 5 days, in 21-day cycles. Control animals received vehicle alone. Points represent the mean of four animals, except for controls, which included seven animals; bars, SD. Animals treated with 3 and 10 mg/kg/day had a significant delay in tumor growth, with significant differences (P < 0.05, Student’s t test) versus control animals at 28 days after implantation and continuing until the control animals were sacrificed because of tumor size requirements.**
began to require sacrifice. None of the animals exhibited >10% weight loss during treatment.

Whereas tumors in the 3 mg/kg/day group grew significantly more slowly than the control tumors, these lesions ultimately reached the size requiring sacrifice, despite retreatment beginning at day 28. In contrast, tumors in the 10 mg/kg/day group demonstrated little, if any, increase in size from the start of treatment, and all four animals remained free of tumor progression for >100 days after implantation. However, they were not rendered disease free by the treatment. After the fourth and final treatment at day 70, discernible tumor growth was not apparent for an additional 30–35 days, but tumors ultimately began to enlarge rapidly thereafter. Large tumors were apparent between 110 and 120 days after implantation, necessitating sacrifice.

**Assessment of Regression of Advanced Tumors in a s.c. Model.**

On the basis of the above results, which showed that a DB67 dose of 10 mg/kg/day was effective in largely eliminating tumor growth but did not induce frank disease remission, we examined whether a higher dose would be capable of inducing regression of large (>1 cm diameter) established tumors. Whereas tumors in control animals rapidly reached a size mandating sacrifice (≥1.5 cm maximum diameter), all four animals treated with a dose of 30 mg/kg/day exhibited rapid tumor regression, with complete tumor disappearance in one and >50% tumor regression in three others after one course of therapy (Fig. 7). Complete regression was achieved in the latter three animals after a second course of treatment, and all four animals remained disease free >120 days after beginning treatment and >90 days after their final treatment. No evidence of weight loss or other toxicity was apparent in the DB67-treated animals.

**Assessment of Antiproliferative Activity in a Nude Mouse Intracranial Glioma Model.**

On the basis of the results of the above studies, a dose of 30 mg/kg/day of DB67 was selected for use in the intracranial model studies. Survival curves for the DB67-treated and control animals are shown in Fig. 8. Median survival was 58 days in the control group, and all animals died by day 70, whereas all six animals in the DB67 group are still alive at >120 days postimplantation. Differences between the respective survival curves were statistically significant (P < 0.001), as were differences in the frequency of 90-day survival (P < 0.00001). No adverse sequelae were apparent in the animals treated with DB67. Animals that died all had evidence of intracranial tumor on examination.

**DISCUSSION**

The first clinically applied topo inhibitor, camptothecin, was extracted from the wood, bark, and fruit of an Oriental tree, *Camptotheca acuminata* (38), which had long been used in Chinese medicine for the treatment of a variety of ailments, including neoplasms. Although studies by the National Cancer Institute established the antitumor activity of this agent in preclinical models (39), clinical testing of this agent demonstrated only modest efficacy with unacceptable toxicity (6, 11). These initial clinical results temporarily quelled enthusiasm for further examination of this compound or its derivatives. However, with the subsequent demonstration that this agent was actually a site-specific inhibitor of the enzyme topo I, which plays an essential role in DNA replication and transcription (10, 11), interest was renewed in developing more effective, better tolerated camptothecin derivatives. Factors that contributed to poor efficacy and unacceptable toxicity of camptothecin were identified, and efforts were made to modify the parent compound to a more suitable derivative. The principal problems included poor solubility and limited bioavailability secondary to albumin binding and rapid acyl cleavage at physiological pH of the α-hydroxy-δ-lactone moiety to a biologically inactive carboxylate form that was nonetheless potentially toxic to normal cells (6, 10, 11, 40). A number of derivatives, such as 9-aminocamptothecin (41), irinotecan (CPT-11; Refs. 12, 42–44), topotecan (13, 45), and 7-ethyl-10,11-methylenedioxy camptothecin (46) were formulated, which overcame some but not all of the above limitations with varying degrees of success.

The synthesis schema that was pursued for the compounds reported here was directed at producing a family of synthetic camptothecin analogues that exhibited improved in vivo stability while preserving or enhancing topo I inhibitory activity. The cascade radical annulation reaction provided a totally synthetic approach to the formulation of such compounds that permitted significant flexibility in drug design to
allow the development of a wide variety of potential analogues. Two general strategies were used to enhance blood stability: (a) structural modifications that eliminated the highly preferential binding of camptothecin carboxylate over camptothecin lactone by human serum albumin (21, 35, 36, 47, 48) were sought. By overcoming the high affinity binding interactions of the carboxylate form to human serum albumin (47, 48), such approaches produce a corresponding improvement in the stability of the active lactone form; and (b) modifications were introduced to enhance lipophilicity to promote reversible partitioning of the camptothecin derivative into lipid bilayers (49, 50), thereby protecting the active lactone forms from hydrolysis.

It was hypothesized that the addition of a highly stable silyl group together with 10-substitution to the parent compound would limit drug inactivation by both protein binding and hydrolysis of the lactone ring and enhance lipophilicity, which would increase in vitro activity, while limiting toxicity. Preliminary studies showed that 7-silyl modification of the B-ring carbon preserved, and in some cases improved, top I inhibitory activity in vitro in conjunction with other structural modifications (30) and appeared to substantially enhance blood stability of the active lactone moiety. Because the enhanced lipophilicity and prolonged blood stability observed for several of the silatecans should theoretically facilitate drug transit across the blood-brain barrier and thus might constitute a particular advantage for the treatment of central nervous system neoplasias, the present study was undertaken to evaluate the functional utility of these agents against human high-grade gliomas both in vitro and in vivo.

Our evaluation of a series of silatecans using a panel of high-grade glioma cell lines in vitro demonstrated profound growth-inhibitory activity with several compounds. The most active agent (DB67) produced nearly complete inhibition of cell growth at concentrations in the low nanomolar range, at least 10-fold more potent than topotecan in our systems. The activity observed for DB67 also exceeded that noted for CPT-11 or its active metabolite SN-38 in the present study and in prior reports (9) and was at least comparable with that observed for 10,11-methylenedioxy camptothecin and its 7-ethyl derivative, which are known to be highly potent inhibitors of cell proliferation (46). Not unexpectedly, DB67 showed significantly less activity against nonneoplastic astrocytes (data not shown), reflecting that these cells proliferate extremely slowly and thus are less vulnerable to the effects of top I inhibition, which selectively “poison” cells that are undergoing DNA replication or transcription. Clonogenic studies confirmed direct cytotoxicity of DB67 against glioma cells in vitro.

In addition to its potent activity against glioma cell proliferation and viability in vitro, DB67 exhibited substantially enhanced blood stability of its lactone moiety in comparison to SN-38, which had previously been noted to have substantially greater blood stability than a variety of other less lipophilic camptothecins, such as 9-amino camptothecin, camptothecin, and topotecan (36). Like SN-38, DB-67 contains a 10-hydroxy group, but a 7-tert-butyldimethylsilyl functionality replaces the 7-ethyl group, which enhances lipophilicity. The overall association constant (K) for SN-38 interacting with small unilamellar vesicles composed of dimyristoylphosphatidylcholine is 260 \( \text{m}^{-1} \), whereas the corresponding association constant for DB-67 is 2500 \( \text{m}^{-1} \) (data not shown). Thus, DB-67 is 10-fold more lipophilic than SN-38 and approximately 15- and 20-fold more lipophilic than the experimental agents 7-ethyl-10,11-methylenedioxy camptothecin and 10,11-methylenedioxy camptothecin, respectively.

More clinically relevant than these in vitro results was the demonstration of profound tumor growth inhibition with DB67 in a series of glioma models. Activity was observed at doses as low as 3 mg/kg/day, whereas doses of 10 mg/kg/day for 5 days in 21-day cycles virtually eliminated the growth of s.c. xenografts. Doses of 30 mg/kg/day produced complete regression of large flank tumors in all treated animals with 100% long-term disease-free survival. In comparison to prior studies using topotecan (51–53), the degree of tumor growth inhibition achieved with DB67 was considerably more dramatic; the effects observed were at least comparable with those achieved with CPT-11 (23, 43, 53–55). Moreover, the rapid regression and ultimate cure of large flank tumors that were achieved with DB67 is unprecedented in our experience using a variety of other conventional chemotherapeutic agents in the U87 s.c. xenograft model system. Finally, the significant prolongation of survival and apparent cure of animals with intracranial xenografts that were treated with DB67 supports the potential applicability of this agent in clinical therapeutics against these tumors.

Because the dosing strategy applied in the present study was selected to conform with those commonly used in previous clinical studies with top I inhibitors and the route of administration was s.c. rather than i.v., it is conceivable that comparable levels of tumor growth inhibition could be achieved using far lower doses of DB67 than were used in the present study. Conversely, because no overt toxicity was detected at a dose of 30 mg/kg/day, it is possible that further dose intensification would be feasible to treat less sensitive tumors in this model system. However, in view of the 100% cure rate for both s.c. and intracranial U87 glioma xenografts that was achieved at this dosage, additional dose escalation to determine a maximum tolerated dose was not pursued in this study.

On the basis of the encouraging results obtained in this initial study, we conclude that silatecans have significant activity against human malignant glioma cell lines in vitro. The potency of the most active derivatives, such as DB67, compares favorably with that of commercially available camptothecins. The enhanced blood stability of DB67 in comparison with other camptothecins may provide a therapeutic advantage for these compounds, an observation that is supported by the significant activity observed in both s.c. and intracranial U87 xenograft models. The present study provides a rationale for proceeding with more extensive efficacy studies comparing the therapeutic activity of DB67 and related silatecans against other highly active camptothecins in a variety of tumor model systems, as well as detailed toxicology and pharmacokinetic studies, to further assess the potential clinical utility of these agents.

REFERENCES

8. Husain, I., Mohler, J. L., Seigler, H. F., and Besterman, J. M. Elevation of topoi-

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EFFECT OF topo I INHIBITION ON GLIOMA PROLIFERATION


Potent Topoisomerase I Inhibition by Novel Silatecans Eliminates Glioma Proliferation in Vitro and in Vivo

Ian F. Pollack, Melanie Erff, David Bom, et al.


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