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ORIGINAL ARTICLE

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The effect of DB-67, a lipophilic camptothecin derivative, on topoisomerase I levels in non-small-cell lung cancer cells

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Abstract *Purpose*: To determine the in vitro drug sensitivity of two non-small-cell lung cancer cell lines after treatment with the novel lipophilic camptothecin derivative, 7-*tert*-butyldimethylsilyl-10-hydroxycamptothecin (DB-67), to determine if topoisomerase I protein levels decrease after treatment with DB-67, and to assess the duration and extent of topoisomerase I modulation after DB-67 exposure, in order to provide information about drug resistance that may be useful in determining an appropriate dosing schedule for DB-67. *Methods*: The growth inhibition of the non-small-cell lung cancer cell lines A549 and H460 after exposure to DB-67 was evaluated with the MTS assay. A549 and H460 cells were treated for various times with DB-67 and topoisomerase I levels were determined by western blot analysis. In addition, A549 and H460 cells were treated with DB-67 for 24 h and topoisomerase I levels were determined by western blot analysis daily for 1 week after drug removal. *Results*: DB-67 inhibited the growth of both A549 and H460 cells grown in culture; the A549 cells were more resistant to the cytotoxic effects of DB-67 than H460 cells. Notably, A549 cells had approximately one-half the baseline topoisomerase I than H460 cells. Topoisomerase I protein levels significantly decreased after 8–18 h of exposure to DB-67. Both A549 and H460 cells treated with DB-67 for 24 h had only negligible amounts of topoisomerase I at the end of treatment. However, within 24 h of drug removal topoisomerase I levels returned to near baseline levels in both cell lines. *Conclusions*: The decrease in topoisomerase I levels caused by DB-67 may represent a mechanism of resistance to this novel camptothecin derivative. Dosing DB-67 once every 48–72 h may maximize the interaction of the drug with topoisomerase I and should be considered as a potential dosing schedule in the preclinical and clinical development of this compound.

Keywords DB-67 · Topoisomerase I · Camptothecin · Resistance

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Introduction

Camptothecin is a plant alkaloid extracted from *Camptotheca acuminata* and is cytotoxic to a wide range of human cancers [44]. Currently two derivatives of camptothecin, irinotecan and topotecan, are FDA-approved for the treatment of cancer. Despite the promise of this class of agents, the effectiveness of camptothecins is limited by their poor stability in blood and their propensity to bind to human serum albumin (HSA) [11]. Many camptothecin derivatives have a lactone moiety in the E ring, which is required for biological activity. At physiological pH, the lactone is readily hydrolyzed to the corresponding carboxylate. The carboxylate form preferentially binds to HSA, shifting the equilibrium away from the active lactone species to the inactive carboxylate [9, 10].

A novel, highly lipophilic camptothecin derivative, 7-*tert*-butyldimethylsilyl-10-hydroxycamptothecin (DB-67) (Fig. 1), has been developed that has improved blood stability. The addition of a lipophilic *t*-butyldimethylsilyl group at the 7 position and a hydroxyl at the 10 position of the E ring decreases protein binding and improves the stability of the lactone [6–8]. The lipophilic character of DB-67 enables it to cross the blood–brain barrier, making it a promising therapy for the treatment

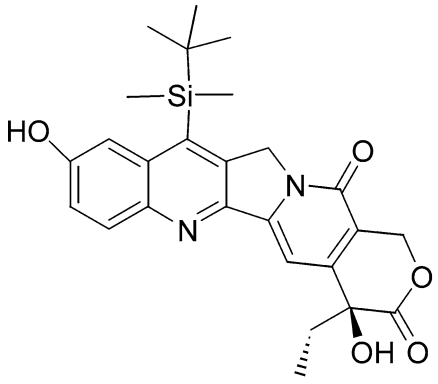

Fig. 1 Structure of 7-*tert*-butyldimethylsilyl-10-hydroxycamptothecin (DB-67)

of high-grade gliomas and other CNS cancers [33]. Additionally, DB-67 is cytotoxic to leukemia, melanoma, colon, ovarian, renal, prostate, non-small-cell lung (NSCLC), and breast cancer cells grown in culture [8]. This therapeutic agent is in preclinical development and will be entering phase I clinical trials.

Camptothecin derivatives, including DB-67, exert their cytotoxic effect by interacting with topoisomerase I, which results in DNA double-strand breaks [24]. Topoisomerase I, an essential nuclear enzyme, relieves torsional strain caused by advancing DNA replication forks during the S phase of the cell cycle. Topoisomerase I preferentially binds to DNA when supercoiling is present, inducing a single-strand break, which allows the cleaved strand to rotate and relieve torsional strain. Camptothecins associate with the DNA-topoisomerase I complex, stabilize the topoisomerase I-DNA interaction, and prevent topoisomerase I from annealing the DNA strand break and dissociating from DNA. This leads to double-strand breaks in DNA and cellular death [16, 34, 39].

Cellular resistance to camptothecins develops through a variety of mechanisms [2, 12, 15, 21, 26, 38, 42]. One mechanism of resistance to camptothecin and camptothecin derivatives is a decrease in topoisomerase I protein levels [45]. In in vitro models, topoisomerase I levels are decreased after exposure to camptothecin derivatives [3, 4, 17, 20]. In addition, patients treated with a camptothecin derivative have reversible decreases in topoisomerase I protein levels [22, 23, 32]. Since topoisomerase I is essential for camptothecin derivatives to exert their cytotoxic effect [5], and since cells with lower levels of topoisomerase I protein are more resistant to the cytotoxic effects of the camptothecins [19, 40], topoisomerase I may be an important determinant in predicting the efficacy of topotecan or irinotecan in cancer patients [1, 37, 41]. Consequently, characterization of the topoisomerase I protein levels after treatment with a camptothecin derivative is important to determine a dosing schedule that results in maximal new interaction of the drug with the topoisomerase I-DNA complex. Since DB-67 will soon be entering clinical trials, the objective of this work was to measure topoisomerase I levels in NSCLC cells after treatment with DB-67 to provide information that may help determine an appropriate dosing schedule for this promising anticancer agent.

Materials and methods

Drug and cell culture reagents

DB-67 was provided by Dr. Thomas G. Burke (University of Kentucky) and determined to be greater than 99% pure by HPLC. The A549 and NCI-H460 (H460) cell lines were obtained from the American Type Culture Collection (Manassas, Md.) and grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml), 2 m*M* glutamine and 1 m*M* sodium pyruvate. All cell culture reagents were purchased from Invitrogen (Carlsbad, Calif.).

DB-67 cytotoxicity

Exponentially growing A549 and H460 cells were plated in 96-well microtiter plates at 5×10^3 cells/well for the 4, 6, 8, 18, and 24-h time-points and at 3×10^3 cells/well for the 48 and 72-h time-points. The cells were allowed to adhere overnight. Serial dilutions of DB-67 (0.5 p *M* to 50 μ*M*) were added to the appropriate well. Six replicates of each concentration were analyzed. After the cells were exposed to drug for the specified time (4, 6, 8, 18, 24, 48, or 72 h), the drug was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Supplemented DMEM was added to the cells and after an additional 24 h of growth in drug-free medium, the MTS cell proliferation assay (Promega, Madison, Wis.) was performed as previously described [14]. KC4 Software (Bio-Tek Instruments, Winooski, Vt.) was used to determine the IC_{50} value at each time-point. Each experiment was conducted in triplicate.

Determination of topoisomerase I content after continuous exposure to DB-67

Exponentially growing A549 or H460 cells were seeded in 100-mm dishes (A549 at 7.5×10^5 cells/dish and H460 at 1×10^6 cells/dish), and allowed to adhere overnight. DB-67 was added to each dish such that the final concentration in the dish was 1 μ*M* for H460 cells and 25 μ*M* for A549 cells. At the specified times (2, 4, 8, 18, 24, 48, or 72 h), the cells were harvested with trypsin, counted to normalize cell numbers, lysed with Laemmli buffer (63 m*M* Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol) and heated at 100°C for 5 min to ensure the dissociation of DNA/protein complexes. Baseline controls were made by harvesting exponentially growing A549 or H460 cells that had not been treated with drug. The cells were counted and lysed in an

analogous manner to the drug-treated cells. The samples were stored at −20°C until analysis by western blotting. Each experiment was conducted in quadruplicate.

Duration and extent of effect of DB-67 on topoisomerase I levels

Exponentially growing A549 or H460 cells were seeded into 150-mm dishes (A549 at 4×10^6 cells/dish and H460 at 1×10^7 cells/dish), and allowed to adhere overnight. DB-67 (H460 0.5 μ*M*; A549 2.5 μ*M*) was added to the cells on day 0. After 24 h of drug exposure, the cells were harvested (day 1) and seeded into 100-mm dishes at a concentration of 5×10^5 and 3×10^5 cells/dish in 10 ml medium for H460 and A549 cells, respectively. On each successive day (days 2–7) cells were harvested, lysed in Laemmli buffer, heated, and stored at −20°C until analysis by western blotting. Each experiment was conducted in quadruplicate.

Western blot analysis

Aliquots containing equal amounts of total cell extract were resolved by a 6% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PDVF membrane. The membrane was blocked with 5% nonfat dried milk in Tris-buffered saline with Tween (TBST) for 1 h at room temperature. The membrane was then incubated with scleroderma antibody to human topoisomerase I antibody (1:1000 dilution; Topogen, Columbus, Ohio) and murine monoclonal β-actin antibody (1:10,000 dilution; Chemicon, Temecula, Ohio) for 2 h at room temperature. The membrane was washed three times for 10 min each with TBST and then incubated for 1 h at room temperature with goat-antihuman (1:2500 dilution; Chemicon) and goat-antimouse antibody (1:10,000 dilution; Chemicon) conjugated to horseradish peroxidase. After washing the membrane three times with TBST (10 min each washing), the proteins were visualized using the ECL detection system (Amersham, Arlington Heights, Ill.). The bands were quantified using GeneSnap software (Synoptics, Cambridge, UK) and protein loading equivalence was assessed by β-actin.

Results

DB-67 was cytotoxic to both A549 and H460 cells (Fig. 2). The IC_{50} values rapidly decreased between 8 and 24 h of exposure before plateauing at a constant value. A549 cells were more resistant to the cytotoxic effects of DB-67 than H460 cells as evidenced by their higher IC_{50} values at every time-point evaluated. Even after 18 h of drug exposure, only minimal cell death was observed in A549 cells, while significant cytotoxicity was observed in H460 cells. Notably, this was not attributable to a difference in cell doubling (23 h vs 24 h), but may have been associated with baseline levels of topoisomerase I (A549 cells had 54 ± 8% of the topoisomerase I found in H460 cells). These data are consistent with previously reported results indicating that baseline levels of topoisomerase I predict sensitivity to camptothecin and camptothecin derivatives [1, 36, 41].

Since the amount of topoisomerase I protein is an important parameter influencing the cytotoxic potential of topoisomerase I inhibitors, topoisomerase I protein levels were measured after A549 and H460 cells were exposed to DB-67 for various amounts of time. Protein levels of topoisomerase I rapidly decreased in A549 and H460 cells exposed to DB-67 (Fig. 3). Less than 50% of the baseline topoisomerase I level was detectable after 2 h of exposure and by 24 h less than 10% of the baseline topoisomerase I was observed. The time course and extent of the decrease in topoisomerase I levels was similar in both cell lines. Prior to analysis, the cells were diluted in drug-free medium and heated as described in "Materials and methods". Both dilution and heating promote the dissociation of topoisomerase I from DNA [25, 30]. Consequently, the protein observed on the gel represents total topoisomerase I (DNA-bound and free topoisomerase I). Additionally, it is important to note

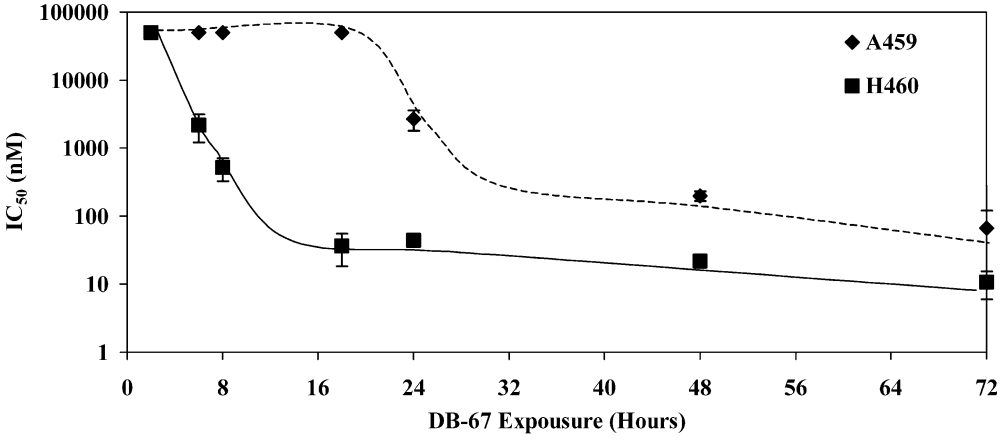

Fig. 2 Cytotoxicity of A549 and H460 cells after exposure to DB-67 over time. The results are expressed as the IC_{50} value at each time and the cytotoxi city was determined using an MTS assay. Each point represents the mean value ± SEM for three to five replicates

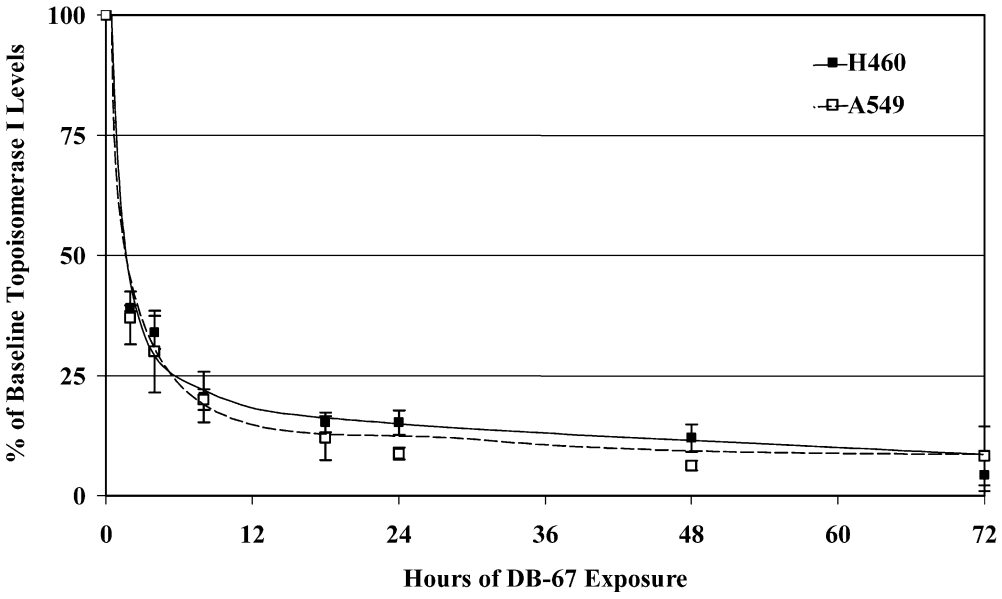

Fig. 3 Topoisomerase I levels, as determined by Western blotting, in NSCLC cells after continuous exposure to DB-67. A549 and H460 cells were treated with 25 μ*M* and 1 μ*M* DB-67, respectively. The results are expressed as percent of control baseline topoisomerase I in each respective cell line and are the means ± SEM from quadruplicate experiments

that equal amounts of protein were loaded onto the gel and the topoisomerase I bands were normalized to actin, indicating that the decrease in topoisomerase I was not simply attributable to DB-67-induced cellular death.

The data shown in Fig. 3 clearly indicate that topoisomerase I levels decreased after exposure to DB-67. In order to provide information that may help establish an optimal dosing regimen of DB-67, the amount of time required for topoisomerase I to recover to baseline levels after drug removal was assessed. As seen in Fig. 4, exposure to DB-67 for 24 h resulted in a significant decrease in topoisomerase I. After DB-67 was removed from the cells, topoisomerase I rapidly increased to near baseline levels. One day after DB-67 was removed A549

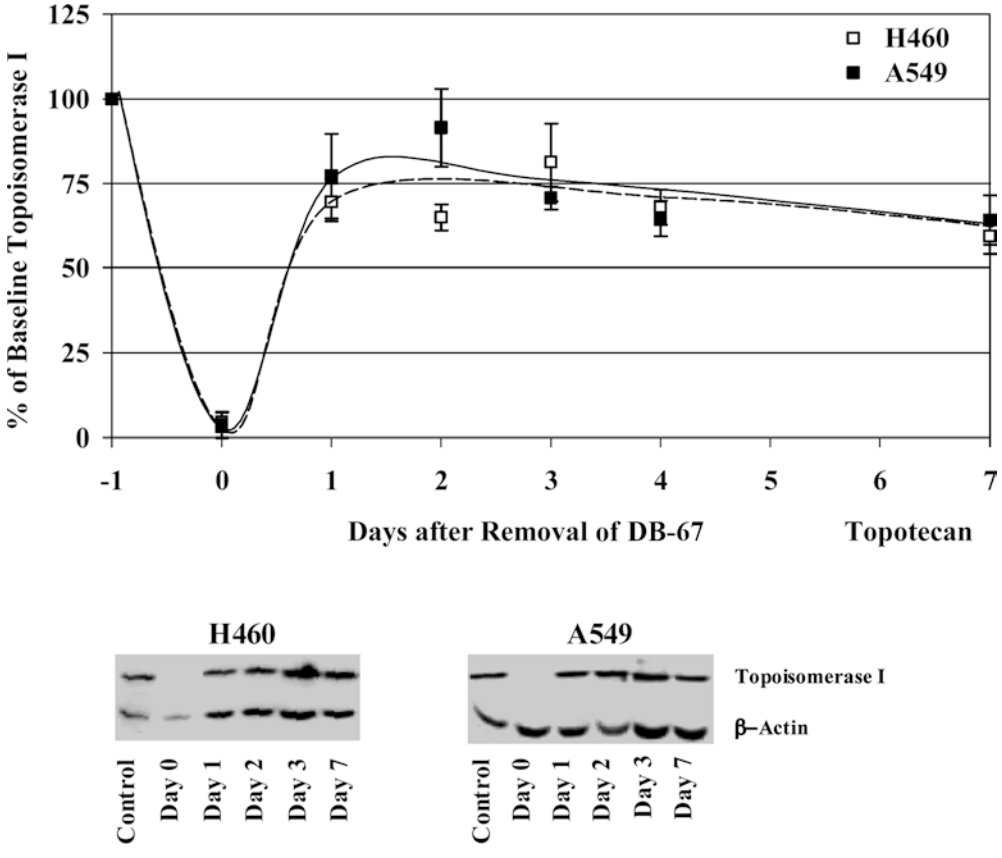

Fig. 4 Topoisomerase I levels in NSCLC cells. The results are expressed as the mean ± SEM percent from quadruplicate experiments of control baseline topoisomerase I in each respective cell line. A549 and H460 cells were treated with 2.5 μ*M* and 0.5 μ*M* DB-67, respectively, for 24 h. The drug was removed and the topoisomerase I content was determined on each subsequent day by Western blotting. Representative Western blots of H460 and A549 cells are shown

and H460 had 77 ± 13% and 70 ± 6% of baseline topoisomerase I, respectively. Although the mean level of topoisomerase I appeared to decrease after day 1, the topoisomerase I level on day 7 was not significantly different from the value measured on day 1, indicating that topoisomerase I returned to maximal levels within 24 h of drug removal.

Discussion

DB-67 is a promising lipophilic camptothecin derivative that, like other camptothecin derivatives, is excepted to exert its effect in the S phase of the cell cycle and, consequently, should be most efficacious with continuous exposure. However, it has previously been demonstrated in our laboratory and others that continuous exposure to topotecan or camptothecin results in a reversible decrease in topoisomerase I protein levels [3, 4, 17, 20]. Since topoisomerase I is required for topoisomerase I inhibitors to have activity [5], we have postulated that bolus dosing, rather than continuous exposure may maximize the interaction of the camptothecin derivatives with topoisomerase I and lead to improved clinical outcomes [4]. Consistent to what has been observed with other camptothecin derivatives, continuous exposure to DB-67 resulted in a rapid and significant decrease in topoisomerase I levels that was reversible upon drug removal.

Although both A549 and H460 cells had analogous topoisomerase I level profiles after treatment with DB-67, A549 cells were appreciably more resistant to DB-67, having a higher IC_{50} value at every time-point analyzed. This is consistent with a published report indicating that topotecan is active against H460 xenografts, but not A549 xenografts [28]. A reasonable explanation for the differential cytotoxic effects of DB-67 in A549 and H460 cells is their baseline levels of topoisomerase I. Untreated A549 cells have approximately one-half the topoisomerase I protein of untreated H460 cells. Since both cell lines are p53- and Rb-positive [27] and they both express multidrug resistance proteins [35, 43], it is unlikely that the differences in cytotoxicity can be explained solely by these mechanisms. Although H460 cells are a large-cell carcinoma from a pleural effusion, while A549 cells were derived from an adenocarcinoma, and A549 cells can synthesize lecithin with a high percentage of desaturated fatty acids utilizing the cytidine diphosphocholine pathway [29], it is unclear how these differences would contribute to the differential effects of DB-67 on A549 and H460 cells.

The data from these experiments suggest that intermittent bolus administration of high concentrations of DB-67 may both maximize the interaction of DB-67 with topoisomerase I and improve its cytotoxic potential. We assumed that the administration of DB-67 would result in detectable concentrations in the serum for approximately 24 h (similar to both topotecan and irinotecan). Consequently, we evaluated the time required for topoisomerase I protein levels to rebound to near baseline levels after exposure to DB-67 for 24 h. These data will be helpful in determining the proper dosing interval. Since the maximal rebound in topoisomerase I levels are observed 1 to 2 days after removal of DB-67 (48–72 h after initiation of exposure), it is reasonable to suggest that dosing DB-67 once every 48–72 h may maximize the interaction of DB-67 with topoisomerase I. However, it is important to note that even at 1 week after drug removal topoisomerase I levels were still slightly below baseline levels, indicating that there may be lasting effects on topoisomerase I levels after exposure to DB-67. Since topoisomerase I protein levels are an important determinant of the activity of camptothecin derivatives and since topoisomerase I levels are modulated after exposure to DB-67, topoisomerase I may be a useful pharmacodynamic endpoint to evaluate in preclinical and clinical studies to validate the optimal dosing regimen of DB-67. An additional benefit of pulsed dosing with high concentrations of drug is the likelihood of DB-67 achieving deeper penetration into large, poorly vascularized tumors. It is possible, however, that this same result could potentially be achieved with other dosing regimens such as continuous administration.

The reduction in topoisomerase I protein levels after exposure to DB-67 may be a result of cellular responses to the formation of the DB-67-topoisomerase I-DNA complex. Decreases in topoisomerase I protein levels after exposure to other camptothecin derivatives have been previously reported and have been attributed to degradation of topoisomerase I. For instance, in breast, colorectal, and epidermoid carcinoma cells lines, topoisomerase I is degraded by the ubiquitin-dependent 26S proteasome pathway [17, 20]. After treatment with camptothecin, topoisomerase I cleavable complexes are rapidly conjugated with SUMO (small ubiquitin-related modifier) proteins [18, 31]. However, their precise role in topoisomerase I degradation has yet to be defined. Another mechanism of degradation has been described in proliferating human T lymphocytes, where topoisomerase I is degraded by a Mg^{2+}-dependent trypsin-like serine protease [13]. Our data, as seen in Fig. 3, clearly show that after exposure to DB-67 topoisomerase I is depleted. The data do not, however, permit speculation on whether the depletion is due to decreased synthesis of topoisomerase I or increased degradation. Although it is likely that DB-67 may induce topoisomerase I degradation in an analogous manner to other camptothecin derivatives, the fate of topoisomerase I after exposure to DB-67 has yet to be characterized. These experiments indicate that dosing DB-67 once every other day or once every third day may maximize the interaction of DB-67 with topoisomerase I and result in greater cytotoxicity. These data will be useful in determining the optimal dosing schedule to be used in the preclinical and clinical development of this promising, highly lipophilic camptothecin derivative and indicate that pharmacodynamic measurements of topoisomerase I may be warranted in the development of DB-67.

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