Intermittent Exposure of Medulloblastoma Cells to Topotecan Produces Growth Inhibition Equivalent to Continuous Exposure

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ABSTRACT
Camptothecin analogues such as topotecan increase the number of covalent topoisomerase I-DNA complexes, which, in turn, have been proposed to initiate apoptosis. If induction of apoptosis by the camptothecins is, in fact, dependent on the formation of topoisomerase I-DNA complexes, then it would be of clinical relevance to identify schedules of exposure to the camptothecins that maximize the formation of these complexes but minimize the total amount of the drug administered. The time and dose dependence of topoisomerase I-DNA complex formation was determined by incubating Daoy pediatric medulloblastoma cells in vitro with topotecan at concentrations equivalent to those achievable in the plasma clinically (10, 50, or 200 nM) and measuring the number of complexes present in cells incubated for 15 min to 48 h with the drug. Regardless of the concentration of topotecan used, covalent topoisomerase I-DNA complexes were maximal within 15 min following addition of the lactone form of topotecan to the tissue culture medium. After 2 h of exposure to topotecan, complexes had decreased from maximum to approximately half of that value. Few, if any, complexes were detectable with topotecan incubations of 24–48 h. Growth inhibition studies showed that the IC_{50} of topotecan for the Daoy cell line (2.2 × 10^{-9} M) and for a second pediatric medulloblastoma cell line, SJ-Med3 (3.6 × 10^{-9} M), exposed to topotecan 8 h daily for 5 days or continuous exposure were equivalent. The decrease in topoisomerase I-DNA complexes between 15 min and 1 h was consistent with a pH-dependent re-equilibration of topotecan to the less active hydroxyacid form of the drug. The decrease in complexes after a 2–48-h incubation with the drug was attributable neither to biological inactivation of topotecan as shown by sequential growth inhibition studies nor to a decrease in amount of topoisomerase 1 in the drug-treated cells. Indirect immunofluorescence labeling of topoisomerase 1 in Daoy cells incubated for 48 h with 10 nM topotecan showed a redistribution of nucleolar topoisomerase 1. We are currently evaluating the antitumor effect of intermittent repetitive exposures to topotecan in mice bearing Daoy cells as a xenograft. The clinical utility of each effective schedule of exposure will depend on whether the therapeutic index of repetitive intermittent exposure to the drug is more or less favorable than the therapeutic index of continuous exposure.

INTRODUCTION
The antitumor effect of the camptothecins has been postulated to be mediated by an increase in covalent topoisomerase I-DNA complexes (reviewed in Ref. 1). If this hypothesis is correct, then schedules of exposure to the camptothecins that maximize the total number of these complexes in tumor cells should have the greatest antitumor effect. Several reports document that in vitro topoisomerase I-DNA complexes reverse rapidly when camptothecins are removed from tissue culture medium (2–4), but few studies describe the time course of these complexes in the continuous presence of the drug (3). Of particular interest is the recent observation of Beidler and Cheng (3) that preincubation of KB cells with 5 μM camptothecin for 24 h made the cells refractory to the formation of additional complexes by a second pulse of the same concentration of the drug. Also, we reported recently (5) that micromolar concentrations of topotecan or SN-38, both camptothecin analogues, produced a transient increase and subsequent decrease in covalent protein-DNA complexes in anaplastic astrocytoma cells in vitro. Micromolar concentrations of topotecan induced a redistribution of topoisomerase 1 in the anaplastic astrocytoma cells. A question raised by that study was the extent to which the biphasic increase/decrease of topoisomerase I-DNA complexes and redistribution of topoisomerase I occurred in cells treated with more clinically relevant, nanomolar concentrations of topotecan.

Also unknown was the extent to which metabolic or chemical inactivation of topotecan contributed to the decrease in complexes and the effect of this decrease on the cytotoxicity of topotecan.

Therefore, we examined in Daoy pediatric medulloblastoma cells in vitro the time and dose dependence of these complexes produced by concentrations of topotecan achievable in the plasma clinically (6–9). We also compared the growth-inhibitory potential of several different schedules of exposure to topotecan, based on the duration for which complexes could be detected. Data in this article document that covalent topoisomerase I-DNA complexes show a biphasic increase/decrease in...
Daoy cells exposed to nanomolar or micromolar concentrations of topotecan. The data also provide evidence that continuous exposure to clinically achievable levels of topotecan does not maximize topoisomerase I-DNA complex formation in tumor cells in vitro and that intermittent exposure to a given concentration of topotecan produces growth inhibition comparable to continuous exposure.

**MATERIALS AND METHODS**

**Cell Lines and Chemicals**

The Daoy pediatric medulloblastoma cell line (10) was obtained from the American Tissue Type Collection (Rockville, MD). A subclone isolated from the parent cell line was used for experiments reported in this paper. The SJ-Med3 pediatric medulloblastoma cell line was established from tumor tissue obtained at diagnosis from a St. Jude patient; characterization of this cell line will be described elsewhere. The sample was obtained after informed consent had been given, in accordance with policies of the St. Jude Children’s Research Hospital Institutional Review Board.

Each cell line grows as a monolayer in DMEM (BioWhittaker, Inc., Walkersville, MD) supplemented with 15% fetal bovine serum (HyClone Laboratories, Logan, UT). All experiments were done with cells in logarithmic growth. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

**Intermittent Exposure to Topotecan**

Sequential Growth Inhibition Assays

Daoy cells were seeded in 35-mm tissue culture dishes at a concentration of $2 \times 10^5$ cells/dish and allowed to attach overnight. Topotecan solutions (pH 7.4) were prepared the day before the drug was added to the tissue culture dishes, and the solutions allowed to equilibrate overnight at 4°C. Aliquots of stock solutions were added to the tissue culture dishes containing cells to achieve final concentrations of topotecan in log increments from $10^{-10}$ to $10^{-5}$ M in each dish of triplicates (day 1). Forty-eight h after addition of the drug (day 3), the medium containing topotecan was harvested sterilely, and the cells remaining in the dishes were trypsinized and counted using a Coulter Multisizer II cell counter. Results were expressed as number of cells in topotecan-treated dishes/number of cells in control dishes. The medium containing topotecan was then used for a second growth inhibition assay from days 3–5, using a second set of dishes containing Daoy cells. The number of cells remaining in the second set of dishes was determined on day 5. The concentrations of topotecan required to inhibit growth by 50% compared to vehicle-treated controls (IC$_{50}$) were calculated using GraphPad Prism software.

**Comparison of the Inhibition of Growth of Medulloblastoma Cells Produced by Continuous and Intermittent Exposure to Topotecan**

To compare the growth-inhibitory effect of intermittent and continuous exposure to topotecan, we incubated Daoy or SJ-Med3 medulloblastoma cells with topotecan 0, 4, 6, 8, or 24 h daily for 5 days. Each day, the drug and medium from the previous day were removed, and fresh drug and medium were added to each tissue culture dish. The number of cells remaining in each dish on day 7 was determined. Results were expressed as number of cells in drug-treated cultures/number of cells in vehicle-treated controls and are reported as the concentration of the drug required to inhibit growth 50% (IC$_{50}$).

**Immunofluorescence Staining for Topoisomerase I**

Topoisomerase I was detected by indirect immunofluorescence staining with a rabbit polyclonal antiserum generated with a 21-amino acid synthetic peptide complexed to keyhole limpet hemocyanin. Details of the production and characterization of the antiserum and the staining procedure have been published (11).

**Image Cytometry**

**Image Acquisition and Processing: Quantitation of DNA and Topoisomerase I.** Photomicrographs included in this study are images of indirect immunofluorescence stains of DNA topoisomerase I (see Fig. 3) in which binding of primary antibody was detected with a FITC-conjugated antirabbit IgG. The DNA of the cells was stained with Hoechst 33342 (not shown). Images were acquired and stored for analysis as described previously (5).
DNA ploidy was determined by constructing a DNA histogram with the values obtained from Hoechst 33342 fluorescence. Image cytometry produces DNA histograms comparable to flow cytometry (11, 12). The mean fluorescence units (DNA content) of the G1 peak was designated as a ploidy of 2 N, and the phase of the cell cycle for each cell was identified by comparing the units of Hoechst fluorescence of each cell with that of the 2 N population. G1-phase cells are defined as those having a 2 ± 0.5 N DNA content; S-phase cells are defined as those having a 3 ± 0.5 N DNA content; and G2-M-phase cells are defined as those having a 4 ± 0.5 N DNA content.

The area of DNA fluorescence was then used to define the nucleus of each cell, generate a binary mask of this area, and quantitate the amount of topoisomerase I in each nucleus. Methods for quantitation of topoisomerase I by immunofluorescence methods with the T1-1 polyclonal antiserum have been corroborated by immunoblot techniques in previously published studies (11). The amount of topoisomerase I was defined as the total FITC fluorescence units in each nucleus. Values for amount of topoisomerase I in individual nuclei were then binned based on DNA content, and mean values for topoisomerase I levels were determined for cells having either a G1, S-, or G2-M-phase DNA content. Amount of topoisomerase I in nuclei of control cells with a G1 DNA content was normalized to a value of 1.0, and means and SD of amounts of topoisomerase I calculated for cells in each phase of the cell cycle at each time point. Calculations were done with GraphPad Prism software on an IBM-compatible personal computer.

To circumvent investigator bias, fields of cells for analysis were chosen using only the DNA filter, when protein fluorescence was not visible. Fields of cells containing 3–12 nonoverlapping and intact nuclei were imaged. After recording the DNA image, the filter was changed to allow image acquisition of the indirect immunofluorescence stain for topoisomerase I of that field. All images recorded were included in analyses.

**Determination and Identification of Cells That Are Representative of Changes Characteristic of Daoy Cells Exposed to Topotecan.** Using image cytometry, characteristics of individual cells identifiable with specific fluorochromes can be evaluated and quantitated. Nuclei shown in Fig. 3 are representative of those seen in cells treated with 10 nm topotecan for 0, 4, or 48 h. These cells were determined to be representative by the following seven parameters: sum of nucleolar fluorescence, sum of nuclear fluorescence, mean nucleolar fluorescence, mean nuclear fluorescence, nucleolar area, nuclear area, and the ratio of topoisomerase I:DNA fluorescence. Representative cells had values within 1 SD of the mean for all seven parameters.

**Drug**

Topotecan was obtained from SmithKline Beecham. The drug was solubilized in sterile water at a concentration of 10⁻² m; this solution was stored at −20°C without loss of potency for several months. Working stock solutions were made by serially diluting the 10⁻² m solution with 10 mm Tris-HCl. Depending on the objective of an individual experiment, 10-fold dilutions of the stock solution were made with 10 mm Tris-HCl (pH 4.0, 7.4, or 9.0). The serial dilutions were stored at 4°C and used for a maximum of 5 days. Working dilutions of the drug were allowed to equilibrate overnight before use to ensure complete conversion of topotecan to the active lactone form (pH 4.0) or hydroxyacid form (pH 9.0).

**RESULTS**

**Time-dependent Changes in Covalent Topoisomerase I-DNA Complexes in Daoy Cells Exposed to Topotecan.** DNA and protein in Daoy pediatric medulloblastoma cells were radiolabeled metabolically with [³H]thymidine and [14C]leucine, respectively, and then the cells were incubated with 10 nm, 50 nm, or 2.5 μm topotecan (pH 4.0) for the times indicated in Fig. 1. At each time point, topoisomerase I-DNA complexes were quantitated by the K⁺-SDS precipitation method, which was modified for use with nanomolar concentrations of topotecan. Results in Fig. 1 are expressed as the ratio of DNA:protein precipitated at each time point, with untreated controls normalized to a value of 1.0. Topotecan was present continuously and was not removed from the medium prior to denaturation of topoisomerase I by SDS. At each concentration of topotecan, complexes were maximal 15 min after topotecan was added to the medium and decreased thereafter. Cells exposed to 10 nm topotecan for 8–48 h contained fewer protein-DNA complexes than the untreated control cells (Fig. 1). Additionally, few complexes (1.1-fold and 1.2-fold above control values) were detectable when 50 nm or 2.5 μm topotecan had been present in the medium for 48 h. Similar results were also obtained with Rh30 rhabdomyosarcoma cells, SJ-Med3 medulloblastoma cells, and GC3 colon adenocarcinoma cells (data not shown).

The decrease in topoisomerase I-DNA complexes after 15 min of exposure to topotecan (Fig. 1) could be explained in one of three ways: (a) topotecan could be metabolized to an inactive compound, (b) the active lactone form of topotecan might re-equilibrate in a pH-dependent manner over time to the inactive hydroxyacid form, or (c) intracellular events following exposure to topotecan may modify cellular responses to this drug in a time-dependent manner.

**Stability of Topotecan in Tissue Culture Medium.** We addressed the first of these possibilities by carrying out sequential growth inhibition studies using the same topotecan/tissue culture medium for both studies. The objective of the sequential growth inhibition assays was to determine whether topotecan was present and biologically active after 48–96 h in the medium of tissue culture dishes containing Daoy cells. Cells were plated in tissue culture dishes, and the drug was added on day 1. Percentage of inhibition of cell growth of drug-treated cells compared to untreated cells was determined by quantitating cell number on day 3.

The medium and serum containing topotecan from this first growth inhibition study was then reused for a second growth inhibition study done on days 3 through 5, as detailed in "Materials and Methods." The growth-inhibitory potency of solutions of log increments of topotecan decreased 12 ± 1% in the assay counted on day 5 compared to that counted on day 3. We concluded that topotecan was still present and active after 48 h in culture and that metabolic inactivation did not account for the time-dependent decrease in topoisomerase I-DNA complexes seen in Fig. 1.
Comparison of the Effect of Addition of Either the Lactone or Hydroxyacid Form of Topotecan on the Formation of Topoisomerase I-DNA Complexes. To address the possibility that changes in levels of covalent topoisomerase I-DNA complexes were due to a pH-dependent shift in equilibrium between the active lactone and the inactive hydroxyacid forms of topotecan, we compared the levels of topoisomerase I-DNA complexes formed after adding either the lactone form of topotecan (pH 4.0) or the hydroxyacid form of topotecan (pH 9.0) to cultures of Daoy cells for 15 min–48 h. The experiment was done as detailed in Fig. 1. Data in Fig. 2 show that 15 min following addition of the lactone form of the topotecan, the number of protein-DNA complexes had increased 5.9-fold over untreated controls and then decreased to 1.4-fold by 4 h. A small, transient increase in complexes at the 6–8-h time point was observed consistently, with 200 nm (Fig. 2) and 50 nm (Fig. 1) topotecan in both Daoy cells and Rh30 cells (data not shown). When the hydroxyacid form of the drug was used, protein-DNA complexes were maximal (a 1.6-fold increase) 1 h after the drug had been added, and thereafter, complexes were equivalent to those produced when topotecan (pH 4.0) had been added. Few complexes (1.25-fold above control levels) were detected 24 h after the addition of either form of the drug. The decrease in complexes seen between 15 min and 1 h following addition of topotecan lactone is consistent with previous studies showing that the time required in monolayer cultures to achieve an intracellular steady-state level of topotecan lactone is ~60 min (13). As expected, the number of topoisomerase I-DNA complexes produced by the two preparations was equivalent between 2 and 24 h. We confirmed by high-performance liquid chromatography analysis of the tissue culture medium that the concentration of topotecan lactone was constant 2 h after addition of the drug. It is unlikely, therefore, that changes in levels of covalent topoisomerase I-DNA complexes after ≥2 h exposure to topotecan are due to pH-dependent changes in intracellular levels of topotecan lactone.

Fig. 1 Quantitation of covalent topoisomerase I-DNA complexes in Daoy medulloblastoma cells incubated with 10 nm, 50 nm, or 2.5 μM topotecan (pH 4.0). The protein and DNA of Daoy cells were labeled metabolically by an overnight incubation with [3H]thymidine and [14C]leucine. Cells were then incubated with the indicated concentrations of topotecan for times ranging from 15 min to 48 h. Covalent protein-DNA complexes were precipitated by the K⁺-SDS method, and radioactivity in the precipitate was quantitated by liquid scintillation counting. Results were normalized to the ratio of DNA:protein precipitated in the control (0 h) cells. The cpm of the internal control [14C] precipitated in each sample analyzed varied among experiments; the minimum cpm precipitated at time 0 was 5000. Means from a representative experiment of two to five experiments are shown (bars, SD).

Fig. 2 Quantitation of covalent topoisomerase I-DNA complexes in Daoy cells incubated with 200 nm topotecan lactone (pH 4.0) or hydroxyacid (pH 9.0). The procedure used was similar to that described in the legend to Fig. 1. The cpm precipitated at time 0 were ~1500 and ~3500 for thymidine and leucine, respectively. The experiment shown is representative of three experiments. Data are means; bars, SD.

Comparison of the Effect of Addition of Either the Lactone or Hydroxyacid Form of Topotecan on the Formation of Topoisomerase I-DNA Complexes. To address the possibility that changes in levels of covalent topoisomerase I-DNA complexes were due to a pH-dependent shift in equilibrium between the active lactone and the inactive hydroxyacid forms of topotecan, we compared the levels of topoisomerase I-DNA complexes formed after adding either the lactone form of topotecan (pH 4.0) or the hydroxyacid form of topotecan (pH 9.0) to cultures of Daoy cells for 15 min–48 h. The experiment was done as detailed in Fig. 1. Data in Fig. 2 show that 15 min following addition of the lactone form of the topotecan, the number of protein-DNA complexes had increased 5.9-fold over untreated controls and then decreased to 1.4-fold by 4 h. A small, transient increase in complexes at the 6–8-h time point was observed consistently, with 200 nm (Fig. 2) and 50 nm (Fig. 1) topotecan in both Daoy cells and Rh30 cells (data not shown). When the hydroxyacid form of the drug was used, protein-DNA complexes were maximal (a 1.6-fold increase) 1 h after the drug had been added, and thereafter, complexes were equivalent to those produced when topotecan (pH 4.0) had been added. Few complexes (1.25-fold above control levels) were detected 24 h after the addition of either form of the drug. The decrease in complexes seen between 15 min and 1 h following addition of topotecan lactone is consistent with previous studies showing that the time required in monolayer cultures to achieve an intracellular steady-state level of topotecan lactone is ~60 min (13). As expected, the number of topoisomerase I-DNA complexes produced by the two preparations was equivalent between 2 and 24 h. We confirmed by high-performance liquid chromatography analysis of the tissue culture medium that the concentration of topotecan lactone was constant ≥2 h after addition of the drug. It is unlikely, therefore, that changes in levels of covalent topoisomerase I-DNA complexes after ≥2 h exposure to topotecan are due to pH-dependent changes in intracellular levels of topotecan lactone.

From data in Figs. 1 and 2 and sequential growth inhibition studies, we conclude that the decrease in detectable topoisomerase I-DNA complexes after a 2–48-h incubation with topotecan was not due to metabolic or chemical inactivation of the drug, and that a cellular response to the drug most likely accounts for the decrease in topoisomerase I-DNA complexes.

Subnuclear Localization of DNA Topoisomerase I after Incubation of Daoy Cells with 10 nm Topotecan. We have shown previously that micromolar concentrations of topotecan
induce a redistribution of immunodetectable topoisomerase I in SJ-G5 anaplastic astrocytoma cells. In that report, we postulated that the redistribution could be responsible for the biphasic response in levels of topoisomerase I-DNA complexes after cells have been exposed to topotecan. We (5) and others (1, 13–17) have also suggested that fluctuations in nuclear or whole-cell levels of topoisomerase I might have impact on the number of topoisomerase I-DNA complexes. Therefore, we determined the effect of 10 nM topotecan on the amount and subnuclear distribution of topoisomerase I in Daoy cells. Topotecan (10 nM) is achievable in the plasma of patients and is the IC_{99} for Daoy cells incubated for 5 days with topotecan in vitro.

Daoy cells were fixed and stained by indirect immunofluorescence methods for DNA topoisomerase I before and after exposure of cells to 10 nM topotecan for 0, 2, 4, 8, 24, or 48 h. Analyses of amount and subcellular distribution of topoisomerase I were done by fluorescence image cytometry as reported previously (5). An average of 123 cells per time point were analyzed. Nuclei from cells representative of those exposed to 10 nM topotecan for 0, 4, or 48 h, according to the criteria detailed in “Materials and Methods,” are shown in Fig. 3.

In the absence of the drug (Fig. 3), topoisomerase I was associated with small, discrete, irregularly shaped nucleoli. After 4 h of exposure to 10 nM topotecan, topoisomerase I staining appeared to be somewhat more dispersed throughout the nucleoplasm compared to untreated cells. After 48 h, the topoisomerase I of the topotecan-treated cells was associated with a few very large spherical bodies (18), more uniform in shape than the nucleoli seen in the untreated controls.

In addition to these morphological changes, visual inspection of the nuclei of Daoy cells incubated for 4 h with 10 nM topotecan showed an apparent decrease in nuclear and nucleolar topoisomerase I content. The observed decrease was not due to the inability of the antiserum to recognize DNA-bound topoisomerase I, because maximum binding of topoisomerase I to DNA occurred within 15 min after addition of the drug, and the apparent decrease in topoisomerase I was not observed until 4 h after the drug was added. Inspection of representative nuclei of Daoy cells incubated for 48 h with 10 nM topotecan revealed no obvious change in fluorescence intensity of immunodetectable topoisomerase I, compared to nuclei of untreated control cells. Notably, untreated controls included cells in each phase of the cell cycle; cells exposed to 10 nM topotecan for 4 h were predominantly G_1; and cells exposed for 48 h were predomi-

Fig. 3 Photomicrographs of topoisomerase I in nuclei of Daoy cells exposed to 10 nM topotecan for 0, 4, or 48 h. DNA topoisomerase I was stained by indirect immunofluorescence methods with a FITC-conjugated secondary antibody, and fluorescence units were quantitated by image cytometric methods. Nuclei shown have values within 1 SD of the seven parameters describing topoisomerase I and DNA amount and distribution detailed in “Materials and Methods.”

Fig. 4 Quantitation of nuclear topoisomerase I in Daoy cells exposed to 10 nM topotecan for 0, 4, 8, 24, or 48 h. Topoisomerase I and DNA in the nucleus of each of an average of ~150 cells in each phase of the cell cycle in a total of six experiments were quantitated. Results (means; bars, SD) were normalized to the mean fluorescence units of topoisomerase I in untreated cell in the G_1 phase of the cell cycle.
Intermittent versus Continuous Exposure to Topotecan

**Table 1** IC₅₀ of topotecan for different durations of repeated exposures in Daoy and SJ-Med3 medulloblastoma cells

Topotecan was present in the tissue culture medium for the indicated number of hours, daily for 5 consecutive days.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Exposure to topotecan h/day for 5 days</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daoy</td>
<td>4</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2.2</td>
</tr>
<tr>
<td>SJ-Med3</td>
<td>4</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*IC₅₀ is defined as the concentration of topotecan required to inhibit growth by 50%.

**Table 2** Comparison of the IC₅₀ of two different schedules of exposure to topotecan in Daoy and SJ-Med3 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Exposure to topotecan h/day for 5 days</th>
<th>IC₅₀ (nM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daoy</td>
<td>8</td>
<td>2.20 ± 0.84</td>
</tr>
<tr>
<td>SJ-Med3</td>
<td>8</td>
<td>3.65 ± 1.70</td>
</tr>
</tbody>
</table>

*IC₅₀ is defined as the concentration of topotecan required to inhibit growth by 50%.

**Effect of 10 nM Topotecan on the Amount of Topoisomerase I in Daoy Cells.** Although the above images suggested that 10 nM topotecan produced a transient decrease in nuclear/nucleolar topoisomerase I as well as a redistribution of the protein, it is important to note that the changes seen were estimated visually on a per-nucleus basis and not normalized to cellular protein or DNA content. Because topotecan causes perturbations in cell cycle distribution, we next quantified the amount of topoisomerase I per nucleus of cells incubated for 0, 2, 4, 8, 24, or 48 h with 10 nM topotecan and normalized the topoisomerase I level to cellular DNA content by expressing the results as topoisomerase I content per nucleus of cells having either a G₁-, S-, or G₂-M-phase DNA content (Fig. 4). Analysis of ~150 cells/phase of the cell cycle in a total of six experiments showed that, as anticipated, nuclei with a G₂-M DNA content had higher levels of topoisomerase I than cells with a G₁ DNA content. The apparent transient decrease between 4 and 8 h was observed in cells in all three phases of the cell cycle; however, the apparent decreases were not statistically significant. We conclude that in addition to changes in cell cycle distribution, 10 nM topotecan for 48 h also alters the nuclear distribution, but not the amount, of topoisomerase I in Daoy medulloblastoma cells.

It is important to note that after exposure to 10 nm topotecan for 48 h, Daoy nuclei and nucleoli contain as much topoisomerase I as untreated cells, and the topotecan is still biologically active, yet no covalent topoisomerase I-DNA complexes can be detected in these cells. The amount of nuclear topoisomerase I does not reflect the number of complexes present.

We then proposed that if the cytotoxicity of topotecan is, in fact, dependent on the formation or presence of topoisomerase I-DNA complexes and that these complexes are present for only 4–8 h following addition of topotecan (10 nm; Fig. 1), then intermittent 8-h exposures to topotecan might produce growth inhibition similar to that seen following continuous exposure to the drug.

**Comparison of Intermittent and Continuous Exposure to Topotecan.** Data from the experiment testing the hypothesis discussed above showed no difference between the IC₅₀ of Daoy or SJ-Med3 cells exposed to topotecan continuously or 8 h/day for 5 days. The data also suggested that daily exposures of 6 or 4 h/day appeared to be progressively less effective in inhibiting the growth of Daoy cells.

On the basis of the data in Table 1, we compared more rigorously the growth-inhibitory effect of exposure to topotecan 8 h daily for 5 days or 24 h daily for 5 days. Four to six experiments were carried out with each of the two pediatric medulloblastoma cell lines (Table 2). Exposures of 8 h/day for 5 days to topotecan produced the same growth inhibition in both cell lines when compared to continuous exposure, although the total drug exposure of cells incubated intermittently with the drug was one-third that for cells incubated continuously with the drug. These experiments suggest that it may be possible to identify several efficacious schedules of exposure to topotecan.

**DISCUSSION**

Data in this article show that continuous exposure of Daoy pediatric medulloblastoma cells to topotecan lactone produces a biphasic effect on topoisomerase I-DNA complexes. These complexes are maximal after a 15-min exposure to the drug and decrease to less-than-control or near-control levels by 8–48 h (Fig. 1), in spite of the continuous presence of the biologically active drug. The lack of detectable topoisomerase I-DNA complexes following continuous exposure to topotecan is not accompanied by a decrease in topoisomerase I. Of note, in growth inhibition assays, exposure of Daoy and SJ-Med3 medulloblastoma cells to topotecan 8 h/day for 5 days is equitoxic with continuous exposure to the drug.

Previously published studies have focused on the relative growth-inhibitory potential of different lengths of exposure to camptothecins (20, 21). Each of these studies showed that for a given concentration of the drug, a single short exposure to the drug produced less growth inhibition than a single longer exposure. Our study is the first to compare the growth inhibitory effect of repeated exposures to topotecan. Additionally, Beidler and Cheng (3) recently reported the effect of camptothecin on levels of "topoisomerase I-DNA adducts." In that study, KB human nasopharyngeal carcinoma cells were preincubated with 7 nm–5 μM camptothecin and then uniformly challenged with 5 μM camptothecin to determine the effect of preincubation of a range of concentrations on the number of covalent topoisomerase I-DNA complexes induced by 5 μM camptothecin. In contrast, the experiments reported in our study show the effect of a single concentration of topotecan on topoisomerase I-DNA complexes following intermittent 8-h exposures to topotecan.
complexes over time in an effort to mimic an in vivo exposure to the drug given as an i.v. infusion.

Several observations reported here are in apparent contrast to other reports in the literature. Our image cytometry study detected little change in the amount of topoisomerase I in Daoy cells incubated with 10 nm topotecan for 0–48 h. It is likely that the apparent discrepancy between our results and those describing a more pronounced decrease in the amount of topoisomerase I following exposure in vitro or in vivo of cells to camptothecins (3, 22) can be attributed to the concentrations of the drug used and to the manner in which the amount of topoisomerase I is expressed. Because it is well documented that the camptothecins produce cell cycle perturbations (1, 19), levels of topoisomerase I following exposure to topotecan are comparable only if first normalized to cellular protein or DNA content. Furthermore, both DNA-bound and free enzyme must be included in the quantitation. The antibody used in our experiments recognizes both the DNA-bound and free forms of the enzyme as shown by its equal reactivity with nuclear and nucleolar topoisomerase I in control cells and in cells incubated with 0.25 and 1 μM topotecan for 0.25–1 h (Ref. 5 and data not shown). Finally, because cell density has a profound effect on topoisomerase I:DNA ratio, cell density must be carefully controlled to ensure logarithmic growth. Our data show little effect of 10 nm topotecan on the amount of topoisomerase I in Daoy cells in vitro (Fig. 4).

We do show in Fig. 3, however, that consistent with previous reports with micromolar concentrations of camptothecin derivatives (5, 23) 10 nm topotecan for 48 h induced a redistribution of DNA topoisomerase I. The pattern of redistribution is strikingly similar to that shown for another nucleolar protein RNA polymerase I in cells incubated with concentrations of D-galactosamine that inhibited RNA synthesis (24), suggesting that redistribution of nucleolar proteins is a nonspecific cellular response not unique to the camptothecins. Obvious changes in nucleolar topoisomerase I distribution were not evident until after 24–48 h of exposure to topotecan, whereas the decrease in complexes was evident within 2 h after addition of the drug. Therefore, it is unlikely that redistribution of nucleolar protein contributes directly to the decrease in complexes seen after incubation with 10 nm topotecan for 2–24 h. Possibly, redistribution of topoisomerase I prevents or minimizes the formation of additional complexes by distancing topoisomerase I from the DNA sequence to which it binds. Recent reports of an enzymatic activity that excises covalent 3′-DNA-protein complexes are of interest; we speculate that a repair activity such as that described by Yang et al. (25) could account for the observed decline in topoisomerase I-DNA complexes at later time points in spite of persistent levels of topoisomerase I and biologically active topotecan.

Furthermore, the cytotoxicity of the camptothecins has been reported to be dependent on ongoing DNA synthesis (1). Potentially, if only cells in S phase are sensitive to the cytotoxicity of topotecan, then as the cells traverse the cell cycle, long infusions as have been used in several clinical trials would ensure that the drug would be present as additional tumor cells entered S phase. This may, in fact, be a correct hypothesis. However, hematopoietic toxicity and mucositis may limit the amount of topotecan that can be given to patients (6, 8, 9). As an alternative strategy to identify effective schedules of exposure to the camptothecins, we reasoned that if the complexes that mediate or initiate the cytotoxic effect of topotecan are present only for 8 h regardless of the duration of exposure, or if the transient increase in complexes at 6–8 h were important, perhaps an 8-h exposure to the drug would be as effective as continuous exposure. Growth inhibition studies substantiated this hypothesis in the Daoy and SJ-Med3 pediatric medulloblastoma cell lines in vitro (Table 2) and in SJ-G5 anaplastic astrocytoma cells (data not shown). We are currently evaluating the antitumor effect of intermittent repetitive exposures to topotecan in mice bearing Daoy cells as a xenograft.

The clinical utility of each effective schedule of exposure to topotecan will depend on whether the therapeutic index of repetitive intermittent exposure to the drug is more or less favorable than the therapeutic index of continuous exposure. Because hematopoietic toxicity is most frequently the dose-limiting toxicity of topotecan, in vitro studies are currently being done in our laboratory to compare the effect of continuous and intermittent exposure schedules on tumor cells and human bone marrow progenitor cells in vitro. Ultimately, understanding cellular response to the camptothecins may help to identify schedules of intermittent exposure that maximize the therapeutic index of this class of drugs in the clinic. Ongoing experiments focus on determination of the optimal interval between the 8-h exposures to topotecan and on comparison of the toxicity of different schedules of exposure of topotecan to human tumor and bone marrow progenitor cells.

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