Effect of Prolonged Topotecan Infusion on Topoisomerase 1 Levels: A Phase I and Pharmacodynamic Study

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ABSTRACT

Topoisomerase 1 (topo-1) inhibitors act on the target enzyme by forming “cleavable complex,” a high molecular weight DNA protein adduct. The formation of such cleavable complexes results in depletion of the M, 100,000 “free” topo-1 band detectable by Western blot. The objectives of this study were to determine the maximally tolerated dose of prolonged topotecan infusion in previously untreated and minimally pretreated patients. A secondary objective was to measure the effect of prolonged topotecan infusion on topo-1 levels in peripheral blood mononuclear cells (PBMCs) as a pharmacodynamic end point.

In a prior Phase I study of 21-day topotecan infusion (H. Hochster et al., J. Clin. Oncol., 12: 553–559, 1994), the maximum tolerated dose for patients treated previously was 0.53 mg/m²/day for 21 days every 28 days. In this study, patients with no prior therapy were treated similarly at 0.7 mg/m²/day for 21 days, and doses were escalated in 0.1 mg/m²/day increments. Patients who had one prior chemotherapy regimen or radiation therapy to a portal of ≤20 cm² were entered at the 0.6 mg/m²/day level. Cohorts of four patients were entered until the maximum tolerated dose was determined. Peripheral blood was sampled weekly to obtain plasma topotecan drug levels and topo-1 levels in PBMCs by Western Blot.

For previously untreated patients, the dose-limiting toxicity was myelosuppression at the dose of 0.8 mg/m²/day. Anemia was seen as a cumulative effect. Unexpected nonhematological toxicity was not observed. topo-1 level analysis by Western blot showed progressive decrement in the percentage of free topo-1 (compared to baseline value) during weeks 1, 2, and 3. The median percentage of decrease from baseline was 26% (P, not significant; Wilcoxon signed rank test) at week 1, 45% (P = 0.10) at week 2, and 77% (P = 0.016) at week 3. At week 4, off drug treatment, the median percentage of decrease from baseline was only 14%. Additional analysis of free topo-1 level as a function of both area under the curve (P = 0.005) and day of infusion (P = 0.003) demonstrated a significant relationship by regression analysis using a linear mixed effects model.

In this Phase I study of topotecan prolonged infusion, hematological toxicity remained dose limiting without evidence of previously described nonhematological toxicity. The recommended Phase II dose is 0.7 mg/m²/day for 21 days every 28 days for previously untreated patients and 0.6 mg/m²/day for those with limited prior therapy. Western blot analysis of noncomplexed topo-1 in PBMCs sampled weekly showed a progressive depletion of free topo-1 over the 21 days of infusion, which reached statistical significance by week 3. Within 1 week of stopping infusion, topo-1 levels return to baseline. A strong correlation of topo-1 level with area under the curve and duration of infusion was demonstrated. These data suggest that prolonged administration of topo-1 inhibitory drugs results in sustained depletion of free topo-1 enzyme as measured by Western Blot analysis, which may be an important consideration in the clinical use of these agents. Direct randomized, comparative trials will be necessary to determine whether such schedules will improve therapeutic index and efficacy.

INTRODUCTION

topo-1 is a unique target for cancer chemotherapy. This intranuclear enzyme, involved in unwinding of supercoiled DNA, is integrally involved in a host of essential cell housekeeping functions including replication and transcription (2–4); therefore, it serves as an attractive target for chemotherapy compounds that inhibit its function. topo-1 is a monomeric M₉ 100,000 protein encoded by a single gene located on chromosome 20 at the q12–13.2 band (5). The intranuclear enzyme functions to relax supercoiled DNA and induces a temporary single-stranded DNA break, allowing DNA strand passage in the process. This unwinding makes the freely swiveling DNA strand available for replication and repair.

The active site for topo-1 activity is located at the tyrosine moiety of amino acid residue 723 of the human enzyme (3). The tyrosine group participates in a nucleophilic attack on the phosphodiester linkage of DNA, resulting in covalent binding of topo-1 to the broken DNA strand (6). The opposite phosphodiester linkage undergoes strand passage to accomplish the un...
winding function. Following this step, a reannealing of the DNA strand occurs via another nucleophilic attack by the 5' hydroxyl group on the DNA-enzyme complex. Although many effective chemotherapeutic agents inhibit topo-2, an enzyme involved in double-strand DNA unwinding and breakage, only camptothecin and its analogues inhibit topo-1. The reannealing step is delayed by the interaction between topo-1 and camptothecins and forms a DNA:topo-1:camptothecin “cleavable complex” detectable in the laboratory as high molecular weight, protein-associated DNA adducts. This unique mechanism deserves emphasis due to two important properties: (a) because the interaction does not rely on inhibition of a target substrate regulated by feedback processes, cytotoxicity by topo-1 inhibitors is proportional to the amount of topo-1 enzyme present in the cell; and (b) cell death is caused by the conversion of the normal housekeeping protein into a “cellular poison.”

In a prior Phase I study, we reported on the feasibility of prolonged low-dose topotecan administration in patients treated previously (1). Our hypothesis was that prolonged administration of camptothecin analogues would prove more effective than other schedules and achieve a greater therapeutic index. Animal experiments using camptothecin analogues suggested that prolonged s.c. depot administration to mice bearing human tumor xenografts could cure these tumors with minimal toxicity (7). In the prior study, we began at the very low dose of 0.2 mg/m2/day for 7 days and escalated in cohorts of four patients to achieve a well-tolerated dose of 0.53 mg/m2/day for 21 days, repeated every 28 days. This group of patients was heavily pretreated with a mean of three prior chemotherapy regimens. Additional observations suggested that tolerance of this regimen was affected by the extent of prior therapy.

The purpose of this study was to extend these observations to previously untreated patients or minimally pretreated patients, having no more than one prior chemotherapy regimen or minimal radiation therapy (with a portal ≤20 cm2). The untreated group began at a dose of 0.7 mg/m2/day for 21 days, and the patients with prior therapy began at a dose of 0.6 mg/m2/day for 21 days. In both cases, the dose was escalated in 0.1-mg/m2/day increments.

Concurrent with initiation of this study, we began to investigate the utility of Western blot determination of topo-1 as a clinical monitoring tool. Through a series of assay modifications and sensitivity improvements, we were able to quantitate the free (noncomplexed) topo-1 in tumor tissues and normal tissues.4 We selected PBMCs as a normal tissue that would be readily available for serial sampling and analysis. In this group of 17 patients, we obtained peripheral blood once during “steady-state” level initially and then weekly (in the last eight patients) to determine the course of inhibition of the topo-1 enzyme in a readily available normal tissue. This analysis was a secondary end point of the current study.

**PATIENTS AND METHODS**

**Clinical.** Seventeen patients were entered into this study using standard Phase I eligibility criteria including: (a) histologically confirmed malignant solid tumor refractory to standard therapy, or for which no established therapy existed; (b) measurable or evaluable disease; (c) Eastern Cooperative Oncology Group performance status grade 0 or 1; (d) adequate organ function with WBCs >3,000/mm3, platelets >100,000/mm3, bilirubin <2.0 mg/dl, liver function test (transaminases and alkaline phosphatase) less than three times the upper limit of normal, and creatinine ≤2.0 mg/ml; (e) no prior chemotherapy for one cohort or, for the other cohort, no more than one prior chemotherapy or radiation to a port of ≤20 cm2. Patients with both measurable and evaluable disease were eligible for this study.

On-study data included physical examination, chest X-ray, complete blood count, serum chemistries, and computerized axial tomography scans to document extent of disease. Complete blood counts with differentials were performed twice weekly, and radiographic studies were repeated every two cycles to evaluate response. Standard Eastern Cooperative Oncology Group response criteria were used as well as National Cancer Institute Common Toxicity Criteria for grading toxicity. Simultaneous entry in parallel dose levels was carried out for the nonpretreated and minimally pretreated groups of patients. Four patients were to be entered in each nontoxic dose level. Up to two additional patients were added to each toxic dose level. An additional three patients were added at the MTD to better define the toxicities and their incidence. The MTD was defined as the level prior to that which produced consistent dose limiting toxicity defined as at least two of (a) maximum of six patients having grade 4 hematological or grade 3 nonhematological toxicity.

All patients signed informed consent as approved by the NYU Medical Center Institutional Review Board. This protocol and amendment was approved by the National Cancer Institute, Cancer Therapy Evaluation Program, which supplied topotecan for this study.

**Drug Administration.** Patients were required to undergo placement of a semi permanent venous access device. Topotecan (NSC 609699; SmithKline Beecham, King of Prussia, PA) was provided by the National Cancer Institute, Cancer Treatment Evaluation Program, and administered by CADD ambulatory pump (Sims-Deltec, Minneapolis, MN) as a prolonged 21-day continuous infusion. The preparation used in this study was the initial “AA” formulation, which was approved for 72 h stability only. Cassettes were changed three times weekly according to the sterility and stability data available at the time. Venous access devices were reaccessed weekly with placement of new Huber needles and tubing. Treatment was repeated every 28 days, unless recovery from toxicity was incomplete. For hematological toxicity, recovery was defined as ANC >1,500/mm3 and platelet count >75,000/mm3. Topotecan administration was discontinued early in the event that the WBC count fell below 1,000/mm3 or platelets below 50,000/mm3. In the event of grade 4 hematological toxicity, the dose was reduced one level (by 0.1 mg/m2/day) for subsequent cycles.

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Mononuclear Cell Preparation. Mononuclear cells were prepared from two heparinized tubes, 20 cc of blood, using standard methods (8) with the following modifications to allow for topotecan measurement and the stabilization of the mononuclear preparation for Western blotting analysis. The tubes were centrifuged at 1000 × g for 10 min to separate the plasma from the buffy coat. The separated plasma was mixed with an equal volume of the analysis stabilizing buffer [100 mm (NH₄)₂PO₄, (pH 6.5), 1 mm bis(2-ethylhexyl) sulfosuccinic acid sodium salt]. The reference internal standard [SKF 105107, dissolved in 0.1 m NaH₂PO₄ (pH 3.5); concentration, 1 μg/ml] was also added in sufficient quantity to yield a final concentration of 10 ng/ml of the undiluted plasma prior to the addition of the stabilizing buffer. The remaining buffy coat from each of the heparinized tubes was diluted with 5 ml of RPMI 1640 and then processed through a standard Ficoll-Hypaque gradient centrifugation step (9). The mononuclear cell layer was removed, washed twice with PBS, and centrifuged at 1000 × g. The cells were resuspended in a volume of 0.3 ml, and 0.05 ml was removed and stored frozen for the DNA analysis. Then 0.25 ml of 2× SDS sample buffer [20% glycerol, 10% β-mercaptoethanol, 6% SDS, 125 mm Tris (pH 6.8), and 0.2% bromphenol blue] was added, followed by mixing. This portion of the sample was then boiled for 5 min at 100°C and stored at −70°C until processing for Western blot analysis.

Pharmacokinetics. Plasma specimens were processed according to our modifications of a method described previously (10) using methanol dilution. To allow for better sensitivity given the low doses administered, we used a solid phase extraction technique that concentrated, rather than diluted, the specimens in the protein processing step. Specimens were processed using C₁₈ Solid Phase Extraction columns within 60 min of phlebotomy; specimens were subsequently stabilized in cold methanol as detailed below to preserve the relative ratios of the lactone and open-ring forms of the camptothecin analogue.⁴

Three to five ml of stabilized plasma from clinical samples were centrifuged at 1000 × g for 10 min, followed by the addition of buffers and internal standard (SKF 105107; SmithKline Beecham). These samples were then applied to extraction columns (Fisher Prep Sep; Fisher Scientific, Springfield, NJ). Topotecan and internal standard were then eluted from the column within 5 min. A standard set of plasma containing topotecan ranging from concentrations of 2.5-100 ng/ml was processed and analyzed with each clinical specimen to provide a standard curve. Typically, 0.2 ml of the extract was applied to the high-performance liquid chromatography system for analysis of the closed form. Another 0.1 ml of the extract was mixed with 0.05 ml of 0.05 m H₃PO₄, allowed to sit at room temperature for 1 h, and then analyzed for total drug. The high-performance liquid chromatography system used a 15-cm × 4.6-mm 3-μm C₁₈ column (Phase Separations, Inc., Norwalk, CT) conditioned with 67% methanol containing 0.01 μl bis(2-ethylhexyl) sulfosuccinic acid sodium salt, 1 mm NH₄PO₄ (pH 6.0), and 0.3% triethylamine at a flow rate of 1 ml/min. Detection was used an Applied Biosystems (Perkin-Elmer, Norwalk, CT) 970 fluorometer set at 380 nm excitation with a 470-nm cutoff filter, 0.01 μl full scale. The chromatographic system consisted of a Knauer (Sonntek, Inc., Upper Saddle River, NJ) model 42 pump and a Waters Associates (Milford, MA) model 712 WISP autosampler, controlled by an Axxiom model 747 PC controller/ data system (Axxiom Chromatography, Inc., Moorpark, CA).

Each sample was run in duplicate, and the results were expressed as an average of the values. Blood levels were modeled using PC-NONLIN version 4.2 (Scientific Consulting, Inc., Apex, NC) with a steady-state infusion model.

Topo-I Determination by Western Blot. Protein mixtures obtained from the cell lysates were separated by 6% PAGE with 50 μl of each sample loaded per well. Duplicate analyses were run for each sample, and adequate sample was left over for reanalysis at a later date if necessary. The gel was run at a constant current of 40 mA for 6 h. The proteins were then transferred onto a NEN Duroapore membrane (Dupont Biotechnology Systems, Boston, MA) by electroblotting at 4°C at a constant current of 850 mA for 22 h. The membrane was air dried for two min, reactivated with methanol, and then blocked using 5% milk buffered with TBS (20 mm Tris, 500 mm NaCl, and 0.03% Tween 20, pH 7.4) for at least 1 h.

The membranes were next incubated overnight with the primary antibody suspended in 5% buffered milk at a dilution of 1:1000. Following this incubation, the membranes were washed twice (6 min/wash) with 5% buffered milk, three times (10 min/wash) with TBS, and again two times (6 min/wash) with 5% buffered milk.

After these washing steps, the membranes were exposed to horseradish peroxidase-conjugated protein A (1:1000 dilution; Amersham Life Sciences, Arlington Heights, IL) in 5% buffered milk for 1 h, washed twice (6 min/wash) with 5% buffered milk, followed by six washes (10 min/wash) with TBS. The amplification of the topo-I signal by the Enhanced Chemiluminescence system (ECL; Amersham Life Sciences) involved the addition of 12 ml of the ECL reagents for 1 min with constant agitation, drying with blotting paper, and then wrapping in a polyethylene film. Exposures to XAR-5 X-ray film (Kodak, Rochester, NY) were conducted with an initial 10-s exposure to assess the strength of the signal, followed by additional exposures ranging from 0.5–15 min. Quantitation of Western Blot scans was accomplished using a GS-2 densitometer (Hoeffer, San Francisco, CA) linked to our laboratory PC, equipped with an Axxichrom chromatography system (Axxiom Chromatography, Inc., Moorpark, CA). The densitometric areas were then normalized for the amount of DNA measured from the companion sample subjected to diphenylamine DNA analysis. In repeat sampling of three normal volunteers over 5 weeks, we found the mean (± SD) copy number to be 20.9 ± 3.1 × 10⁴ copies/cell. Within individuals, assay variability showed a SD of 13–34%.

Peripheral blood was initially sampled once per cycle for steady-state levels. After initial analysis in the first 10 patients, it became apparent that PBMC topo-I levels did not remain at a constant steady-state level but varied through the treatment cycle (data not shown). Therefore, we began to sample blood weekly in the latter patients enrolled and in the earlier patients who were still in treatment. These patients’ samples yielded a total of 11 cycles with baseline and additional data points during the cycle.

DNA Analysis. PBMC aliquots from the same samples as used in the Western Blot assay were analyzed for DNA content using a modification of the diphenylamine colorimetric determination (11) for use in a microtiter plate reader. Three
Table 1  Patient characteristics

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<td>Primary tumor</td>
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<td>Pancreas</td>
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hundred-μl aliquots were used in each well with the absorbance determined at 570 nm.

**Antibody Procurement.** Serum samples for SCL-70 antibody screening were obtained from patients with scleroderma (12). To obtain a supply of sera rich in SCL-70 antibody, a panel of patient serum specimens was analyzed by Western blot with varying concentrations of HeLa cell extracts, which are known to have a high level of topo-1 expression (13). Comparisons made between various samples yielded a patient with a high titer of highly specific SCL-70 antibody. Aliquots of 80 μl of this serum were stored at −80°C. This reagent was used for all studies in which we report the topo-1 protein copy numbers.

**Statistical Analysis.** For cycles in which topo-1 levels were measured at baseline (day 0), as well as at some other time points during that cycle, the percentage of change from baseline was calculated at each time point. The Wilcoxon signed rank test was used to assess whether the median percentage of changes at weeks 1, 2, and 3 significantly differed from baseline. To account for multiple comparisons conducted, a Bonferroni correction was used, i.e., the significance level for individual comparisons was set at $P = 0.017$, rather than the conventional 0.05.

To assess the relationship between topo-1 level and duration of infusion or AUC, a mixed effects linear model with subject as a random effect was used to account for the correlation between repeated observations on the same subject. The effect of dose and cycle number on the decrease in topo-1 level during a cycle was also assessed.

**RESULTS**

Seventeen patients were entered in this trial including seven patients at the 0.6 mg/m²/day dose level with prior therapy and ten with no prior therapy, five at 0.7 mg/m²/day and five at 0.8 mg/m²/day. Patient characteristics of this group are reported in Table 1. The median age was 59 years, and 12 (71%) of these patients had a diagnosis of gastrointestinal malignancy. The seventeen patients were treated with 37 cycles of therapy for 764 patient-days of topotecan infusion. The median number of cycles per patient was two (range, 1–5). Two patients each received five cycles of therapy.

**Hematological Toxicity.** Table 2 reports on the hematological toxicity seen for each group, including grade of toxicity and median nadir counts and ranges for the first cycle and for all cycles. The dose-limiting toxicity was hematological, as seen in our prior study with heavily pretreated patients. For the group with no prior therapy, five patients were treated at 0.7 mg/m²/day. There was one episode of grade 3 leukopenia, neutropenia, and thrombocytopenia. Five more patients were, therefore, entered at 0.8 mg/m²/day, resulting in two episodes of grade 4 neutropenia, both associated with grade 4 thrombocytopenia. Both required hospitalization for prolonged myelosuppression.

For the group with prior treatment, four patients were originally entered. One of these patients refused further therapy on day 5 and had no hematological toxicity. Of the other three patients, one developed grade 4 neutropenia and thrombocytopenia. Three more patients were entered at this dose level with one grade 3 and one episode of grade 4 neutropenia. Of these seven patients, two patients required early discontinuation of infusion in their second cycles due to low counts. One patient was escalated to 0.7 mg/m²/day in this group, which he tolerated with grade 2 neutropenia only. We did not formally test the 0.7 mg/m²/day dose in this group despite true lack of dose-limiting toxicity at the 0.6 mg/m²/day level. Nonetheless, our experience to date convinced us that we would see sufficient dose-limiting toxicity at the 0.7 level to meet the criteria of a toxic level exceeding the MTD. We conclude that 0.6 mg/m²/day can be considered a Phase II dose for minimally pretreated patients.

Other hematological toxicity included a significant degree of anemia, with 10 patients requiring transfusions, including four with grade 3 anemia. A total of three cycles were interrupted early due to decreasing blood counts. No delays in resuming therapy were necessitated at the 0.6 mg/m²/day level. Two patients in the previously untreated cohort, treated at 0.7 mg/m²/day, required delays in resuming treatment on the 28th day, including 3 of 14 cycles. At the 0.8 mg/m²/day level, only 2 of 14 cycles required delay. Median delay was 8 days. The recommended Phase II dose for untreated patients is 0.7 mg/m²/day for 21 days, and for those minimally pretreated, 0.6 mg/m²/day for 21 days.

**Nonhematological Toxicity.** Nonhematological toxicity was minimal in this patient population. One patient developed grade 3 nausea and vomiting and requested discontinuation of study drug within the first 5 days of therapy. This was a unique circumstance among 61 patients treated with low-dose topotecan infusion in both this study and the prior study. Three other patients developed grade 2 nausea and vomiting, and nine had grade 1 nausea, easily controlled with oral antiemetics. One patient had grade 3 and one grade 4 diarrhea, both in the setting of grade 4 neutropenia and hospitalization with antibiotic therapy. Fatigue was described as a very common symptom in the majority of the patients continuing on more than one course of therapy, usually described as grade 1. One patient developed a local port infection (grade 2), and one had a biliary tract infection (grade 2) due to the presence of a stent tube.

**Response.** In this group of patients, only one partial response was seen in a patient who had a primary pulmonary sarcoma. This patient had a near-complete response and went on to surgical resection of the residual lung nodule after five cycles at 0.8 mg/m²/day.
Results of topo-1 Determination. Peripheral blood mononuclear cells were isolated from 20 ml of heparinized blood, initially once per cycle for the first 10 patients enrolled and then weekly in the final seven patients enrolled. Analysis of the "steady-state" topo-1 levels in the first 10 patients showed significant variation, depending on the day of sampling (data not shown). Therefore, we began sampling blood weekly for PBMC topo-1 levels. For 11 cycles in nine patients sampled repeatedly, one or more determinations of PBMC topo-1 levels were available in addition to the baseline measurement. The topo-1 levels were quantitated by scanning densitometry of the Mr 100,000 band, where the density of the band reflects the amount of "free" noncomplexed topo-1 present. Each weekly value was compared to the baseline (day 0) sample and expressed as a percentage of baseline value. Fig. 1 demonstrates the results of Western blot analysis of PBMCs obtained in a typical patient cycle. Fig. 1A illustrates the results of Western blot analysis of PBMCs in a typical patient cycle. The results of the quantitation by scanning densitometry for this Western blot are shown in Fig. 1B. The blot demonstrates decreasing topo-1 intensity with increasing treatment time in the cycle.

Representative individual curves for topo-1 depletion are shown in Fig. 2A, which display the absolute copy number with day of treatment within each cycle. These two representative curves show a decrease in free topo-1 throughout the course of treatment in each case. For patient no. 58, data are shown through two consecutive cycles. This curve demonstrates progressive inhibition of topo-1 through the 21 days of therapy in the first cycle. When the topotecan infusion is stopped, the level returns to baseline on day 28. With retreatment in the second cycle, progressive inhibition is seen again over the 21-day period.

Fig. 2B summarizes the median percentage of change in topo-1 levels observed at each week of the cycle, relative to the baseline (day 0) level, for the 11 cycles analyzed in nine patients for which measurements were available at baseline as well as some other time point during the cycle. The median topo-1 level at baseline (day 0) was 20.0 × 10^6 copies/cell (SE, 2.2 × 10^6). A decrease in topo-1 level from baseline was observed at each time point; the median percentage of decreases were 26% (P, not significant; Wilcoxon signed rank test), 45% (P = 0.10), and 77% (P = 0.016) at weeks 1, 2, and 3, respectively. Data on topo-1 level at week 4 were available for three patients only.
Topoisomerase 1 Inhibition with Prolonged Topotecan Infusion

DISCUSSION

Topotecan and other camptothecin analogues now in the clinic function by the unique mechanism of topo-1 inhibition. topo-1 is an essential intranuclear enzyme with important DNA "housekeeping" functions. The interaction of the camptothecin analogue with the topo-1 enzyme stabilizes a DNA-protein adduct state termed the "cleavable complex." When monitored using the Western blot technique, this interaction results in depletion of detectable free topo-1 in the M1, 100,000 band. We have used this technique as a means of measuring the effect of topo-1 inhibitors over a prolonged period of topotecan administration. The presence of such cleavable complexes can be demonstrated by brief heating to 60°C, which restores free topo-1 by breaking apart the complexes (see below).

In this Phase I study, we recommend a Phase II dose for previously untreated patients of 0.7 mg/m²/day and 0.6 mg/m²/day for those with one prior therapy. As expected from our prior experience with more heavily pretreated patients, the dose-limiting toxicity was hematological, with one patient uniquely intolerant to this regimen due to persistent nausea and vomiting. Cumulative anemia and the requirement for red cell transfusion was also significant in this group of patients.

At present, Phase II studies of the 21-day infusion are being conducted by SmithKline Beecham and the Eastern Cooperative Group in ovarian, colon, breast, and lung cancers to further gauge the activity of this schedule in the Phase II setting.

In this study, we have also investigated the interaction of topotecan with its target enzyme, topo-1, as a clinical correlate and pharmacodynamic end point. This was accomplished by serially sampling PBMCs as a readily available normal tissue. We isolated mononuclear cells and performed Western blot analysis for the amount of free (or noncomplexed) topo-1 present in the M1, 100,000 band. These experiments demonstrate a progressive inhibition of topo-1 through the 3 weeks of infusion, which returns to baseline once the drug has stopped. This phenomenon is best illustrated by patient no. 58 through two cycles of therapy (Fig. 2a). For the entire data set, consisting of 11 cycles in nine patients, the median depletion of topo-1 compared to baseline progressively increased from 26% at week 1 to 45% at week 2 and 77% at week 3 (Fig. 2b). The change in topo-1 level reaches statistical significance only at week 3, but this may be due to the small number of values in this exploratory data set. Likewise at week 4 (day 28), the median depletion is only 14%, but only three values were available for this time point. These relationships will be explored in greater depth in on-going Phase II studies.

Depletion of the topo-1 signal shown here is due in part to formation of cleavable complexes that remain at the top of the gel and are not visualized. These cannot be detected by a direct assay in a quantitative way (data not shown). One method of demonstrating their presence is to break such complexes apart in a parallel specimen by brief boiling to 60°C. This phenomenon is shown in Fig. 4 for two patients treated with topotecan continuous infusion. The enhancement of the M1, 100,000 band after this heat "reversal" step demonstrates a large proportion of the topo-1 to be tied-up in high molecular weight complexes. We have found the phenomenon of signal restoration with brief boiling to be present in most PBMC specimens we have treated.
Fig. 3  Plot of topo-I protein levels as a function of AUC. A multiple regression analysis (mixed effect linear model) demonstrates significant correlation with AUC ($P = 0.005$). The line represents a simple regression line of the data.

Fig. 4  Western blot of PBMCs in two patients treated with topotecan infusion. Samples were treated in parallel with "heat reversal" to release topo-I from high molecular weight cleavable complexes, restoring intensity of the Mr 100,000 "free" topo-I band. A: Lanes 1 and 2, $1 \times 10^6$; Lane 3, $2 \times 10^6$ HeLa cells, which serve as controls for copy number determination. PBMC preparation from patient no. 1020 before (Lanes 12 and 13) and after (Lanes 14 and 15) heat treatment at 60°C for 1 min to release topo-I from cleavable complexes is shown (Lanes 4–11 show topo-I levels from other patients not subject to heat reversal). The copy number increased from barely detectable to $5.9 \times 10^5$ copies/cell after heat reversal. B: Lanes 1–3 same as A; Lanes 4–7, PBMC preparation from patient no. 1014 before (Lanes 4 and 5) and after (Lanes 6 and 7) heat treatment. Densitometry of the Mr 100,000 band shows a similar increase from barely detectable to $1.9 \times 10^5$ copies/cell with heat reversal.

in this way, suggesting that a large proportion of the observed topo-I depletion is due to cleavable complex formation. Unfortunately, this assay cannot be quantitatively reproduced in large numbers of clinical specimens, probably due to degradation of liberated topo-I by heat-activated proteases. In our hands, this heat-reversal assay cannot be used for a pharmacodynamic end point as reliably as the more reproducible Mr 100,000 band depletion.

Additional evidence for this mechanism of high molecular weight complex formation was shown by Subramanian et al. (14) using another assay (In Vivo Complex of Enzyme) demonstrating DNA-associated topo-I. This group reports formation of the complexes in PBMCs shortly after the end of 30 min of topotecan infusion with rapid return to baseline. These data are consistent with those reported here.

Another possibility for the observed decrease in topo-I levels, besides formation of high molecular weight complexes, includes down-regulation of enzyme production. The data shown here cannot demonstrate what proportion of the topo-I depletion is due to that mechanism versus cleavable complex formation. However, the heat-reversal experiments described above demonstrate that high molecular weight complex formation is a major mechanism of band depletion here. Danks et al. (15) have also described the translocation of topo-I from the nucleus to cytoplasm in the presence of low doses of topotecan. This movement would prevent formation of high molecular weight complexes with DNA. Such a phenomenon would lead to constant topo-I levels (lack of depletion) in our Western blot assay but would falsely suggest that cleavable complexes were not being formed. We also cannot eliminate the possibility that this phenomenon occurs in our patient specimens. Nonetheless, because we have observed a statistically significant depletion of the free topo-I with week of treatment and with duration of infusion, and we can demonstrate that complex formation occurs regularly with heat reversal, we do not believe this phenomenon has a major role in this setting.

The pharmacodynamic data show a clear relationship between topotecan-lactone AUC and topo-I copy number when
Topotecan is administered by the prolonged infusion schedule. Such a relationship has not been described previously in a patient population undergoing therapy with a topo-1 inhibitor on any schedule. These data give further credence to the hypothesis that schedule dependency of topo-1 inhibitor therapy is an important clinical factor. Prolonged infusion of topotecan appears to produce more sustained depletion of “free topo-1” in PBMCs and, at the same time, allows dose intensity at least equal to bolus schedules using growth factors. Comparative clinical trials will be necessary to directly address the question of whether prolonged topotecan infusion results in greater therapeutic benefit or improved therapeutic index.

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