Sequential topoisomerase targeting and analysis of mechanisms of resistance to topotecan in patients with acute myelogenous leukemia

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Resistance to topoisomerase I (TOP1)-targeting drugs such as topotecan often involves upregulation of topoisomerase II (TOP2), with accompanying increased sensitivity to TOP2-targeting drugs such as etoposide. This trial was designed to investigate sequential topoisomerase targeting in the treatment of patients with high-risk acute myelogenous leukemia. An initial cohort of patients received topotecan and cytosine arabinoside daily for 5 days. Serial samples of circulating mononuclear cells were examined to evaluate peak elevations of TOP2-α protein expression. In subsequent cohorts, etoposide was administered daily for 3 days, beginning 6 h after initiation of the topotecan infusion. The etoposide dose was escalated to determine a maximum-tolerated dose. Circulating mononuclear cells were analyzed for TOP1 mutations and ABCG2 protein expression. In addition, systemic and intracellular topotecan concentrations were measured. Thirty-one patients were enrolled. On the basis of TOP1-α protein levels in three patients with peripheral blast counts greater than 50%, etoposide administration began 6 h after initiation of the topotecan/cytosine arabinoside infusion. Using this schedule of administration, the maximum-tolerated dose of etoposide was 90 mg/m^2. No TOP1 mutations were identified, but increases in ABCG2 expression during the infusion were observed in mononuclear cells from two of four evaluable patients. Administration of etoposide 6 h after initiation of a topotecan/cytosine arabinoside infusion is feasible and is associated with clinical activity. Analysis of TOP2-α protein levels in this small number of patients indicated that peak increases occurred earlier than expected based on earlier publications. Upregulation of ABCG2 was detected in circulating cells and may represent an inducible form of drug resistance that should be investigated further.
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Introduction

Topoisomerase I and II (TOP1 and 2)-targeting drugs exhibit significant single-agent activity in the treatment of patients with acute myelogenous leukemia (AML) [1,2]. Induction regimens for the treatment of patients with this disease commonly involve combinations of cytosine arabinoside with a TOP2-targeting drug such as daunorubicin, or a TOP1-targeting drug such as the camptothecin (CPT) analog topotecan. Although these cytosine arabinoside/topoisomerase-targeting combination regimens induce complete remissions in 50–70% of patients, a minority of patients are cured. Treatment of AML with any of the characteristics associated with a poor prognosis (age greater than 60, preceding myelodysplasia, high circulating blast counts, or poor prognosis cytogenetic abnormalities) rarely results in disease-free survival greater than 2 years [3]. New therapeutic approaches to the treatment of poor prognosis of AML are needed.

One approach to enhance the activity of TOP1-targeting drugs such as topotecan involves sequential administration of a TOP2-targeting drug such as etoposide. Preclinical studies indicate that resistance to CPTs is often accompanied by upregulation of TOP2 isozymes, with resultant hypersensitivity to TOP2-targeting drugs [4–13]. In addition, animal models indicate that the sequential administration of TOP1-targeting and TOP2-targeting drugs can result in additive or synergistic antitumor activity [14–16]. Furthermore, immunofluorescent analyses of leukemic cells obtained from patients treated with continuous infusion of topotecan

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demonstrated increase in TOP2-α protein levels [17], and in a resistant K562 leukemia cell model sequential topotecan–etoposide was effective in overcoming resistance to topotecan [18].

Although these studies provide a rationale for the sequential administration of topotecan and etoposide, the timing of the administration of etoposide to patients may be a critical variable in schedule design, as cell culture studies indicate that antagonistic effects may result if CPT and etoposide are administered simultaneously [16,19]. Furthermore, the effects of concurrent administration of cytosine arabinoside on topotecan-induced alterations in topoisomerase protein levels have not been studied. In this regard, cytosine arabinoside was shown to induce formation of TOP1–DNA and TOP2–DNA complexes *in vitro* and in cells [20,21]. Although the significance of these interactions for the antileukemic effects of cytosine arabinoside is not clear, it is possible that cytosine arabinoside alters TOP1 or TOP2 protein levels in patient tissues. Therefore, we designed a clinical trial to investigate the effects of topotecan/cytosine arabinoside-induction therapy on circulating leukemic cell TOP2-α protein expression. The trial was also designed to identify dose-limiting toxicities and the maximum-tolerated dose (MTD) of etoposide, when this drug was administered after initiation of a topotecan/cytosine arabinoside infusion. Furthermore, as clinical resistance to topotecan and other CPTs is poorly understood, a secondary goal of this protocol was to investigate potential mechanisms of resistance to topotecan in patients enrolled in the trial.

Patients and methods

Study design and eligibility criteria

On the basis of earlier studies suggesting that TOP2-α protein levels were increased in leukemia cells on day 3 during a 5-day continuous infusion of topotecan [17], an initial cohort of patients received a 5-day topotecan/cytosine arabinoside regimen, with etoposide administered on days 3, 4, and 5. After TOP2-α protein levels were evaluated in peripheral mononuclear cells (which were 86% blasts at baseline) obtained from a patient in this cohort, however, the protocol was amended to include a cohort in which patients received topotecan/cytarabine alone, allowing an extended temporal evaluation of TOP2-α protein changes in peripheral leukemia cells in the absence of etoposide. Data from this 'pharmacodynamic' cohort were used to select the timing of initiation of the 3-day etoposide administration, as described in statistical methods. The original plan was to enroll a maximum of 14 patients in the pharmacodynamic cohort before beginning dose escalation of etoposide. Given the low frequency of patients with peripheral blast counts $\geq 50\%$, after enrolling the 11th patient in the pharmacodynamic cohort, however, a decision was made to use the data from the three evaluable patients (peripheral blast count $\geq 50\%$) in this cohort to select the time for initiation of etoposide administration. Additional cohorts were included to determine the MTD of etoposide using this schedule of administration.

Eligibility requirements included: (a) histologically documented AML with at least one factor associated with poor prognosis [age > 60 years, high-risk cytogenetics (e.g. monosomy 5, 5q–, monosomy 7, 7q–, 11q23, complex abnormalities), presence of an antecedent hematologic disorder, earlier leukemogenic therapy, relapsed or refractory AML] or chronic myelogenous leukemia in myeloid blast crisis; (b) preserved hepatic, renal, and cardiac function (bilirubin $< 2.0 \times$ upper limit of normal, aspartate aminotransferase $< 3 \times$ upper limits of normality, creatinine < 1.8 mg/dl, left ventricular ejection fraction $> 40\%$); (c) negative pregnancy test for women of childbearing potential; and (d) no uncontrolled psychiatric illness.

The study was approved by the Robert Wood Johnson Medical School Institutional Review Board. All patients were informed of the investigative nature of the study and gave informed consent in accordance with institutional and federal guidelines.

Drug administration and dose escalation

On the basis of published tolerable doses of cytosine arabinoside administered over 2 h daily five times combined with a 5-day continuous infusion of topotecan [22], the initial cohort received topotecan 1.5 mg/m^2/day as continuous infusion over 24 h on days 1–5, cytosine arabinoside 1 g/m^2/day as a 2 h infusion on days 1–5, and etoposide 60 mg/m^2/day as a 1 h infusion on days 3–5 (Table 1). The next cohort received cytarabine and topotecan alone, to evaluate TOP2-α protein increases on days 3–5 in the absence of etoposide. The topotecan infusion was started first, 2 h later followed by initiation of the cytarabine infusion. In subsequent cohorts etoposide was administered on days 1–3 intravenously over 1 h, beginning 6 h after the start of the topotecan administration. The initial etoposide dose was 60 mg/m^2, and was increased in cohorts of patients as listed in Table 1.

Dose-limiting toxicity (DLT) was defined as any irreversible grade 2 and any grades 3–4 nonhematologic toxicity (National Cancer Institute Common Toxicity Criteria, version 2.0). If all three patients treated in a cohort were observed for 28 days and did not experience DLT then the etoposide dose was escalated. If two of three patients experienced DLT then the previous dose was considered the MTD. If one of three patients experienced DLT then three more patients were enrolled at that dose level. If none of these patients experienced DLT then the dose was escalated; otherwise the previous dose was considered the MTD. At least six patients were to be accrued at the etoposide dose identified as the MTD.

Table 1 Etoposide dose levels

Cohort	Etoposide dose (mg/m^2/day)	No. of patients
0	60 (day 3)[a]	3
1	0	11
2	60	3
3	75	3
4	90	7
5	100	4

[a]Cohort 0 received etoposide starting on day 3, whereas for subsequent cohorts in which etoposide was administered, the etoposide infusion began 6 h after initiation of topotecan/cytarabine and was administered daily three times.

Supportive treatment measures included administration of granulocyte colony-stimulating factor 5 μg/kg/day subcutaneously starting on day 7 and continuing until absolute neutrophil count was greater than 1000. All patients received prophylaxis with acyclovir and fluconazole. For the initial cycle of therapy, patients also received a sodium bicarbonate infusion and allopurinol in anticipation of hyperuricemia. Transfusions, antibiotics, and additional cytokine support were administered as indicated. Patients received only a single course of protocol therapy. After completion of a monitoring period (28 days from initiation of treatment), further therapy was administered at the discretion of the treating physician.

Clinical evaluation

Pretreatment evaluation included a history, physical exam, complete blood count, serum chemistries, lactate dehydrogenase, electrocardiogram, urinalysis, chest radiograph, and multiple gated acquisition scan. All patients were treated as inpatients and were evaluated daily with history, physical exam, complete blood count, and serum chemistries. A bone marrow examination was performed before chemotherapy administration as well as between days 14 and 28–35. Toxicities were classified using the National Cancer Institute Common Toxicity Criteria, version 2.0. Responses were evaluated using a modification of previously published guidelines [23]. A complete remission was defined as recovery of blood counts (white blood cells $> 2500/mm^3$ with a normal differential count, hemoglobin > 10 g/dl, platelets $> 100\,000/mm^3$ in the absence of transfusion or cytokine support) in association with a bone marrow aspirate and biopsy that does not have evidence of leukemia by morphologic, immunophenotypic, or cytogenetic criteria. A partial remission was defined as a bone marrow aspirate and biopsy that failed to reveal any morphologic, immunophenotypic, or cytogenetic criteria of leukemia, without improvement of blood counts to the values described above for complete remission.

Analysis of topoisomerase and ABCG2 protein levels

Mononuclear cells were obtained from approximately 8 ml of blood using cell preparation tubes (CPT; Becton Dickinson, Franklin Lakes, New Jersey, USA). Two tubes were obtained at 1, 3, 6, 24, 48, 72, 96, and 120 h after initiation of the topotecan infusion. Mononuclear cells obtained from the tubes were used to assay both cellular protein levels and intracellular topotecan concentrations. In cases where few cells were obtained, prioritization was given to protein analyses. In some cases, there were insufficient cells for either analysis. Samples from patients with pretreatment peripheral blast counts of $\geq 50\%$ were considered evaluable for analysis of topoisomerase and ABCG2 protein levels in peripheral blasts. The cells were lysed in a buffer containing 1% sodium dodecyl sulfate, 50 mmol/l Tris–Cl pH 7.4, 150 mmol/l NaCl, 1 mmol/l dithiothreitol, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mmol/l phenylmethylsulfonyl fluoride. Equal amounts of protein from each sample were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membranes. TOP2-α, TOP1, ABCG2, and actin immunoblotting were performed sequentially on the blots using TOP2-α (Neomarkers, Fremont, California, USA), TOP1 [8], and ABCG2 (Alexis Biochemicals, San Diego, California, USA) antibodies.

NIH 3T3, FBCL, WI38, U937, RPMI 8402, and CPT-K5 [24] cell lines were used to assess the effects of topotecan exposure on ABCG2 protein expression. The cell lines were maintained at 37°C in the presence of humidified 5% CO_2. Exponentially growing cells were exposed to 0.1 or 1 mmol/l topotecan for 6 or 24 h before lysis and analysis of ABCG2 protein expression using immunoblotting as described [25].

Plasma and intracellular topotecan concentrations

For determination of plasma topotecan concentrations, heparinized blood samples were obtained immediately before initiation of topotecan therapy and at 1, 2, 3, 6, 24, 48, 72, 96, 120, and 144 h after the start of infusion on day 1. The samples were processed promptly to obtain plasma and subsequently stored at −80°C until analysis. Working topotecan standards were prepared in human plasma. One hundred microliters of the standards and patient plasma were precipitated with methanol (200 μl) and centrifuged at 20 000*g* for 10 min to obtain clear supernatant. The supernatant was diluted (1 : 3) with mobile phase and analyzed for total topotecan concentration (lactone and carboxylate) in a Hitachi (New York, New York, USA) D7000 series high-performance liquid chromatography system using fluorescence detection (excitation wavelength 380 nm and emission wavelength 527 nm) as described previously [26]. Plasma topotecan steady-state concentration (C_{ss}) was estimated as the average of concentrations obtained at 48, 72, and 96 h. Area under the concentration time (144 h) was calculated using the linear-logarithmic trapezoidal method [27]. Total body clearance (CL) was estimated according the equation $CL = R_0/C_{ss}$, where R_0 is the rate of drug infusion.

For determination of intracellular topotecan concentrations, peripheral blood mononuclear cells isolated from

CPT tubes were washed with ice-cold phosphate-buffered saline then lysed with 200 µl methanol. The resulting supernatants were diluted in mobile phase, and intracellular topotecan was measured by high-performance liquid chromatography as described above. To account for differences in cell number, intracellular topotecan concentrations were normalized to sample protein content as determined by the Bradford method [28].

Topoisomerase I and ABCG2 gene sequencing

DNA was isolated from leukemic blast cells using an isopropanol-based procedure (Puregene DNA isolation; Gentra, Minneapolis, Minnesota, USA). Primers for PCR were designed based on complementary DNA sequences obtained from GenBank (primer sequences listed in Supplemental Material). For TOP1, primers were designed to amplify regions previously implicated as conferring drug resistance when mutated, including: (a) phenylalanine 361 (encoded in exon 12); (b) arginine 488, glycine 503, aspartic acid 533 (all encoded in exon 15); and (c) asparagine 722 (exon 20). In addition, primers were designed to amplify four additional regions reported to contain single nucleotide polymorphisms (SNPs) (codons 214, 349, 517, and 591). For ABCG2, primers were designed to amplify regions containing the following four SNPs: G34A (V12M, exon 1), C376T (stop, exon 3), A616C (I206L, exon 5), C421A (Q141K, exon 12), and A1768T (N590Y, exon 14). PCR reactions were performed using a high-fidelity polymerase (GC genomic PCR polymerase; Clontech, Mountain View, California, USA). Five microliters of the PCR products were run on agarose gels to confirm amplification and the remaining product was purified (GenElute PCR Clean-Up Kit; Sigma, St Louis, Missouri, USA) and sequenced using an automated sequencer (ABI-3100 Genetic Analyzer; Applied Biosystems, Foster City, California, USA). Electropherograms were inspected manually to evaluate possible double peaks not identified by automated sequence calling.

Statistical methods

Pharmacodynamic data obtained during the treatment of patients with topotecan/cytarabine alone were used in an attempt to establish an optimal timing of etoposide treatment. The optimal timing was defined as the three-consecutive-day range (in the first week) during which more than 66% of future patients will have peak leukemic blast TOP2-α levels, or the predictive probability is the largest. Sequential prediction probability was to be calculated and updated for every three to five patients recruited to the 'pharmacodynamic' cohort until the prediction probability in a three-consecutive-day was more than 66% or a maximum of 14 patients were recruited. In either case, the three consecutive days with the highest prediction probability was considered as the optimal time of the administration of etoposide treatment for subsequent cohorts of patients.

Probabilities were calculated as follows. Let $X = (x_1, x_2, \ldots, x_7, x_8)$, where x_i is the observed total number of patients having their peak blood cell TOP2 levels at day i if $1 \leq i \leq 7$, and not within the first week (including never increasing) if $i = 8$. The prediction probability that the peak is at day $\underline{t}$ given the observed data X is defined as follows:

$$P(t|X) = \frac{\int_\Theta p(t|\theta)p(X|\theta)p(\theta)\mu(\mathrm{d}\theta)}{\int_\Theta p(X|\theta)p(\theta)\mu(\mathrm{d}\theta)},$$

where $t = 1, 2, 3, 4, 5, 6,7$, or greater than 7 if for some patient whose TOP2 level never increases within the first week, $p(X|\theta)$ is the density function of the multinomial distribution with parameters n and $\theta = (\theta_1, \theta_2, \ldots, \theta_8)$ (assuming that n patients are observed), $p(t|\theta) = \theta_t$, $p(\theta)$ is the earlier distribution of θ on $\Theta = \{(\theta_1, \theta_2, \ldots, \theta_8) \,|\, \Sigma\theta_i = 1, 0 \leq \theta_i \leq 1\}$.

The trial also included correlative analyses between response to treatment and plasma topotecan pharmacokinetic parameters. Logistic regression was used for these analyses. In addition, a two-sample t-test was used to analyze etoposide treatment effect on topotecan pharmacokinetic parameters.

Results

Patient characteristics and determination of an administration time for etoposide

A total of 16 women and 15 men were enrolled in the study (Table 2). Most of the patients were 60 years of age or older (median 69), and all patients had AML. Seven patients had received chemotherapy previously for AML or myelodysplasia, and six patients had a high-risk cytogenetic profile. Among the first three patients (who received etoposide 60 mg/m^2/day on days 3–5), only one patient had a baseline peripheral blast count of $\geq 50\%$ (86%). Analysis of TOP2-α protein levels in this patient indicated that relative to pretreatment, the level was increased at 48 and 72 h after treatment initiation, raising the possibility that peak TOP2-α protein increases might occur beyond the 72 h time point at which etoposide administration began (Fig. 1). Therefore, the protocol was amended to include a cohort of patients who did not receive etoposide, allowing a more complete evaluation of TOP2-α protein levels during the 5-day topotecan/cytarabine infusion (Table 1). Three among the 11 patients in this cohort had peripheral blast counts $\geq 50\%$. Among these patients, peak TOP2-α levels were observed at 6 h in two patients (Fig. 1; patients 4 and 13) and at the pretreatment time in the third (Fig. 1; patient 8).

Given the low frequency of patients with peripheral blast counts $\geq 50\%$, based on these limited data, a decision was made to initiate the etoposide infusion 6 h after the beginning of the topotecan infusion. Using the results from the one patient in cohort 0 and the three patients

in cohort 1 with peripheral blast counts $\geq 50\%$, the probability that a peak in peripheral blast TOP2-α protein levels occurs between days 1 and 3 is 54.5%.

Toxicities and etoposide dose escalation

Consistent with earlier reports [29,30], severe (grade 3 or 4) nonhematologic toxicities were observed in four of the 11 patients treated with topotecan/cytarabine alone. In patients who received etoposide starting 6 h after the beginning of the topotecan infusion, the etoposide dose was escalated in sequential cohorts to 100 mg/m^2, where two of four patients experienced dose-limiting toxicities (Table 3). One of these toxicities consisted of grade 3 delirium and mood changes beginning on day 2 of treatment. Another patient had grade 3 aspartate aminotransferase and alanine aminotransferase elevations starting on day 2 and lasting for 5 days. No grade 4 nonhematologic toxicities were observed. None of the seven patients administered 90 mg/m^2 etoposide experienced dose-limiting toxicities. Therefore, the MTD of etoposide combined with topotecan/cytosine arabinoside using this schedule of administration was defined as 90 mg/m^2.

Antileukemia activity

Complete and partial responses were observed in patients treated with topotecan/cytarabine alone, as well as in patients who were given topotecan starting 6 h after the topotecan infusion (Table 4). Although this study was not designed to compare response rates among the cohorts, the overall response rate was 64% for patients who did not receive etoposide, compared with 59% for patients who received etoposide (any dose) starting 6 h after topotecan.

Investigation of potential resistance mechanisms to topotecan

Several mechanisms of resistance to CPTs have been defined using cell culture models [31]. These mechanisms can be grouped into three categories: (a) drug efflux (e.g. by certain membrane transporters, most notably ABCG2 and not ABCB1); (b) alterations in the target (e.g. mutations or posttranslational modifications of TOP1); or (c) 'downstream' mechanisms that are involved after formation of drug-stabilized TOP1–DNA complexes (e.g. upregulation of DNA repair or antiapoptotic proteins). It is not known which, if any, of these

Table 2 Patient characteristics

Characteristic	No. of patients ($n=31$)
Sex	
Women	16
Men	15
Median age (range)	69 (16–82)
WHO diagnosis	
AML with recurrent genetic abnormalities	2
AML with multilineage dysplasia (after MDS, or without MDS)	11
AML with MDS, therapy related	2
AML therapy related	1
AML not otherwise categorized	15
Number of earlier AML regimens	
0	24
1	6
≥ 1	1

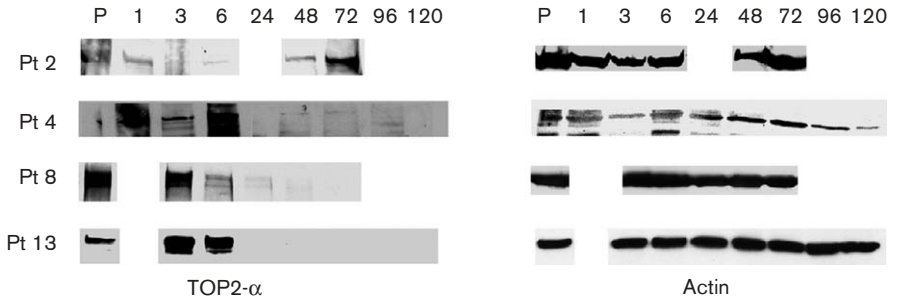
AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome.

Table 3 Severe nonhematological toxicities attributed to protocol treatment

Cohort	Etoposide dose (mg/m^2)	No. of patients	No. of patients with $\geq$ grade 3 toxicity
0	60 (day 3)[a]	3	0
1	0	11	4 (2 epistaxis; 2 bacteremia, mucositis, elevated AST)
2	60	3	1 (hallucinations)
3	75	3	0
4	90	7	0
5	100	4	2 (1 elevated AST, ALT, LDH; 1 hallucinations)

ALT, alanine amino transferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.
[a]The first cohort received etoposide starting on day 3, whereas for subsequent cohorts in which etoposide was administered, the etoposide infusion began 6 h after initiation of topotecan/cytarabine.

Fig. 1



Analysis of topoisomerase II-α (TOP2-α) protein levels in circulating mononuclear cells. Mononuclear cells were purified from patient peripheral blood cells obtained at the indicated hours after initiation of the 5-day infusion of topotecan/cytarabine. The samples were processed for immunoblotting as described in Patients and methods. A β-actin immunoblot is shown as a loading control. Pretreatment percentage of blast counts in peripheral blood cells for patients 2, 4, 8, and 13 were 86, 54, 86, and 70%, respectively. For patient 2, etoposide was administered after collection of the 72-h blood sample. None of the other patients received etoposide. P, pretreatment; Pt, patient.

Table 4 Complete and partial responses according to cohort

Cohort	Etoposide dose (mg/m^2)	No. of patients	Response (CR, PR)	Response (%)
0	60 (day 3)[a]	3	0.0	0
1	0	11	7.0	64
2	60	3	0.2	67
3	75	3	1.2	100
4	90	7	1.2	43
5	100	4	1.1	50

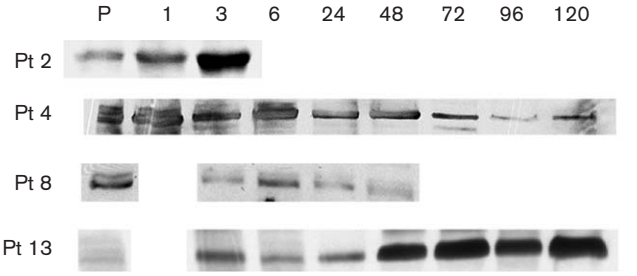
CR, complete remission; PR, partial remission.
[a]The first cohort received etoposide starting on day 3, whereas for subsequent cohorts the etoposide infusion began 6 h after initiation of topotecan.

mechanisms are, however, operative in the de-novo or acquired resistance to CPTs that is observed in patients with hematologic or nonhematologic malignancies. In this study, we focused on the efflux protein ABCG2, TOP1 mutations, and TOP1 downregulation as potential mechanisms of resistance to topotecan. It should be noted that ABCG2 expression confers resistance to both topotecan [32,33] and etoposide [34] in preclinical models, and was implicated as a clinically relevant resistance mechanism in patients with leukemia [35] and small cell lung cancer [36].

ABCG2 expression was analyzed in circulating mononuclear cells obtained from the four patients in cohorts 0 and 1 with peripheral blast counts $\geq 50\%$ (note that none of the patients in cohorts 2–5 had peripheral blast counts $\geq 50\%$). In two patients, ABCG2 protein expression was detectable in pretreatment cells and exhibited a decrease during the topotecan/cytarabine infusion (Fig. 2, patients 4 and 8). By contrast, in the other two patients (2 and 13), little ABCG2 expression was evident in pretreatment cells, but protein levels were strikingly increased at 3 and 48 h, respectively (Fig. 2). Patients 4 and 13 experienced a complete response to the topotecan/cytarabine infusion, whereas patient 8 experienced a partial response, and patient 2 experienced disease progression. Therefore, an increase in ABCG2 expression (as observed in patient 13) does not preclude at least an initial clinical response to a topotecan/cytarabine infusion. We note, however, that the patient with the most dramatic and rapid upregulation of ABCG2 expression (patient 2) experienced disease progression.

To investigate potential mechanisms underlying the increase in ABCG2 expression observed in these patients, both malignant (U937, RPMI 8402 and a CPT-resistant subline, CPT-K5) and nonmalignant, immortalized cell lines (NIH 3T3, FBCL, WI38) were used to investigate whether exposure to topotecan induces ABCG2 protein expression in cultured cells. No increase in ABCG2 protein expression was observed at 6 or 24 h in experiments using either 100 nmol/l or 1 μmol/l topotecan (data not shown) in any of the cell lines, suggesting that topotecan does not induce ABCG2 expression within this time frame in clonal cell populations.

Fig. 2



Analysis of ABCG2 protein levels in circulating mononuclear cells. Mononuclear cells were purified from peripheral blood cells obtained at the indicated hours after initiation of the 5-day infusion of topotecan/cytarabine. The samples were processed for immunoblotting with an ABCG2 antibody as described in Patients and methods. Actin immunoblotting was used as a loading control, with the relevant actin blots shown in Fig. 1. Pretreatment blast percentages for patients 2, 4, 8, and 13 were 86, 54, 86, and 70%, respectively. P, pretreatment; Pt, patient.

We also analyzed ABCG2 polymorphisms in DNA obtained from circulating mononuclear cells from 12 patients. A SNP was identified in two patients (enrolled in cohorts 2 and 5, respectively), involving a coding region G-to-A substitution that yields a V12M substitution in the ABCG2 protein (refSNP ID: rs2231137). This relatively common polymorphism was reported to have little effect on ABCG2 protein function [37]. The two patients with this polymorphism were not clearly distinguishable from other patients with regard to either response to treatment or topotecan pharmacokinetic parameters. Although the patient in cohort 5 experienced dose-limiting hepatic toxicity, this patient received a dose of etoposide ($100\ mg/m^2$) that was determined to be above the MTD.

With regard to TOP1 mutations, we focused on three sites implicated previously as 'hot spots' for the occurrence of resistance-conferring mutations in cell culture models [31]: residues 361, 488, 503, 533, and 722. Using PCR, these regions were sequenced from DNA obtained from peripheral mononuclear cells in 10 patients. In eight patients, samples were available both before and 28 days after treatment initiation. Peripheral blast counts were less than 50% in all of these patients. No sequence alterations in these regions were detected in any of these samples. Somewhat surprisingly, we also did not observe any polymorphisms [relevant to the GenBank sequence (accession number NM_003286)] in these regions of TOP1 among the 10 patients. Moreover, we did not detect any sequence variations at the four reported coding region SNPs for TOP1 in the dbSNP database (rs6029542, rs6029545, rs6129757, rs1061982). On the basis of close inspection of the dbSNP entries, however, we note that with the exception of rs6029542, which is a synonymous SNP at codon 349 and has

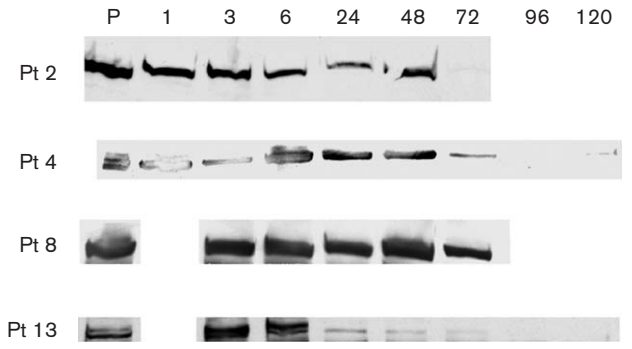
an allelic frequency of only 2%, these SNPs may be sequencing error artifacts.

With regard to TOP1 protein level alterations, significant decreases in TOP1 at the 72-h time point were observed in mononuclear cells obtained from three of the four patients with peripheral blast counts $\geq 50\%$ (Fig. 3; patients 2, 4, and 13). None of these patients received etoposide before 72 h. In one case (patient 2), the decrease in TOP1 occurred concurrently with an increase in TOP2-α levels (compare Figs 1 and 3).

Plasma and intracellular topotecan concentrations were also analyzed in this study. Complete plasma concentration–time profiles were available for 16 patients in three different cohorts (cohorts 1, 2, and 5; Table 5). Plasma topotecan pharmacokinetic parameters were similar to those observed previously using a 5-day infusion of topotecan combined with carboplatin [38]. Furthermore, there was no significant difference between steady-state topotecan concentrations in patients who did not receive etoposide (cohort 1) versus those who did receive etoposide (cohorts 3 and 5) ($P \geq 0.30$). No significant associations between response and topotecan pharmacokinetic parameters, such as C_{ss}, T_{max}, area under the concentration–time curve, and CL, were found.

Intracellular topotecan concentrations were obtained in three of the four patients with peripheral blast percentages $\geq 50\%$ (patients 4, 8, and 13) over the initial 48 h of the topotecan infusion (Fig. 4). In all three patients, intracellular concentrations rose during the initial hours of the infusion and appeared to reach a similar steady-state level of approximately 1 ng/mg protein by 24 h (Fig. 4).

Fig. 3



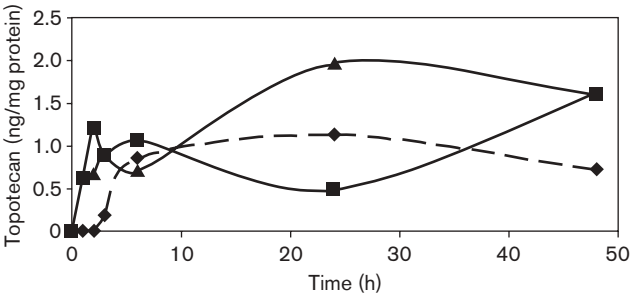
Analysis of topoisomerase I protein levels in circulating mononuclear cells. Mononuclear cells were purified from peripheral blood cells obtained at the indicated hours after initiation of the 5-day infusion of topotecan/cytarabine. The samples were processed for immunoblotting as described in Patients and methods. As a loading control, the relevant actin immunoblotting is shown in Fig. 1. Pretreatment blast percentages for patients 2, 4, 8, and 13 were 86, 54, 86, and 70%, respectively. P, pretreatment; Pt, patient.

Table 5 Plasma topotecan pharmacokinetic parameters ($n=16$)

Parameter	Cohort (*n*)		
	1 (11)	2 (3)	5 (2)
C_{ss} (μg/l)	6.2 ± 2.7[a]	5.6 ± 1.3	8.7 ± 4.0
T_{max} (h)	76.4 ± 36.9	88.0 ± 55.4	36.0 ± 17.0
AUC (μg·h/l)	653.8 ± 163.7	663.0 ± 148.4	932.5 ± 437.7
CL (l/h)	10.7 ± 3.3	11.5 ± 2.7	8.2 ± 3.7

AUC, area under the concentration–time curve; CL, total body clearance.
[a]Values are expressed as mean ± SD.

Fig. 4



Intracellular topotecan concentrations in circulating mononuclear cells. Peripheral blood mononuclear cells were obtained before and at various times after initiation of the topotecan/cytosine arabinoside infusion in three patients with initial peripheral blast percentages greater than 50%. Cells were lysed and intracellular topotecan concentrations measured as indicated in Patients and methods. To account for differences in cell number, concentrations were normalized to sample protein content. Triangle, diamond, and square symbols indicate results from patients 13, 4, and 8, respectively.

Discussion

The concept of sequential topoisomerase targeting is based on a strong preclinical rationale, but it is unknown whether this strategy improves clinical outcomes relative to single topoisomerase targeting. This trial was designed to investigate administration of etoposide, a TOP2-α inhibitor, after initiation of a TOP1-targeting topotecan/cytosine arabinoside infusion. The finding that only four of 11 patients treated in initial cohorts had sufficient circulating leukemic blasts to be evaluable for assessment of leukemia cell topoisomerase protein levels highlights the difficulties of obtaining temporal data regarding topoisomerase alterations. TOP2-α protein levels were observed to peak at 6 h after the initiation of the topotecan/cytosine arabinoside infusion in two evaluable patients, and at 72 h in a third evaluable patient. Given these small numbers, our results need to be interpreted with caution. To our knowledge, however, our study represents the only analysis of cellular topoisomerase protein levels earlier than a 24-h time point after initiation of a topotecan infusion. Using a quantitative immunofluorescence technique rather than immunoblotting, an earlier study of a 5-day continuous infusion of topotecan in patients with leukemia reported peak

TOP2-α protein levels at 72 h in peripheral mononuclear cells obtained from nine patients [17]. The earliest assessment, however, occurred at 24 h after infusion initiation. Notably, etoposide was administered starting on day 6 in this trial, a time at which TOP2-α protein levels were at or below pretreatment levels in the bone marrow and peripheral blood cells of most patients [17]. In addition, in another study of sequential topotecan and etoposide in patients with leukemia, in which topotecan was administered as a 72-h continuous infusion followed by five daily doses of etoposide, TOP2 catalytic activity was unchanged (relative to baseline) or decreased in most bone marrow aspirates obtained at the initiation of etoposide treatment on day 4 [39]. Similarly, our results indicate that among three of four evaluable patients, TOP2-α protein levels in peripheral blasts were at or below baseline levels from 24 to 120 h after initiation of the topotecan/cytosine arabinoside infusion. By contrast, Mainwaring *et al.* [40] reported increases in TOP2-α in leukemic bone marrow cells after a 3-day infusion of topotecan, and found that increased TOP2-α levels on day 4 correlated with response to a sequential topotecan/etoposide and mitoxantrone strategy. Together, these results suggest that upregulation of TOP2-α protein levels in leukemic blasts occurs relatively early after administration of TOP1-targeting drugs to patients. These results should, however, be interpreted with caution, given the relatively small number of evaluable patients in our trial and others employing a sequential topoisomerase-targeting strategy. Furthermore, we note that randomized trials evaluating the clinical benefit of employing a sequential topoisomerase-targeting strategy have not been reported. Indeed, in our trial, the addition of etoposide to a topotecan/cytosine arabinoside infusion was not associated with a striking improvement in response rate. Therefore, the clinical usefulness of sequential topoisomerase targeting remains to be established.

With regard to dose escalation of etoposide, we identified 90 mg/m^2/day × 3 days as the MTD for this combination regimen. Interestingly, 100 mg/m^2/day × 3 days was identified as the MTD for etoposide in a sequential topoisomerase-targeting strategy involving administration of etoposide after a continuous infusion of topotecan alone at 1.5 mg/m^2/day × 5 days [17]. The nonhematologic DLTs in our trial (transaminitis, central nervous system toxicity) are similar to those observed previously in studies of sequential topotecan/etoposide [17,39].

Regarding potential mechanisms of resistance to topotecan, we note that the steady-state plasma topotecan concentrations of about 15 nmol/l observed in this trial are similar to typical IC_{50} values of 20–40 nmol/l obtained in cell culture experiments of leukemic cell lines [41,42]. We did not observe any mutations in TOP1 either before or after drug administration. As the peripheral blood cell samples used for this analysis, however, contained less than 50% blasts, it is possible that mutations present in blast cells were not detectable on sequencing. Furthermore, our sequencing method would not be able to detect a small population of cells harboring a TOP1 mutation. Notably, a similar lack of TOP1 mutations in tumor specimens was reported previously [43,44]. Indeed, there is only one study that reported a mutant TOP1 sequence in a clinical specimen, and this occurred in a single patient with lung cancer [45]. These findings suggest that mutation of TOP1 is unlikely to be a common mechanism of clinical drug resistance.

By contrast, our results suggest that ABCG2 may be upregulated in leukemic blast cells during chemotherapy administration and thus contribute to clinical resistance. To our knowledge, upregulation of ABCG2 protein expression after initiation of chemotherapy has not been reported previously. As ABCG2 protein expression in peripheral blast cells was considered evaluable in only four patients, we cannot correlate this expression with clinical response. Furthermore, with upregulation identified in only two of the four patients, this finding requires additional study before it can be concluded that ABCG2 levels are upregulated after topotecan/cytarabine treatment.

With regard to the mechanisms of ABCG2 upregulation, although transcriptional induction by substrates is well established for certain efflux transporters such as P-glycoprotein [46]; ABCG2 expression is not upregulated by xenobiotics that activate nuclear receptors such as liver X receptor (LXR) or pregnane X receptor (PXR) [47]. Indeed, as we were not able to induce ABCG2 protein expression in leukemic or normal cell lines with topotecan, we conclude that the clinical observation relates to other potential mechanisms of upregulation, including: (i) an effect of cytosine arabinoside (not known to be an ABCG2 substrate) on BCRP protein expression; (ii) an effect of topotecan on the leukemia cell microenvironment *in vivo*, thus affecting blast cells indirectly; or (iii) a selection effect of the topotecan/cytosine arabinoside regimen on leukemic cell populations. As hematopoietic stem cells express ABCG2 constitutively [47], it is possible that selection occurs in patients who are treated with topotecan. It seems, however, unlikely that a selection phenomenon can explain our results, as total cell counts would not be expected to change significantly during the initial 3 h of the topotecan/cytosine arabinoside infusion. Accumulating evidence that implicates ABCG2 in resistance to combination chemotherapy used in leukemia [35] and in resistance to cisplatin in lung cancer patients is present [36]. In a recent trial of infusional topotecan combined with carboplatin in patients with leukemia, however, pretreatment ABCG2 expression in bone marrow mononuclear cells did not correlate with response [38].

Similar to earlier studies using topotecan alone [39], we observed decreases in TOP1 protein expression in peripheral blasts obtained from three of four evaluable patients. Although relevant signaling pathways have not been identified, this downregulation is likely related to ubiquitin-mediated degradation of TOP1 [48,49]. Reduction in TOP1 expression is typically associated with resistance to CPTs [4]. Although in our study the number of evaluable patients was too small for correlative analyses, in their study of sequential topotecan/etoposide, Cooper *et al.* [39] found that patients with a greater than 70% decrease in TOP1 catalytic activity after topotecan administration had a higher bone marrow response rate ($P = 0.045$). Together with our results, this finding indicates that downregulation of TOP1 may be useful as a pharmacologic marker for TOP1 targeting, indicating that the drug interacts with the target in cells. Nevertheless, this kind of posttreatment assessment in cancer tissues (i.e. requiring serial biopsies) will be difficult to employ in practice.

Other, 'downstream', proteins may also be relevant as potential biomarkers for sensitivity to CPTs. For example, Kaufmann *et al.* [38] found that low pretreatment BCL-2 protein levels in bone marrow mononuclear cells correlated with response to infusional topotecan/carboplatin. Additional studies are needed to confirm this finding as well as to identify other predictive biomarkers for TOP1-targeting drugs.

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