Cancer Chemother Pharmacol (2002) 49: 347–355
DOI 10.1007/s00280-002-0423-9

ORIGINAL ARTICLE

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# Topotecan-induced topoisomerase IIα expression increases the sensitivity of the CML cell line K562 to subsequent etoposide plus mitoxantrone treatment

Received: 11 June 2001 / Accepted: 24 December 2001 / Published online: 6 March 2002


**Abstract** *Purpose:* A new chemotherapy regimen was designed for leukemia to improve response to therapy and elucidate the possible underlying mechanisms responsible for its efficacy. *Methods:* Cells of the chronic myelogenous leukemia (CML) cell line, K562, were treated singly, in combination, and in sequence with clinically equivalent dosages of topotecan, which targets topoisomerase I (Topo I), and etoposide and mitoxantrone, which target topoisomerase II (Topo II), to determine the best treatment. Apoptosis, early cell deaths, and cell cytotoxicities in drug-treated cells were determined with annexin V and propidium iodide staining and MTT assays, respectively. Confocal microscopy and RT-PCR showed the cellular locations and relative increases in Topo IIα in topotecan-treated cells. The double comet assay of individual cells showed simultaneously free Topo proteins, non-Topo-associated DNA, and Topo-DNA complexes in drug-induced DNA fragments. *Results:* Sequential treatment with topotecan on days 1–3, followed by etoposide + mitoxantrone on days 4, 5, 9 and 10 resulted in 100% cell death whereas treatments involving administration of drugs singly or simultaneously resulted in less cell kill. The cytotoxicity results in cells treated for fewer days with the same sequential chemotherapy regimen showed the same trend, and adequate surviving cells for the experiments on the cellular and molecular mechanisms of drug action were produced. An increase in Topo IIα mRNA from RT-PCR 1 h after topotecan treatment was observed. Observations on K562 cells treated sequentially with topotecan followed by etoposide, mitoxantrone or etoposide + mitoxantrone were as follows: (1) Topo IIα protein levels increased and relocated from the cytoplasm into the nucleus as detected by confocal microscopy, (2) Topo IIα-DNA complexes increased and were associated with fragmented DNA (positive double comets) as detected by protein-DNA double comet assay, and (3) Topo I and Topo IIβ proteins were not associated with fragmented DNA. Topotecan-induced Topo IIα protein levels correlated with increased numbers of positive double comets and reduction of cell viability. *Conclusions:* Our results showed that Topo IIα protein induction after Topo I-directed drug treatment enhanced the sensitivity of cells to subsequent exposure to Topo II-directed drugs. Timed sequential chemotherapy with topotecan followed by etoposide + mitoxantrone is an effective regimen to ablate CML cancer cells.

**Keywords** Acute leukemia · Topoisomerase II · Sequential chemotherapy · Comet assay · DNA damage

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## Introduction

Chemotherapy is the most commonly used primary treatment for chronic myelogenous leukemia in blast phase (CML-BP), acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL). Unfortunately, only 50–80% of these patients respond to initial chemotherapy [16]. The majority of patients relapse with leukemia cells that are resistant to the standard chemotherapy treatment [6, 12, 13, 14]. This emphasizes the need to develop effective initial chemotherapy treatments to kill all leukemia cells to limit the potential for recurrence of the disease. Two classes of drugs commonly used to treat leukemia target the enzymes topoisomerase I (Topo I), and topoisomerase II (Topo II). These enzymes are essential for DNA unwinding during

replication, transcription, and recombination [7, 19, 21, 22]. Topo I- and Topo II-directed drugs prevent the normal DNA religation of single and double-stranded DNA breaks generated by Topo I and Topo II enzymes, respectively [18]. Poisoning these key DNA metabolic processes promotes apoptosis and eventually leads to cell death [15].

The Topo I-directed drug topotecan and the Topo II-directed drugs etoposide and mitoxantrone are presently used separately [1, 11, 20] or in combination [3, 9] with other classes of drugs to treat hematological malignancies. However, the use of these drugs in sequence has not been studied. We hypothesized that the sequential administration of Topo I- followed by Topo II-directed drugs could synergistically enhance individual drug cytotoxicities. Topotecan treatment alone has been shown to increase Topo IIα levels in leukemia tissue culture cells and in cells of patients with refractory acute leukemia [4, 17, 23]. In addition, a study on Topo IIα gene transfer in breast cancer cells has demonstrated increased levels of Topo IIα mRNA and protein levels that are correlated with enhanced sensitivity to etoposide [24]. A recent clinical study using topotecan and etoposide for treatment of non-Hodgkin's lymphoma has shown that increased Topo IIα mRNA and the formation of Topo IIα-DNA complexes are strongly correlated with clinical response [10].

This study was conducted to determine whether pretreatment of K562 cells with the Topo I-directed drug topotecan would increase the expression of Topo IIα protein that is enzymatically active with DNA, and whether this treatment could sensitize cells to sequential treatment with the Topo II-directed drugs, etoposide + mitoxantrone. The CML cell line K562 was used to determine the efficacy and the underlying cellular and molecular bases of the proposed timed sequential chemotherapy for translation to a clinical treatment for this disease.

## Materials and methods

### Cell line and drug dosages

K-562 cells were grown and maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum plus penicillin and streptomycin, at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Topotecan (SmithKline Beecham Pharmaceuticals, Philadelphia, Pa.), etoposide (Bristol-Myers Squibb Corporation, Princeton, N.J.), and mitoxantrone (Immunex Corporation, Seattle, Wash.) were administered as 1-h bolus treatments at 50 ng/ml, 2.78 μg/ml, and 280 μg/ml, respectively, unless otherwise specified. The clinically equivalent dosages of these drugs were 1.5 mg/m$^2$ per day for topotecan, 100 mg/m$^2$ per day for etoposide, and 10 mg/m$^2$ per day for mitoxantrone.

### Exposure of K562 cells to high doses of topotecan

Topotecan was administered in tenfold increasing dosage steps starting at 50 ng/ml on day 1. The dosage was increased to 0.5 μg/ml for 2 days, then to 5 μg/ml for 3 days and finally, 50 μg/ml for 15 days (1000 times the initial dose). After the last day of treatment, the cells were grown in a drug-free medium for an additional 60 days. The cells were observed for cell survival every day under an inverted microscope (Bausch & Lomb, Germany).

### Cytotoxicity of the sequential drug treatment topotecan followed by etoposide + mitoxantrone in K562 cells

The treatment regimen comprising Topo I- followed by Topo II-directed drugs in K562 cells was conducted to parallel an ongoing clinical trial. Topotecan was given on days 1–3, followed by etoposide + mitoxantrone on days 4, 5, 9 and 10. Topotecan was given as a single drug on days 1–3 and etoposide with or without mitoxantrone on days 4, 5, 9 and 10. Treatment with topotecan on days 1–3 followed by either topotecan, or etoposide with or without mitoxantrone on days 4, 5, 9, and 10 was also evaluated. On day 12, the surviving cells were observed under an inverted microscope (Bausch & Lomb) and quantified by the MTT assay.

### Drug treatment of K562 cells for cellular and molecular mechanism studies

Experiments using the same chemotherapy regimen as above but with fewer days of drug exposure were done to further investigate the cytotoxicity and the cellular and molecular mechanisms of this regimen. The reduced number of days of drug exposure ensured that sufficient viable cells for further laboratory analysis were available. The cells were pretreated or not pretreated with topotecan for 1 h on day 1, followed by etoposide with or without mitoxantrone for 1 h on day 2 (Table 1). Data were obtained 1, 24, and 48 h after the day-2 treatment.

### Apoptosis, early cell death, and cytotoxicity assays

Apoptotic cells are positive for annexin V and negative for propidium iodide (PI), whereas cells that are PI-positive are those that have recently died. Briefly, cell staining was done as follows. Cells were washed twice with cold PBS and resuspended in 300 μl annexin V binding buffer (PharMingen, San Diego, Calif.). To the cell suspension, 5 μl fluorescein isothiocyanate-conjugated annexin V and 5 μl 150 μ*M* PI (Molecular Probes, Eugene, Ore.) were added. The suspension was then vortexed and incubated at room temperature for 30 min. The samples were washed once with the binding buffer, and then observed under a confocal microscope.

Cell cytotoxicities due to drug treatments were determined using the MTT assay according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, Mo.). Only live cells reduce the yellow, water-soluble tetrazolium dye to a purple formazan product that is solubilized by DMSO and glycine. Absorbance at 590 nm was measured in treated and untreated cells with an FL600 microplate fluorescence reader (Bio-Tek Instrument, Winooski, Vt.) to determine the differential survival of treated cells. The absorbance data of the treated cells were normalized against the absorbance of the untreated cells, and are expressed as percentages in relation to live cells.

### Topo IIα protein levels and location in treated K562 cells

Cell cycle analyses were done for all treated and untreated samples (Table 1) to determine whether the increases in Topo II protein levels were due to the drug exposure or to the cell growth phase. Cells were prepared by incubation in Krishan buffer (1% sodium citrate, 20 mg/l ribonuclease A, 3% Nonidet P-40, and 50 mg/l PI, pH 7.4) for 1 h in the dark, followed by centrifugation at 300 *g* (1000 rpm) for 5 min. The pellets were resuspended in Krishan buffer and filtered through a nylon mesh. A FACScan dual wavelength immunocytometry system (Becton Dickinson, San Jose, Calif.) at the University of Florida Core Flow Cytometry Laboratory was used to collect the data. The data were analyzed with

**Table 1.** Individual, simultaneous, and sequential drug exposure studies in K562 cells for the cellular and mechanism studies were performed using topotecan (*T*), mitoxantrone (*M*) and etoposide (E) at various times after treatment (shown in hours as subscript numbers). Pretreatment with topotecan is indicated with a hyphen (*T*-) (*CON* untreated K562 cells)

| Time (h) | Without topotecan pretreatment | | | | | With topotecan pretreatment | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CON | $M_1$ | $E_1$ | $M_1E_1$ | $T_1E_1M_1$ | $T_{24}$ | $T_{24}$-$M_1$ | $T_{24}$-$E_1$ | $T_{24}$-$E_1M_1$ | $T_{24}$-$T_1$ |
| 24 | CON | $M_{24}$ | $E_{24}$ | $M_{24}E_{24}$ | $T_{24}E_{24}M_{24}$ | $T_{48}$ | $T_{48}$-$M_{24}$ | $T_{48}$-$E_{24}$ | $T_{48}$-$E_{48}M_{48}$ | $T_{48}$-$T_{24}$ |
| 48 | CON | $M_{48}$ | $E_{48}$ | $M_{48}E_{48}$ | $T_{48}E_{48}M_{48}$ | $T_{72}$ | $T_{72}$-$M_{48}$ | $T_{72}$-$E_{48}$ | $T_{72}$-$E_{48}M_{48}$ | $T_{72}$-$T_{48}$ |

Lysis II or Mod-Fit software (Verity Software House, Topsham, Me.).

Relative Topo IIα increases at the transcription level in treated K562 cells were determined by RT-PCR (patent pending; Ambion, Austin, Tx.). The total RNA from $2.0\times10^6$ viable K562 cells (determined by annexin V and PI staining) was extracted (AquaPure RNA isolation kit; Bio-Rad, Hercules, Calif.) after 1 h treatment with topotecan (50 ng/ml), and quantified by UV spectrometry. The cDNA from 5% of the total RNA was obtained using the AMV reverse transcriptase and random primers p(dN)6 (Boehringer Mannheim Corporation, Indianapolis, Ind.) in a total mixture volume of 20 µl incubated for 2 h at 42°C. A cDNA fragment corresponding to the C-terminal of Topo IIα was amplified by subsequent PCR using the TaKaRa Ex Taq transcriptase (Ambion, Austin, Tx.) and Topo IIα-specific primers 5′-GTGACAGTGA-AGAAGACAGCAG-3′ and 5′-TTAAACAGATCATCTTCATC-3′ in a total volume of 100 µl. The 18S ribosomal RNA (rRNA) was used as the internal control instead of the more variable constitutively expressed "housekeeping" genes such as *β*-actin, GAPDH, and cyclophilin. Constant amounts of 18S rRNA expression across experimental samples are desired for relevant quantitative comparisons. Each reaction containing gene-specific primers, 18S rRNA primers and 18S rRNA Competimers were used in the PCR reactions. Competimer technology (Ambion, Austin, Tx.) attenuates the 18S rRNA amplification efficiency so that it can be multiplexed effectively with the much less abundant Topo IIα targets. The PCR reaction was run for 30 cycles of 1.5 min at 94°C, 2 min at 60°C, and 2 min at 72°C.

The intracellular location of Topo IIα was determined by confocal microscopy of K562 cells exposed to individual, simultaneous, and sequential drug treatments. Cells were centrifuged, fixed with 3.7% formaldehyde and 1% methanol for 15 min at room temperature, and permeabilized with FACS permeabilizing solution (BD Bioscience, San Diego, Calif.) for 30 min at 4°C. After permeabilization, cells were incubated with monoclonal anti-Topo IIα antibody (Oncogene, Cambridge, Mass.) followed by Cy5-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa.). DAPI was used for nuclear counterstaining. After thoroughly washing with PBS, the cells were mounted between coverslips and glass slides using Fluoromount-G (Fisher Scientific, Pittsburgh, Pa.), and examined using an L510 Zeiss laser scan microscope.

### Topo I- and Topo II-positive comets

Increases in Topo proteins in cells treated with Topo-directed drugs have been shown in the susceptible cell line CEM [19]. These increases have been shown intracellularly and are associated with fragmented DNA (positive comets [2]) indicative of cells undergoing apoptosis. Positive comets showing Topo I- and Topo II-DNA complexes from K562 cells (300–600 cells/µl) after drug treatments were determined using the protein-DNA double comet assay [2]. The human autoantibody against Topo I (TopoGEN, Columbus, Ohio), the monoclonal anti-human Topo IIα (Oncogene, Boston, Mass.), and rabbit anti-human Topo II*β* (kindly provided by Dan Sullivan, Tampa, Fl.) antibodies were used to stain Topo I, IIα, and II*β*, respectively. Cy-3-conjugated donkey anti-human IgG and Cy-5-conjugated donkey anti-mouse IgG were used as secondary antibodies for Topo I and Topo IIα, respectively. The secondary antibody for Topo II*β* was Cy-3-conjugated donkey anti-rabbit IgG and was used separately on samples. Yo-Pro-1 (Molecular Probes, Eugene, Ore.) was used to stain DNA. Topo I- or Topo II-positive comets were expressed as the ratio of comets labeled with the Topo antibodies to the total number of comets. The length of the comet tail [(length–width)/length] was also measured to gauge the amount of degraded genomic DNA for that particular cell. Cell damage due to drug exposures was correlated with the increase in positive comets and the quantity of genomic DNA damage as shown by the length of the comet tail.

### Statistics

The percentage of apoptotic cells, early dead cells, and Topo IIα-positive comets were evaluated with the Chi-square test. All other data were evaluated with Student's *t* test. Data are presented as means ± standard error.

## Results

### Exposure of K562 cells to topotecan

Topotecan as a single agent did not effectively kill all K562 cells even at concentrations up to 1000 times 50 ng/ml, which is the corresponding normal clinical dose. Less than 1 in 1000 cells survived, with viable cells in growth arrest for 30 days after drug removal. Normal growth resumed after 60 days.

### Effect of treatment schedule on cytotoxicity

The percentage of live K562 cells remaining after the following individual, sequential and simultaneous drug treatments are shown in Fig. 1: topotecan on days 1–3 (T); topotecan on days 1–3 followed by topotecan, etoposide, mitoxantrone, or etoposide + mitoxantrone on days 4, 5, 9 and 10 (T-T, T-E, T-M, T-EM); etoposide, mitoxantrone or etoposide + mitoxantrone on days 4, 5, 9 and 10 without topotecan pretreatment (E, M, EM); and topotecan + etoposide + mitoxantrone on days 1–3, 4, 5, 9 and 10 (TEM). Individual treatments with topotecan, etoposide, or mitoxantrone resulted in 17–22% viable cells. Treatments T-T, T-E and T-EM were compared with the simultaneous treatments, EM and TEM. Cells pretreated with topotecan had significantly fewer ($P < 0.01$) live cells than those without pretreatment. The percentages of cells surviving after treatments T-T, T-M and T-E were 8.5%, 1.03%, and 5.2%, respectively. Simultaneous treatment TEM was as effective as sequential treatment T-E (3.6% and 5%,

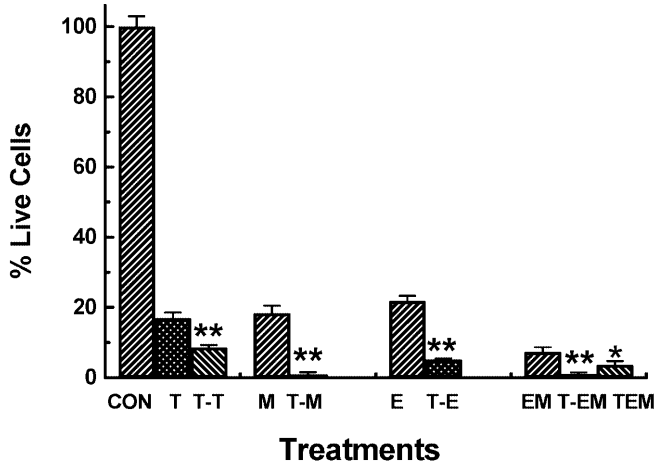


**Fig. 1.** Percentage live K562 cells after treatment with: topotecan days 1–3 (*T*); topotecan days 1–3 followed by topotecan, etoposide, mitoxantrone or etoposide + mitoxantrone days 4, 5, 9, and 10 (*T-T*, *T-E*, *T-M*, *T-EM*); etoposide, mitoxantrone or etoposide + mitoxantrone without topotecan pretreatment (*E*, *M*, *EM*); and topotecan + etoposide + mitoxantrone days 1–3, 4, 5, 9, and 10 (*TEM*). Cells with topotecan pretreatment were compared with the corresponding group without topotecan pretreatment (**$P < 0.01$, *$P < 0.05$)

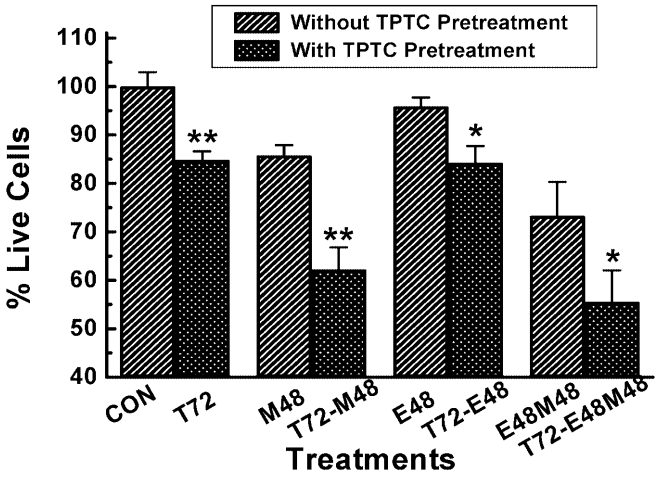


**Fig. 2.** Viability of K562 cells after treatment with the shortened T-EM chemotherapy regimen as measured by the MTT assay 72 h from the start of the experiment (*CON* control, *T* topotecan, *M* mitoxantrone, *E* etoposide, *EM* etoposide + mitoxantrone; the *numbers* indicate the time of measurement in hours after treatment). **$P < 0.01$, *$P < 0.05$, treatment groups with vs those without topotecan pretreatment

respectively). There were no viable cells detected following sequential treatment T-EM as determined both in the MTT assay and by inverted microscope screening.

The results of the cellular and molecular mechanism studies using the same treatment schedules in cells treated for a reduced number of days and drug exposures showed the same trend, with correspondingly lower percentages of killed cells. Cell viability 48 h after individual, simultaneous, and sequential treatments are shown in Fig. 2. All sequential treatments with topotecan pretreatment showed significant to highly significant differences in cell kill compared with treatment regimens without topotecan pretreatment. The greatest reduction in cell numbers was from the sequential treatment $T_{72}$-$E_{48}M_{48}$ (46%), followed by $T_{72}$-$M_{48}$ (39%) (the numbers indicating the time of measurement in hours after treatment). Cell viability 1 h and 24 h after treatment showed similar but less-significant changes.

## Cellular and molecular mechanism studies

### *Apoptosis and early cell death*

The percentages of apoptotic cells (annexin V-positive) and dead cells (PI-positive) measured by flow cytometry at 1 h, 24 h, and 48 h after drug treatments showed the ongoing response of the cells to drug treatment (Fig. 3A). Of all treatments at 48 h after drug exposure, the sequential treatment T-EM resulted in the highest cell kill. Following the single drug treatments E, T, and M, 13% to 29% cells were either dead or committed to apoptosis. The simultaneous treatment EM resulted in an almost twofold increase in cell kill (42%) which was more than that with treatment T-M (39%) but less than that with treatment T-E (55%).

Apoptotic and dead cells were also examined by confocal microscopy from aliquots of samples exposed to individual, simultaneous, and sequential drug treatments at 1 h, 24 h and 48 h. Data for drug exposures at the 48-h time-points are shown in Fig. 3B. The numbers of apoptotic and dead cells were highest with the sequential treatment $T_{72}$-$M_{48}E_{48}$, followed by $T_{72}$-$E_{48}$, then simultaneous treatment $M_{48}E_{48}$. Cells that were annexin V- and/or PI-negative that had intense dark granules in the cells as seen with treatments $E_{48}M_{48}$ and $T_{72}$-$M_{48}E_{48}$ represented late dead cells whose cell membrane had collapsed but remained intact at the time of analysis.

### *Topo IIα protein levels and location in K562 treated cells*

Cell cycle analyses of all treated cells consistently showed $G_2$, M and S phase arrest. Thus, increases in Topo IIα protein levels in all topotecan-treated cells were independent of cell cycle phases.

The relative amount of Topo IIα protein was shown at the level of transcription. An amplified cDNA fragment corresponding to the C-terminal of Topo IIα obtained from the mRNA of topotecan-treated K562 cells increased after 1 h of topotecan treatment (T1) as shown in Fig. 4.

Confocal microscopy images of cells receiving individual ($T_{72}$, $E_{48}$, $M_{48}$), simultaneous ($E_{48}M_{48}$), and sequential ($T_{72}$-$E_{48}$, $T_{72}$-$M_{48}$, $T_{72}$-$E_{48}M_{48}$) drug treatments demonstrated changes in the amounts of Topo IIα proteins, and their cellular location (Fig. 5). Cells treated with the Topo I-directed drug topotecan showed Topo IIα (red) primarily located in the cytosol, whereas cells

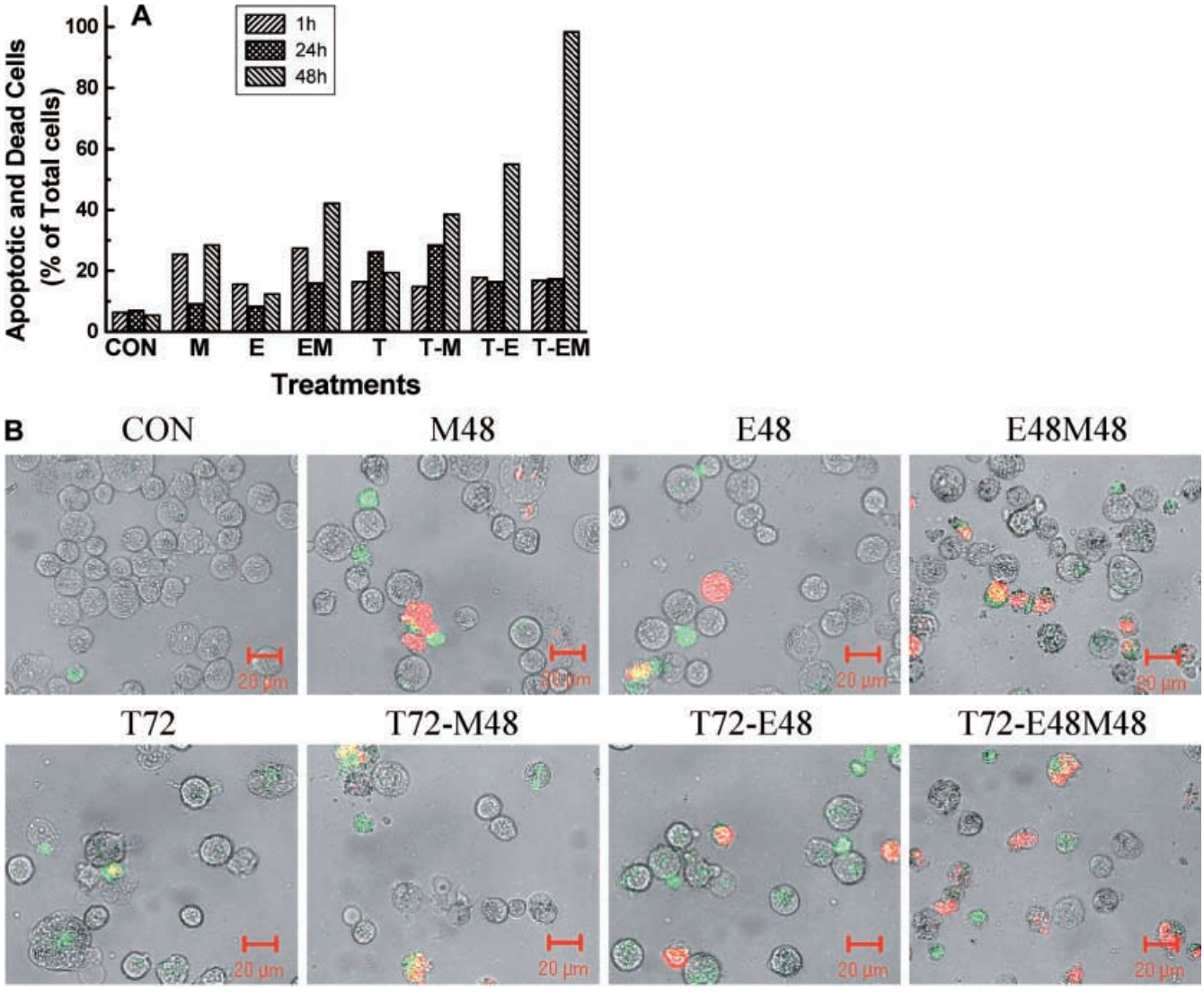


**Fig. 3.** **A** Percentage of apoptotic and dead K562 cells 1 h, 24 h, and 48 h after treatment with topotecan (*T*), etoposide (*E*) or mitoxantrone (*M*) with or without topotecan pretreatment (*T-*) as determined by flow cytometry. **B** Confocal microscopy images of K562 cells treated with mitoxantrone ($M_{48}$), etoposide ($E_{48}$), etoposide + mitoxantrone ($E_{48}M_{48}$), and topotecan ($T_{72}$), compared to samples pretreated with topotecan followed by mitoxantrone ($T_{72}$-$M_{48}$), etoposide ($T_{72}$-$E_{48}$), or etoposide + mitoxantrone ($T_{72}$-$E_{48}M_{48}$). The *numbers* indicate the time in hours after treatment when the images were obtained. Apoptotic cells are annexin V-positive only (*green*), and early dead cells are PI-positive (*red*). Late dead cells with intact cell membranes show an irregular surface and intense dark granules

treated with the Topo II-directed drugs etoposide, and/or mitoxantrone (E, EM, or M) showed Topo IIα located in the nucleus (yellow). Cells treated with topotecan followed by either etoposide (T-E), mitoxantrone (T-M) or etoposide + mitoxantrone (T-EM) showed increased amounts of Topo IIα in the nuclei compared to cells without topotecan pretreatment. Topo IIα protein fluorescent intensity was highest in cells receiving the $T_{72}$-$E_{48}M_{48}$ treatment, followed by those receiving the $T_{72}$-$E_{48}$ and $T_{72}$-$M_{48}$, and then the $T_{72}$, treatments, relative to those receiving treatments without topotecan (M, E, EM). The data from other time-points were similar.

### *Topo I- and Topo II-positive comets*

Single-cell electrophoresis of cells treated with Topo I- and II-directed drugs showed DNA fragmentation as a comet tail. Incubation of these cells with fluorescent

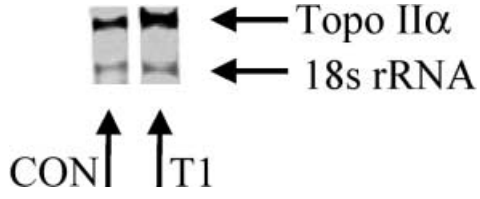


**Fig. 4.** Topo IIα cDNA from K562 cells treated with (*T1*) or without (*CON*) 50 ng/ml topotecan for 1 h was amplified to show differences in the amounts of transcribed Topo IIα. 18s rRNA was used as an internal control

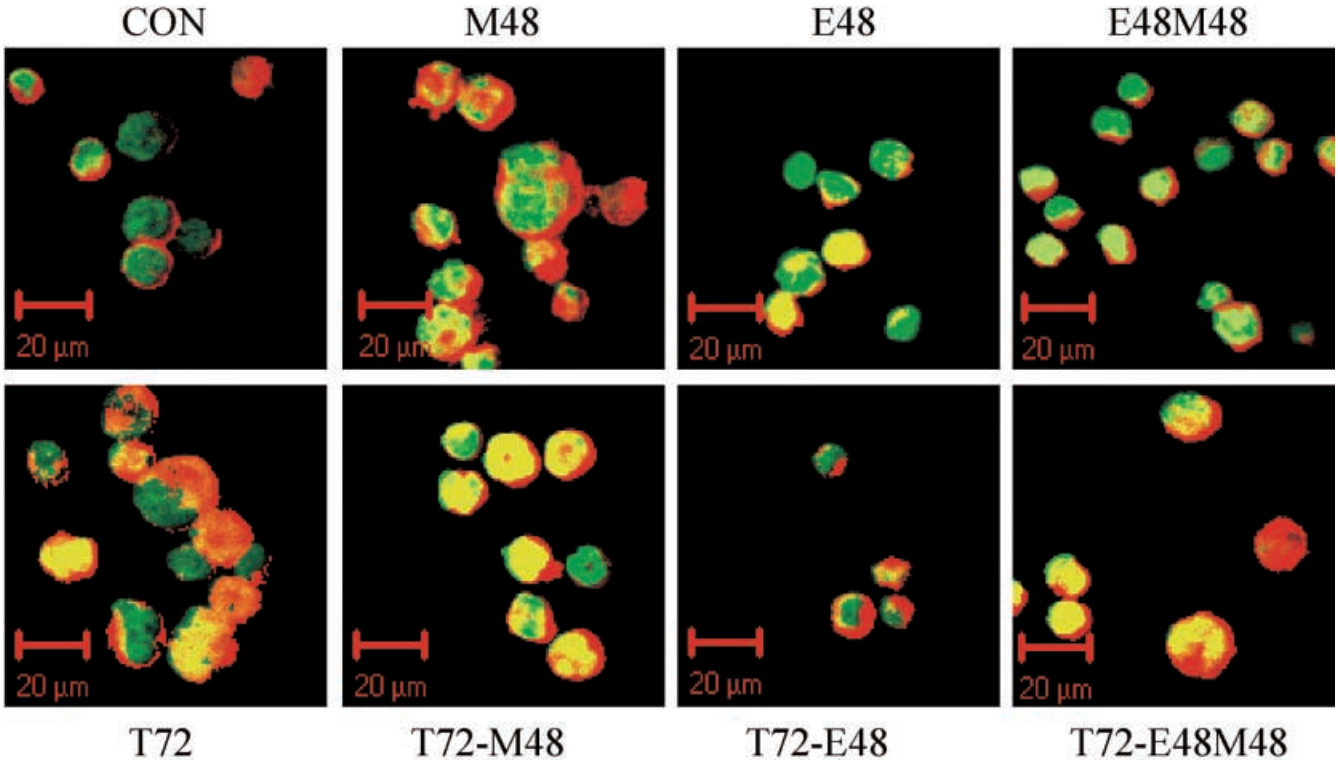


**Fig. 5.** Immunofluorescent confocal microscopy of nuclear DNA (*green*), Topo IIα protein (*red*), and Topo IIα protein associated with nuclear DNA (*yellow*) from K562 cells treated with mitoxantrone ($M_{48}$), etoposide ($E_{48}$), etoposide + mitoxantrone ($E_{48}M_{48}$), and topotecan ($T_{72}$), or pretreated with topotecan followed by mitoxantrone ($T_{72}$-$M_{48}$), etoposide ($T_{72}$-$E_{48}$), or etoposide + mitoxantrone ($T_{72}$-$E_{48}M_{48}$). The *numbers* indicate the time in hours after treatment when the images were obtained

antibodies against Topo I and Topo IIα proteins allowed the identification of Topo-DNA complexes in the intact DNA (comet head) and fragmented DNA (comet tail). Positive comets were those in which fluorescence for Topo I or Topo IIα protein-DNA complexes were detected within the comet tail indicating Topo-mediated DNA fragmentation. Treated K562 cells processed for Topo I-positive comet detection did not show any positive signals within comet tails. However, faint Topo I signals were seen in the comet heads of all treated cells and in the control group, indicating that Topo I was associated only with intact DNA. On the other hand, Topo II-positive comets (TIIPC) were detected in the comet heads and/or tails of treated cells (Fig. 6). The numbers of TIIPC were lowest (2.2%) following treatments $E_1$, $M_1$, $E_1M_1$, and highest following treatments $T_{48}$-$E_{24}$ (77.3%) and $T_{48}$-$E_{24}M_{24}$ (75%). However, the number of TIIPC following all treatments reverted to the control levels (0%) 72 h after administration of the first treatment, except with the sequential treatment $T_{72}$-$E_{48}M_{48}$ where the TIIPC decreased to 15%, a level significantly higher than in all the other treatment groups ($P < 0.01$).

The TIIPC contents in treatment groups with or without topotecan pretreatment within and between each time frame were as follows. No TIIPC increases were found with treatments $T_{24}$-$T_1$ and $T_{24}$-$M_1$, whereas TIIPC increases of 40% and 60% were found with treatments $T_{24}$-$E_1$ and $T_{24}$-$E_1M_1$, respectively. TIIPC increases of only 40% were found with treatments $M_{24}$, $E_{24}$, and $E_{24}M_{24}$, while no significant TIIPC increases were found with treatment $T_{24}$ or with the simultaneous treatment $T_{24}E_{24}M_{24}$. TIIPC increases of 77.3% and 75% were found with treatments $T_{48}$-$E_{24}$ and $T_{48}$-$E_{24}M_{24}$, respectively. These increases were significantly higher than the TIIPC levels with treatments $E_{48}$ and $E_{48}M_{48}$ (i.e., without topotecan pretreatment) (Fig. 7).

## Discussion

A significant number of leukemia patients who respond initially to chemotherapy eventually relapse [16] and those who have refractory and recurrent leukemia respond modestly to one of the accepted "reinduction" chemotherapy regimens using the combination of two Topo II-directed drugs, etoposide and mitoxantrone [9]. Topotecan, a Topo I-directed drug, has shown a modest single-agent activity in the treatment of AML, myelodysplastic syndrome and chronic myelomonocytic leukemia [1, 11]. Continued refractory disease or inevitable relapse with reinduction therapy strongly indicates persistence of resistant tumor clones. In this study, we demonstrated that topotecan, etoposide, or mitoxantrone as single agents were insufficient to kill all CML-BP (K562) cells in culture. Even with topotecan at doses 1000 times normal clinical doses, there was persistence of clones, albeit quiescent clones, that resumed normal growth after a period of time. Similarly, simultaneous

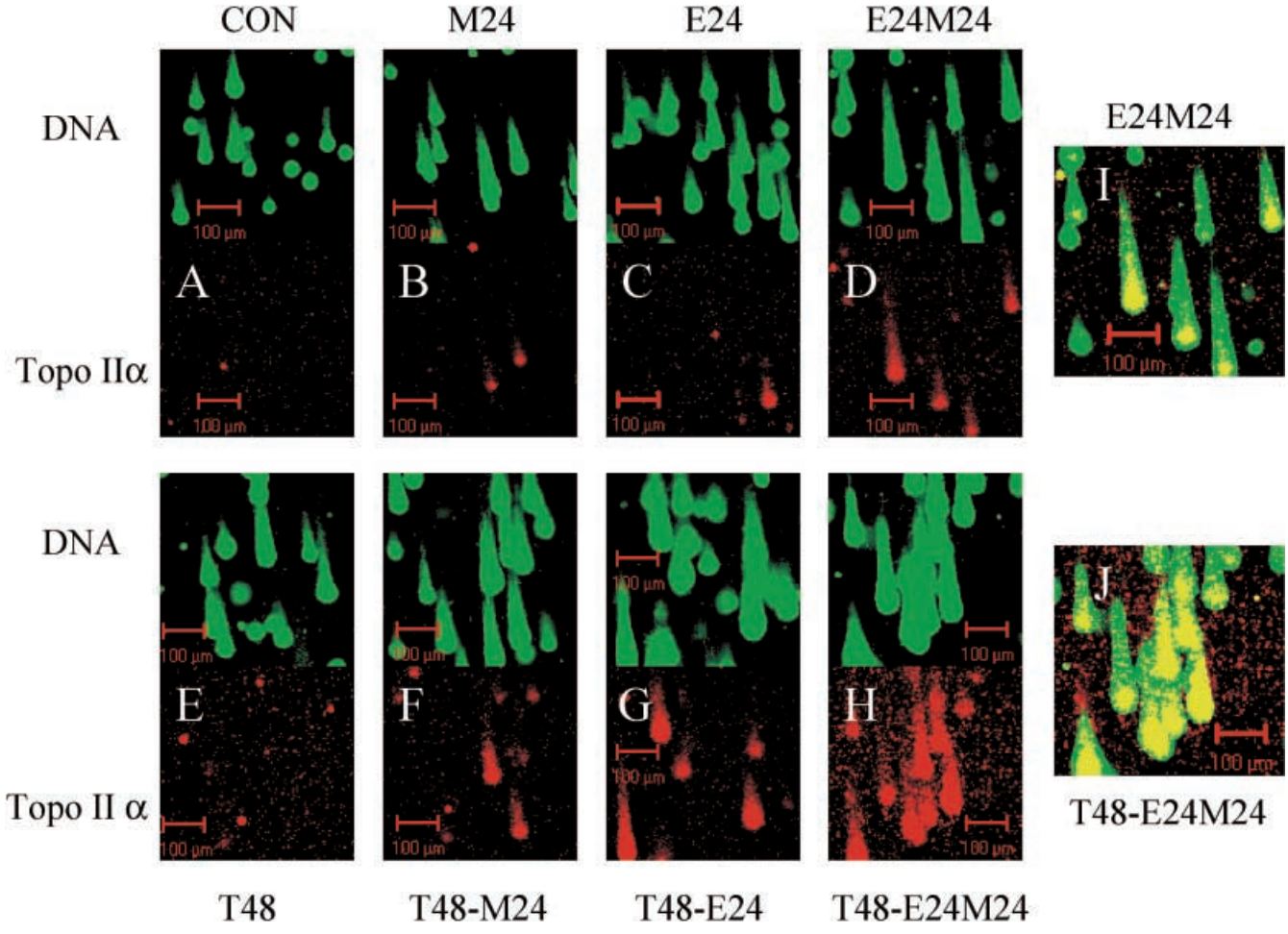


**Fig. 6.** Double comet assay of K562 cells treated with mitoxantrone ($M_{24}$), etoposide ($E_{24}$), etoposide + mitoxantrone ($E_{24}M_{24}$), and topotecan ($T_{48}$), or pretreated with topotecan followed by mitoxantrone ($T_{48}$-$M_{24}$), etoposide ($T_{48}$-$E_{24}$), or etoposide + mitoxantrone ($T_{48}$-$E_{24}M_{24}$). The *numbers* indicate the time in hours of the assay after treatment. DNA is shown in *green* (upper half of each image) and Topo IIα in the same cell is represented in *red* (lower half). Positive double comets refer to cells with Topo IIα proteins associated with fragmented DNA (*yellow*) seen in the comet tail

treatments with topotecan and etoposide or topotecan and mitoxantrone were incapable of eradicating K562 cells.

Initial treatment with topotecan was highly effective in increasing the sensitivity of the K562 cells to subsequent etoposide and/or mitoxantrone treatments. After treatment with the regimen that paralleled an ongoing clinical trial where topotecan was administered on days 1–3, followed by etoposide + mitoxantrone on days 4, 5, 9, and 10, no viable cells were observed (Fig. 1). Repeated use of topotecan or simultaneous use of topotecan and mitoxantrone or etoposide was not as effective as when the drugs were used sequentially. The results of the cellular and molecular studies (Table 1) where the same chemotherapy regimen was administered for a shorter period of time also showed the same trend. As in the former chemotherapy regimen, the highest reduction in cell numbers, and percentages of apoptotic and dead cells were observed in cells treated sequentially with topotecan, followed by etoposide + mitoxantrone.

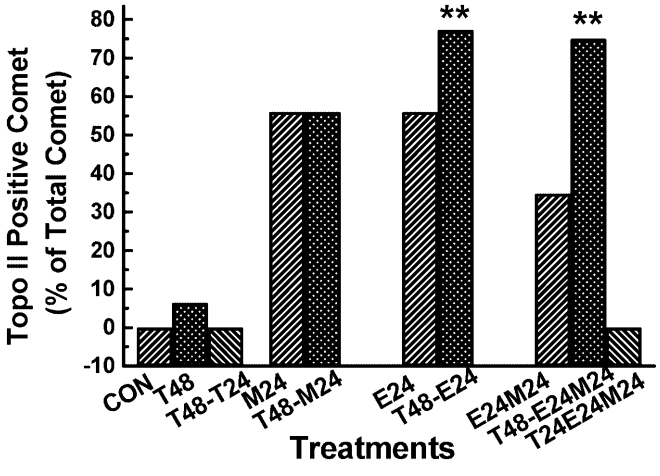


**Fig. 7.** Topo II-positive comets of K562 cells treated with topotecan (*T*), etoposide (*E*), mitoxantrone (*M*), etoposide + mitoxantrone (*EM*) with or without topotecan pretreatment (*T*-), or topotecan + etoposide + mitoxantrone (*TEM*). The *numbers* indicate the time in hours of the assay after treatment. $^{**}P < 0.01$, $E_{24}$ vs $T_{48}$-$E_{24}$, and $E_{24}M_{24}$ vs $T_{48}$-$E_{24}M_{24}$

Amplified Topo IIα cDNA fragments from cells treated with topotecan showed an increase in Topo IIα compared to those from untreated cells, indicating the effect of topotecan at the level of translation and transcription. Confocal microscopy studies confirmed increases in Topo IIα following topotecan pretreatment. Differences in Topo IIα cellular distribution between treatment groups were also found. Treatment with Topo II-directed drugs alone increased the distribution of Topo IIα in the nucleus without changing the total cellular concentration. In contrast, topotecan treatment increased total Topo IIα proteins and these were primarily localized within the cytosol. Sequential treatment with topotecan followed by etoposide, mitoxantrone or etoposide + mitoxantrone resulted in increased Topo IIα protein levels and an enhanced intensity primarily within the nucleus. Interestingly, DNA staining (DAPI) of samples treated with topotecan followed by etoposide + mitoxantrone was notably diminished and the cell size was increased compared to other treated samples, indicating extensive DNA damage and cellular swelling. This degree of drug-induced DNA fragmentation was confirmed by the double comet assay.

The double comet assay (designed in our laboratory) is a simple method that detects simultaneously in individual cells free Topo proteins, non-associated DNA, and Topo-DNA complexes in drug-induced DNA fragments (positive double comets) in individual cells. Positive double comets were seen with greater frequency and with longer comet tails in samples pretreated with topotecan followed by etoposide or etoposide + mitoxantrone, when compared to control samples or those treated with the single agents alone. The sequential treatment topotecan followed by etoposide + mitoxantrone resulted in the greatest intensity of Topo IIα-DNA complexes that were also associated with smaller DNA fragments resulting in longer comet tails. The Topo IIα-positive comets were observed up to 48 h after treatment, indicating the existence of Topo IIα-DNA complexes long after removal of drugs poisoning the Topo IIα enzyme religation function. Topo II$\beta$- and Topo I-DNA complexes were observed but were not associated with fragmented DNA.

Induction of Topo IIα protein was directly associated with increased DNA fragmentation and decreased cell viability, supporting the proposed mechanism for the efficacy of the topotecan followed by etoposide + mitoxantrone treatment regimen. This regimen showed very promising results in increasing the efficiency of initial chemotherapy and this may have the potential to decrease refractory or recurrent leukemia in the clinical setting. The formation of Topo IIα-DNA complexes in samples from patients being treated for non-Hodgkin lymphoma with topotecan and etoposide has been reported to be strongly correlated with clinical response [10]. Enhanced Topo II drug-induced DNA fragmentation occurred with increased Topo IIα and indicated a direct causal link between the downstream events and the antiproliferative and cytotoxic activity of Topo II-directed drugs, as reported by others [5, 8].

**Acknowledgements** The support of a Veteran Affairs Career Development Award to M.G.M. is gratefully acknowledged.

## References

1. Beran M, Kantarjian H, O'Brien S, Koller C, al-Bitar M, Arbuck S, Pierce S, Moore M, Abbruzzese JL, Andreeff M, Keating M, Estey E (1996) Topotecan, a topoisomerase I inhibitor, is active in the treatment of myelodysplastic syndrome and chronic myelomonocytic leukemia. Blood 88:2473
2. Chen S, Gomez S, Mainwaring MG (2001) Detection of protein-DNA complexes using the double comet assay (abstract no. 8319). American Association for Cancer Research Proceedings
3. Cortes J, Estey E, Beran M, O'Brien S, Giles F, Koller C, Keating M, Kantarjian H (2000) Cyclophosphamide, ara-C and topotecan (CAT) for patients with refractory or relapsed acute leukemia. Leuk Lymphoma 36:479
4. Crump M, Lipton J, Hedley D, Sutton D, Shepherd F, Minden M, Stewart K, Beare S, Eisenhauer E (1999) Phase I trial of sequential topotecan followed by etoposide in adults with myeloid leukemia: a National Cancer Institute of Canada Clinical Trials Group Study. Leukemia 13:343
5. Davis SM, Robson CN, Davies SL, Hickson ID (1988) Nuclear topoisomerase II levels correlate with the sensitivity of mammalian cells to intercalating agents and epipodophyllotoxins. J Biol Chem 263:17724
6. Estey EH (2000) Current therapy and outcome of patients with relapsed acute myeloid leukemia. Biol Ther Leukemia 1:2
7. Gangloff S, Lieber MR, Rothstein R (1994) Transcription, topoisomerases and recombination. Experientia 50:261
8. Gorczyca W, Bigman K, Mittelman A, Ahmed T, Gong J, Melamed MR, Darzynkiewicz Z (1993) Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. Leukemia 7:659
9. Ho AD, Lipp T, Ehninger G, Illiger HJ, Meyer P, Freund M, Hunstein W (1988) Combination of mitoxantrone and VP-16 in refractory AML – an active and well-tolerated regimen. J Clin Oncol 6:213
10. Kancherla RR, Nair JS, Ahmed T, Durrani H, Seiter K, Mannancheril A, Tse-Dinh YC (2001) Evaluation of topotecan and etoposide for non-Hodgkin lymphoma: correlation of topoisomerase-DNA complex formation with clinical response. Cancer 91:463
11. Kantarjian HM, Beran M, Ellis A, Zwelling L, O'Brien S, Cazenave L, Koller C, Rios MB, Plunkett W, Keating MJ, Estey EH (1993) Phase I study of topotecan, a new topoisomerase inhibitor, in patients with refractory or relapsed acute leukemia. Blood 81:1146
12. Karanes C, Kopecky KJ, Head DR, Grever MR, Hynes HE, Kraut EH, Vial RH, Lichtin A, Nand S, Samlowski WE, Appelbaum FR (1999) A phase III comparison of high dose ARA-C versus HIDAC plus mitoxantrone in the treatment of first relapsed or refractory acute leukemia. Southwest Oncology Group Study. Leuk Res 23:787
13. Kern W, Schleyer E, Unterhalt M, Wormann B, Buchner T, Hiddemann W (1997) High antileukemic activity of sequential high dose cytosine arabinoside and mitoxantrone in patients with refractory acute leukemia. Results of a clinical phase II study. Cancer 79:59
14. Koller CA, Kantarjian HM, Thomas D, O'Brien S, Rios MB, Kornblau S, Murphy S, Keating M (1988) Combination of mitoxantrone and VP-16 in refractory AML. An active and well tolerated regimen. J Clin Oncol 6(2):213
15. Long BH, Musial ST, Brattain MG (1984) Comparison of cytotoxicity and DNA breakage activity of congeners of podophyllotoxin including VP16-213 and VM-26. Biochemistry 23:1183
16. Lowenberg B, Downing JR, Burnett A (1999) Acute myeloid leukemia. N Engl J Med 341:1051

17. Mainwaring MG, Weeks F, Wingard JW, Lynch J, Rimsza L, Braylan R (1998) Topoisomerase II levels and DNA fragmentation of leukemia blasts from patients treated with timed sequential chemotherapy (abstract). Blood 92:234a
18. Osheroff N, Zechiedrich EL, Gale KC (1991) Catalytic function of DNA topoisomerase II. Bioessays 13:269
19. Roca J (1995) The mechanisms of DNA topoisomerases. Trends Biochem Sci 20:156
20. Rowinsky EK, Adjei A, Donehower RC, Gore SD, Jones RJ, Burke PJ, Cheng YC, Grochow LB, Kaufmann SH (1994) Phase I and pharmacodynamic study of the topoisomerase I inhibitor topotecan in patients with refractory acute leukemia. J Clin Oncol 12:2193
21. Sabourin M, Osheroff N (2000) Sensitivity of human type II topoisomerase to DNA damage: stimulation of enzyme-mediated DNA cleavage by abasic, oxidized and alkylated lesions. Nucleic Acids Res 28:1947
22. Wang JC, Lynch AS (1993) Transcription and DNA supercoiling. Curr Opin Genet Dev 3:764
23. Whitacre CM, Zborowska E, Gordon NH, Mackay W, Berger NA (1997) Topotecan increases topoisomerase II alpha levels and sensitivity to treatment with etoposide in schedule-dependent process. Cancer Res 57:1425
24. Zhou Z, Zwelling LA, Kawakami Y, An T, Kobayashi K, Herzog C, Kleinerman ES (1999) Adenovirus-mediated human topoisomerase IIα gene transfer increases the sensitivity of etoposide-resistant human breast cancer cells. Cancer Res 59:4618