In Vitro and In Vivo Activity of Topotecan Against Human B-Lineage Acute Lymphoblastic Leukemia Cells

By Fatih M. Uckun, Clinton F. Stewart, Gregory Reaman, Lisa M. Chelstrom, Jizhong Jin, Miridula Chandan-Langlie, Kevin G. Waddick, James White, and William E. Evans

Topotecan ([S]-9-dimethylaminomethyl-10-hydroxycamptothecin hydrochloride; SK&F 104864-A, NSC 699699), a water soluble semisynthetic analogue of the alkaloid camptothecin, is a potent topoisomerase I inhibitor. Here we show that topotecan stabilizes topoisomerase I/DNA cleavable complexes in radiation-resistant human B-lineage acute lymphoblastic leukemia (ALL) cells, causes rapid apoptotic cell death despite high-level expression of bcl-2 protein, and inhibits ALL cell in vitro clonogenic growth in a dose-dependent fashion. Furthermore, topotecan elicited potent antileukemic activity in three different severe combined immunodeficiency (SCID) mouse models of human poor prognosis ALL and markedly improved event-free survival of SCID mice challenged with otherwise fatal doses of human leukemia cells at systemic drug exposure levels that can be easily achieved in children with leukemia.

HUMAN DNA topoisomerase I is a nuclear enzyme that plays a critical role in DNA replication by forming transient enzyme-bridged single-strand breaks of duplex DNA. These DNA breaks are believed to function as gates for the passage of the unbroken complementary DNA strands catalyzing relaxation of supercoiled DNA and, hence, the resolution of conformational and topologic problems that occur during DNA replication. Topoisomerase I-targeting anticancer drugs such as camptothecin specifically interfere with the breakage-reunion (resealing) reaction of topoisomerase I, trapping the enzyme in a covalent reaction-intermediate, the cleavable complex. Such stabilized cleavable complexes are believed to be responsible for the antitumor activity of topoisomerase I inhibitors. This notion is predicated on the reported quantitative correlation between drug-stabilized cleavable complex formation and antitumor activity of several camptothecin derivatives and the isolation of a camptothecin-resistant topoisomerase I from a camptothecin-resistant tumor cell line.

Topotecan ([S]-9-dimethylaminomethyl-10-hydroxycamptothecin hydrochloride; SK&F 104864-A, NSC 699699), a water-soluble semisynthetic analogue of the alkaloid camptothecin, is a potent topoisomerase I poison. Topotecan causes cytotoxicity during the course of DNA replication by stabilizing the covalent complex between topoisomerase I and DNA and preventing the religation of enzyme-linked single-strand DNA breaks. The purpose of this study was to examine the antileukemic activity of topotecan against human B-lineage acute lymphoblastic leukemia (ALL) cells. Our findings provide unprecedented evidence that topotecan causes rapid apoptosis of radiation-resistant ALL cells despite their high-level bcl-2 expression and inhibits their in vitro clonogenic growth in a dose-dependent fashion. Furthermore, we demonstrate that topotecan has potent antileukemic activity in three different severe combined immunodeficiency (SCID) mouse models of human poor-prognosis ALL, and at plasma concentrations readily achieved in patients, it markedly improves event-free survival of SCID mice challenged with otherwise fatal doses of human leukemia cells. To our knowledge, this report is the first comprehensive preclinical analysis of the activity of a topoisomerase I inhibitor against human B-lineage ALL cells.

MATERIALS AND METHODS

ALL cell lines. The t(4;11) ALL cell line RS4;11, the t(1;19) ALL cell line LC1;19, and the t(8;14) ALL cell line NALM-6 were used as target cell lines to evaluate the anti-ALL activity of topotecan. These cell lines were maintained by serial passages in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 50 µg/mL streptomycin, 50 IU/mL penicillin, 2 mmol/L L-glutamine, and 10 mmol/L HEPES buffer. Cell cultures were maintained in tissue culture flasks at 37°C in a humidified 5% CO₂ atmosphere. Before injection into SCID mice, cells were washed twice in phosphate-buffered saline (PBS) and resuspended in PBS. SCID mice were inoculated intravenously with 0.2 mL of this cell suspension containing 1×10⁶ NALM-6-UM1 cells, 5×10⁶ RS4;11 cells, or 1×10⁶ LC1;19 cells.

Patients. Four leukemia patients (three boys with ALL and one girl with AML) with a median age of 6 years (range, 4 to 20 years) received topotecan (1.6 to 2.1 mg/m²/d) as a 120-hour continuous infusion on a Phase I Maximum Tolerated Systemic Exposure (MTSE) protocol [National Cancer Institute (NCI) T91-02611] at St. Jude Children’s Research Hospital (Memphis, TN). This Cancer Therapy Evaluation Program-sponsored Phase I study was approved by the St. Jude Institutional Review Board, and informed consent was obtained before patients received topotecan. Consistent with eligibility criteria, patients had normal renal and hepatic function.

From the Biotherapy Program, Departments of Therapeutic Radiology-Radiation Oncology, Pharmacology, Pediatrics, and Laboratory Medicine/Pathology, University of Minnesota, Minneapolis, MN; the Pharmaceutical Department, St. Jude Children’s Research Hospital, Memphis, TN; the Department of Hematology-Oncology, Children’s National Medical Center, Washington, DC; and the Children’s Cancer Group, Arcadia, CA.

Submitted July 28, 1994; accepted December 23, 1994.

Supported in part by U.S. Public Health Service Grants No. CA-42633, CA-51425, CA-42111, CA-21737, CA-61549, CA-21765, CA-60437, CA-57746, CA-20180, and CA-13539 from the National Cancer Institute, Department of Health and Human Services; the American Lebanese Syrian Associated Charities (ALSAC); and special grants from the Minnesota Medical Foundation, the Children’s Cancer Research Fund, the Bone Marrow Transplant Research Fund, University of Minnesota, and the National Childhood Cancer Foundation (NCCF). F.M. U. is a Stohlman Scholar of the Leukemia Society of America.

Address reprint requests to Fatih M. Uckun, MD, PhD, Box 356 UMHC, University of Minnesota, 420 Delaware St SE, Minneapolis, MN 55455.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1995 by The American Society of Hematology.
Fig 1. Expression of bcl-2 protein in human B-lineage ALL cells. Proteins from whole cell lysates were separated on 10.5% polyacrylamide gels, transferred to a 0.45-μm Immobilon-PVDF membrane, and immunoblotted with an antihuman bcl-2 antibody, as described in Materials and Methods.

and received no concomitant drugs that would alter topotecan disposition.

Drugs and drug treatment protocols. Topotecan was obtained (1) from the Division of Cancer Treatment, NCI (Bethesda, MD) in vials containing a lyophilized mixture of 5 mg of topotecan and 100 mg mannitol (for in vitro experiments) and (2) from Dr R. K. Johnson (SmithKline-Beecham, King of Prussia, PA) as pure topotecan powder (for in vivo SCID mouse experiments). For in vitro experiments, the topotecan/mannitol mixture was reconstituted with 2 mL sterile water, diluted in 100 mL of 5% dextrose solution. The pH of the mixture was previously adjusted to 3 to 4 with hydrochloric acid and sodium hydroxide. For SCID mouse experiments, topotecan was carefully weighed and reconstituted with 0.9% normal saline at concentrations of 2 or 4 mg/mL. This solution (100 μL) was then used to fill Alzet 3-day micro-osmotic pumps (Model 1003D, Alza Corp, Palo Alto, CA). The pumps were subcutaneously inserted into SCID mice anesthetized by intraperitoneal injection of 100 mg/kg ketamine (Aveco Co, Fort Dodge, IA), and topotecan was administered as a continuous infusion over 72 hours.

SCID mice. All SCID mice in this study were produced by specific pathogen-free (SPF) CB-17 scid/scid breeders (originally obtained from Dr Melvin Bosma, Fox Chase Cancer Center, Philadelphia, PA) and housed in microisolator cages containing autoclaved food, water, and bedding. Trimethoprim sulfamethoxazole (Biocraft Lab, Elmwood Park, NJ) was added to the drinking water of mice three times per week. Female 8-week-old SCID mice were

Fig 2. Internucleosomal DNA fragmentation in topotecan-treated B-lineage ALL cells. NALM-6 (A) and RS4;11 (B) cells were harvested 4 hours after exposure to topotecan, and DNA was prepared for analysis of fragmentation. DNA was then electrophoresed through a 1% agarose gel, and the DNA bands were visualized by UV light after staining with ethidium bromide.
Anti-ALL Activity of Topotecan

Fig 3. Ultrastructural features of topotecan-treated ALL cells undergoing apoptosis. RS4;11 cells were examined after treatment with 5 μmol/L topotecan for 8 hours by transmission electron microscopy using a Philips 301 electron microscope, as described in Materials and Methods. (A) Minimal changes, including surface blebbing and chromatin condensation along the nuclear membrane. (B) Nuclear fragmentation. (C) Crescents of electron-dense heterochromatin, which result from uniform condensation of chromatin around the inner margin of the nuclear envelope, and masses of fine granular material near the center of the nucleus. (D) Nuclear collapse.

inoculated intravenously with 0.2 mL leukemic cell suspensions containing $1 \times 10^6$ NALM-6-UM1, $1 \times 10^6$ LC1;19, or $5 \times 10^6$ RS4;11 cells. These three SCID mouse models of human ALL have been described in detail in previous reports. Survival of mice was monitored by observation twice per day, and the onset of paraplegia as a result of meningeal leukemia as well as death was recorded daily. Event times were measured from the day of inoculation with leukemia cells to the day of paraplegia or death. The probability of event-free survival was determined, and event-free interval curves were generated using the Kaplan-Meier product limit method, as previously reported. We used the Mantel-Cox log-rank test to assess the effect of drug treatment on the probability of event-free survival.
survival of SCID mice inoculated with leukemia cells, as previously reported. At the time of death, necropsies were performed, and multiple organs of SCID mice were collected for evaluation of their burden of human leukemic cells by histopathologic studies and multiparameter flow cytometric analyses using two-color immunofluorescence staining techniques, as previously described. Human DNA was detected by amplifying a 110-bp fragment from the first exon of the human β-globin gene using two 20-base oligonucleotide primers that flank the region to be amplified, as previously described. Controls included the polymerase chain reaction (PCR) reaction buffer without the genomic DNA as a background control; DNA from bone marrow, liver, spleen, and brain of healthy SCID mice as negative controls; and the PCR reaction mixture with NALM-6-UMI pre-B ALL cell line genomic DNA as a positive control.

**Apoptosis assays.** Topotecan-treated leukemic cells were examined on Wright-Giemsa-stained cytospin slides for morphologic changes characteristic of apoptosis, including nuclear chromatin condensation, segmentation of the nucleus, and plasma membrane blebs. The ultrastructural changes of apoptosis were documented by electron microscopy, as previously described. In brief, cell suspensions were mixed 1:1 with 3% glutaraldehyde in White’s saline [a 10% solution of a 1:1 mixture of (1) 2.4 mmol/L KCl, 46 mmol/L MgSO₄, 64 mmol/L Ca(NO₃)₂, and (2) 0.13 mol/L NaHCO₃, 8.4 mmol/L NaH₂PO₄, and 0.1 g/L of phenol red, pH 7.4]. After 15-minute fixation, the cells were centrifuged, supernatants were removed, and cells were resuspended with 3% freshly prepared glutaraldehyde in White’s saline. Samples resuspended in the aldehyde fixative were maintained at 4°C for 30 minutes, sedimented to pellets, washed in PBS, and then combined with 2% osmic acid with 1.5% potassium ferrocyanide for 2 hours at 4°C. All samples were dehydrated in a graded series of alcohol and embedded in Epon 812. Thin sections cut from the plastic blocks on an ultramicrotome were examined unstained or after staining with uranyl acetate and lead citrate for enhanced contrast. Examination was performed in a Philips (Mahwah, NJ) 301 electron microscope, as described. In addition, cells were harvested 4 hours after exposure to topotecan, and DNA was prepared for analysis of fragmentation, as described. DNA was then electrophoresed through a 1% agarose gel, and the DNA bands were visualized by UV light after staining with ethidium bromide (0.5 μg/mL). Hae III-digested φX174 replicative form DNA mixed 1:1 with HindIII-digested 1 DNA was used for sizing the DNA fragments on the ethidium bromide-stained agarose gels, as previously reported.

**Western blot analysis of bcl-2.** The expression of bcl-2 protein in human leukemia cells was studied by immunoblotting with a monoclonal mouse IgG1κ anti-bcl-2 antibody (anti-human bcl-2 oncoprotein; DAKO-bcl-2, 124, Lot 063; DAKO A/S, Carpenteria, CA), as described previously. This antibody was generated against a synthetic peptide sequence comprising amino acids 41 to 54 of bcl-2 protein and was used at a concentration of 0.5 μg/mL, according to the manufacturer’s instructions. In brief, whole cell lysates were prepared by pelleting 1.0 mL of cell suspension for 8 seconds in a microcentrifuge, aspirating the supernatant, and adding 150 μL of sodium dodecyl sulfate (SDS) lysis buffer (50 mmol/L Tris-HCl, 2% SDS, 10% glycerol) containing 25 mmol/L dithiothreitol, as previously reported. Each sample was boiled for 5 minutes. The DNA was sheared by several passages through a 28-gauge needle, and equivalent amounts of protein in 40 μL SDS reducing sample buffer (50 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 25 mmol/L dithiothreitol) were loaded onto 10.5% polyacrylamide gels and electrophoresed overnight at 4 mA. The proteins were transferred to a 0.45-μm Immobilon-polyvinylidene difluoride (PVDF) membrane (Millipore Corp, Bedford, MA) for 75 minutes at 130 mA using a semidy transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA), and immunoblotting was performed on the PVDF blots with a 1:500 dilution (0.5 μg/mL) of anti-human bcl-2 antibody (260 μg/mL).

![Fig 4](image-url) (A and B) Ultrastructural features of topotecan-treated ALL cells undergoing apoptosis. RS4;11 cells were examined after treatment with 5 μmol/L topotecan for 8 hours by transmission electron microscopy using a Philips 301 electron microscope, as described in Materials and Methods. Two randomly chosen groups of cells are shown in (A) and (B). See text for discussion of the depicted ultrastructural changes.
using our previously described procedures. Prestained molecular weight markers (Amersham, Arlington Heights, IL) were included on each gel as standards.

Clonogenic assay. After treatment with topotecan, cells were washed twice and analyzed for in vitro clonogenic growth by a highly sensitive serial dilution clonogenic assay, as previously described.

Topotecan administration in pharmacodynamic studies and analysis of plasma topotecan levels. Analytical grade topotecan (SmithKline-Beecham, King of Prussia, PA) was reconstituted with 0.9% saline to give a concentration of 4 mg/mL. Alzet 3-day micro-osmotic pumps (Model 1003D, Alza Corp), designed to deliver approximately 1 μL/hr, were filled with 100 μL of this solution (or 400 μg of topotecan). During the 72-hour infusion, 4 μg/hr or a total dose of approximately 290 μg of topotecan was delivered (15.3 mg/m²/day). Pumps were subcutaneously implanted into SCID mice 24 hours after inoculation with 1 × 10⁶ NALM-6-UM1 pre-B ALL cells. Blood samples (150 μL) were collected from mice (four animals per time point) by retroorbital bleeds into heparinized tubes. Sample times included pretopotecan, 29 hours after the start of the infusion, immediately after removal of the infusion pump at 72 hours, and at 1, 1.5, 2, 4, 8, and 24 hours after pump removal. Plasma was collected by immediately centrifuging the blood at 5,000g for 2 minutes, and samples were pooled in 200-μL aliquots. To quantitatively measure the topotecan lactone concentration in these samples, plasma proteins were precipitated by the addition of 200 μL of cold methanol (−30°C). This mixture was vortexed for 10 seconds and centrifuged for 2 minutes at 12,000g in a rapid acceleration/deceleration centrifuge, and the supernatant was decanted into a plastic screw-top tube. A separate 500 μL of the plasma methanol extract was acidified with 20 μL of 20% phosphoric acid for analysis of total topotecan (lactone and hydroxy-acid). Plasma concentrations of topotecan were measured by a reverse-phase isocratic high performance liquid chromatography (HPLC) method using fluorescence detection. Calibration curves were constructed using spiked murine plasma over a range of 0.25 to 10 ng/mL. The lower limit of sensitivity for the assay was 0.25 ng/mL.

Pharmacokinetic analyses. A one compartment model was fit independently to the topotecan plasma concentration-time data by use of maximum likelihood estimation. Estimated parameters included the volume of the central compartment (Vₐ) and elimination rate constant from the central compartment (Kₑ). Other pharmacokinetic parameters calculated included systemic clearance (Vₐ × Kₑ) and half life (0.693/Kₑ). Area under the plasma concentration versus time curve for topotecan (AUC) was calculated by the logarithmic trapezoidal method up to the last measured time point.

RESULTS

Radiation-resistant B-lineage ALL cells rapidly undergo apoptosis after exposure to topotecan. Several cytotoxic agents, including camptothecin, have been reported to induce apoptosis in human acute myelocytic leukemia cells by activation of cytoplasmic endonucleases. As topotecan is a semisynthetic analogue of camptothecin, we sought to determine if topotecan can induce apoptosis in radiation-resistant
B-lineage ALL cells. As shown in Fig 1, RS4;11 cells express higher levels of bcl-2 protein than fresh leukemic cells from two children with newly diagnosed B-lineage ALL (TS and ER) and have been previously shown to be radiation-resistant. NALM-6 pre-B ALL cells known to be not as radiation-resistant as RS4;11 cells expressed less bcl-2 (Fig 1). On agarose gels, DNA from untreated control RS4;11 or NALM-6 cells showed no fragmentation, whereas DNA from NALM-6 cells treated with greater than 1 μmol/L topotecan for 4 hours had a ladder-like fragmentation pattern consistent with an endonucleolytic cleavage of DNA into oligonucleosome-length fragments at multiples of 200 bp (Fig 2A). Unlike NALM-6 cells, RS4;11 cells showed no ladder-like DNA fragmentation after topotecan treatment for 4 hours (Fig 2B) or 8 hours (data not shown). A number of studies have indicated that oligonucleosomal DNA degrada-
tation is a late event in apoptotic cells. DNA from topotecan-treated RS4;11 cells showed a smear of DNA at the top of the gel suggestive of high molecular weight DNA degradation, which may be the earliest event in the apoptotic process (Fig 2B). In some cases of apparent apoptosis, the chromatin may not be degraded into nucleosome-sized fragments, suggesting that this is not an essential component of the apoptotic process. Therefore, the data presented in the agarose gel shown in Fig 2B may underestimate the degree of apoptosis induced by topotecan. To more accurately assess the ability of topotecan to induce apoptosis in RS4;11 cells, we used transmission electron microscopy. As shown in Figs 3 and 4, the examination of topotecan-treated RS4;11 cells by transmission electron microscopy confirmed the presence of multiple apoptosis-associated ultrastructural changes involving both the cytosol and the nucleus. The changes in the cytosol included development of translucent cytoplasmic vacuoles, condensation leading to compaction of organelles, disappearance of microvilli with concomitant formation of blunt protuberances from the cell surface (blebbing), and separation of surface protuberances with plasmalemna sealing to produce membrane-bound apoptotic bodies of spherical or ovoidal shape. Besides condensed cytoplasm, a variety of organelles was included in the apoptotic bodies, including mitochondria and nuclear fragments. Examination of the nuclei from topotecan-treated cells showed chromatin condensation, round masses of fine granular material near the center of the nucleus (Fig 3C), fragmentation of the nucleus (Fig 3B), crescents of electron-dense heterochromatin that result from uniform condensation of chromatin around the inner margin of the nuclear envelope (Fig 3C), nuclear collapse (Fig 3D), and formation of membrane-enclosed apoptotic bodies, some of which contain nuclear fragments (Fig 4). Despite these apoptotic changes in the cytosol and nucleus, the surface membrane and mitochondria remained intact (Figs 3 and 4). Thus, topotecan can cause apoptosis in B-lineage ALL cells, including radiation-resistant (t;4;11) ALL cells with high-level expression of bcl-2 protein, which has been shown to inhibit most types of apoptotic cell death.

Although the experiments shown in Figs 2 and 3 demonstrated that micromolar concentrations of topotecan can cause apoptotic cell death in ALL cells, such concentrations cannot be achieved in patients, and it is believed that topoisomerase I inhibitors will be most effective if used at low doses for extended periods of time rather than at high doses given over a short time. Therefore, it was important to examine the ability of topotecan to induce apoptosis when used at lower concentrations and longer exposure times. As shown in Fig 5, longer treatments with topotecan were more effective, and even 50 nmol/L topotecan caused apoptosis of RS4;11 cells after 2 days. Trypan blue exclusion assays further confirmed that no loss in membrane integrity occurred until 2 days after continuous exposure to topotecan (Fig 5), which is consistent with the published features of the apoptotic death process.

Apoptosis of ALL cells after a 24-hour exposure to nanomolar concentrations of topotecan was also evident from distinctive morphologic changes visualized by light microscopy, including shrinkage of the cell, nuclear chromatin condensation, segmentation of the nucleus, and plasma membrane blebs in greater than 90% of cells (Fig 6). Topotecan-induced apoptosis was not triggered by a decrease of their antiapoptotic bcl-2 oncoprotein levels after drug exposure (Fig 7).

**In vitro antileukemic activity of topotecan against clonogenic ALL cells.** To further assess the activity of topotecan against radiation-resistant B-lineage ALL cells, we next performed clonogenic assays. As shown in Fig 8, topotecan killed clonogenic RS4;11 and NALM-6 cells in a dose- and time-dependent fashion. Notably, topotecan was more effective against RS4;11 cells than against NALM-6 cells, although RS4;11 cells express much higher levels of bcl-2 protein.

**In vivo pharmacokinetic features and anti-ALL activity of topotecan.** We previously reported that RS4;11 [t(4;11) ALL], NALM-6-UM1 [pre-B ALL], and LC1;19 [t(1;19) ALL] B-lineage ALL cells cause disseminated and fatal leukemia in SCID mice. These three SCID mouse models of human B-lineage ALL were used to evaluate the antileukemic efficacy of topotecan administered subcutaneously over 72 hours. The Kaplan-Meier estimate (±SE) of the probability of event-free survival after inoculation with 5 × 10⁶ RS4;11 cells was 52% ± 10% at 2 months, and all 23 of the PBS-treated control SCID mice died within 108 days, with a median event-free survival of 64.5 days (Fig 9A).
Histopathologically, the involvement of bone marrows, spleens, and ovaries of SCID mice was manifested as replacement of normal tissue elements by diffuse sheets of densely packed leukemic cells. Leukemic infiltrates were detected in multiple organs, as previously reported.\(^5\)\(^-\)\(^10\) Multiparameter flow cytometric analyses of bone marrow, liver, spleen, and brain confirmed the presence of human DNA (data not shown). Importantly, treatment with topotecan significantly improved event-free survival of SCID mice challenged with RS4;11 cells. Significantly higher cumulative proportions of topotecan-treated SCID mice remained alive without evidence of leukemia as compared with PBS-treated SCID mice (\(P < .000001\) for 200 \(\mu\)g topotecan as well as 400 \(\mu\)g topotecan). Furthermore, time to event (paraplegia or death) in topotecan-treated groups was significantly delayed as compared with the PBS-treated group (Fig 9A). The Kaplan-Meier estimates (±SE) of the probability of event-free survival at 175 days after inoculation of RS4;11 cells were 40% ± 22% for SCID mice treated with a total of 200 \(\mu\)g topotecan and 50% ± 25% for SCID mice treated with a total of 400 \(\mu\)g topotecan (Fig 9A). Similarly, a dose-dependent improvement in the event-free survival of SCID mice challenged with \(5 \times 10^6\) LC1;19 leukemia cells was observed (Fig 9B). In accord with the in vitro clonogenic assay data, topotecan was most effective against RS4;11 cells with the highest level of bcl-2 protein expression. Figure 9C shows the collective data from all three SCID mouse models. The Kaplan-Meier estimates (±SE) of the probability of event-free survival at 175 days after inoculation with leukemia cells (RS4;11, NALM-6, and LC1;19 cells) were 11% ± 6% for SCID mice treated with a total of 200 \(\mu\)g topotecan (median survival, 45 days) and 57% ± 17% for SCID mice treated with a total of 400 \(\mu\)g topotecan (median survival, greater than 175 days). By comparison, all of the 135 PBS-treated control mice died at a median of 40 days (\(P < .0001\)). As 1,000 leukemia cells caused disseminated and fatal leukemia in these three SCID mouse models within 175 days,\(^6\)\(^-\)\(^10\) the long-term event-free survival of topotecan-treated SCID mice challenged with \(1 \times 10^6\) to \(5 \times 10^6\) cells is consistent with \(\approx 3\) log kill of leukemia cells in vivo by topotecan therapy.

We next examined the pharmacokinetic features of topotecan in SCID mice. A one-compartment model fit the plasma concentration versus time data for topotecan. The apparent subcutaneous clearance for topotecan was 227.8 L/h/m\(^2\), steady-state plasma concentration was 2.6 \(\mu\)g/L, apparent volume of the central compartment was 493.2 L/m\(^2\), (AUC\text{\small{WT8}}) was 137 \(\mu\)g \(\times\) h/L, and \(t\) was 1.5 hours. The median ratio of topotecan lactone to total topotecan (lactone + hydroxy acid) for all samples was 0.38 (range, 0.29 to 0.62). Figure 10 shows the topotecan concentration versus time plot for the SCID mice. For comparison with mouse data, a concentration versus time plot for four children with leukemia receiving topotecan is also presented. The results show that the plasma concentrations of topotecan producing responses in SCID mice are readily achievable in pediatric patients. The subcutaneous dosage administered to the SCID mouse (15.8 mg/m\(^2\)/d) was greater than the intravenous dosage administered to patients (1.75 mg/m\(^2\)/d), which may reflect incomplete subcutaneous bioavailability in the SCID mice.

**DISCUSSION**

ALL is the most common childhood malignancy.\(^26\)\(^-\)\(^30\) In B-lineage ALL, the most prevalent form of ALL, dramatic
Fig 9. Antileukemic activity of topotecan against human ALL cells in SCID mice. SCID mice were inoculated with RS4;11, LC1;19, or NALM-6 leukemia cells, treated with topotecan, and monitored for event-free survival, as described in Materials and Methods. Topotecan group 1 received 200 μg of topotecan as a 72-hour subcutaneous (sc) continuous infusion; topotecan group 2 received 400 μg of topotecan as a 72-hour sc continuous infusion. Controls were treated with PBS instead of topotecan. Results are shown as the cumulative proportion of mice alive and event-free as a function of time after inoculation with leukemia cells. (A) Activity of topotecan against RS4;11 cells in SCID mice. (B) Activity of topotecan against LC1;19 cells in SCID mice. (C) Combined results on the antileukemic activity of topotecan in all three SCID mouse models of human ALL. The indicated numbers of PBS-treated mice are greater than the cumulative number of topotecan-treated mice, as this larger PBS control group was used for examination of the antileukemic efficacy of two other agents (ie, VP-16 and B43-PAP) as well, which are not herein reported.
improvements in multiagent chemotherapy have resulted in cure rates of 70% to 75%. Currently, the major challenges in the treatment of B-lineage ALL are to design more effective front-line therapy for high-risk patients and to cure patients who have relapsed despite intensive multiagent chemotherapy. For B-lineage ALL patients who have relapsed while on therapy or shortly after elective cessation of therapy, the overall survival is very poor. The preferred treatment for these children has generally been intensive chemotherapy to achieve a second remission and subsequent use of ablative radiochemotherapy followed by bone marrow transplantation (BMT).

Although improvements in long-term disease-free survival have been reported, recurrence of leukemia persists as the major dilemma that diminishes the success of BMT for the treatment of high-risk or relapsed B-lineage ALL, with only 10% to 15% of patients becoming long-term disease-free survivors. Recent preclinical studies demonstrated that (1) in 43% of newly diagnosed ALL cases, clonogenic leukemia cells display a marked radiation resistance, and (2) in 70% of newly diagnosed cases, clonogenic leukemia cells are able to repair sublethal radiation damage. Furthermore, certain subsets have been identified among B-lineage ALL patients—such as the immunophenotypically distinct CD34-CD24+ subset, which includes the vast majority of the t(4;11) ALL patients—where ionizing radiation does not trigger apoptotic death of leukemic blasts. Notably, intravenous radiation resistance at the level of clonogenic leukemia cells (also referred to as leukemic progenitor cells) is associated with a high likelihood of relapse after total body irradiation and BMT. Collectively, these preclinical and clinical observations in B-lineage ALL emphasize the need for therapeutic innovation to eradicate radiation-resistant leukemic clones from high-risk ALL patients undergoing BMT.

The enzyme topoisomerase I induces transient single-strand DNA breaks that lead to relaxation of supercoiled DNA, an essential step for DNA replication and RNA transcription. Topotecan causes cytotoxicity during the course of DNA replication by stabilizing the covalent complex between topoisomerase I and DNA, thereby preventing the religation of enzyme-linked single-strand DNA breaks. Topotecan has broad spectrum antitumor activity against murine tumors. Furthermore, it has demonstrated potent antitumor activity in immunocompromised mouse xenograft models of human rhabdomyosarcoma, osteosarcoma, and colon adenocarcinomas. Moreover, some of the solid tumor patients enrolled in ongoing phase I/II clinical trials showed objective clinical responses. However, the clinical potential of this promising new agent for the treatment of ALL has not been fully explored. In the present study, we evaluated the antileukemic activity of topotecan against human B-lineage ALL cells. Topotecan caused rapid apoptosis of B-lineage ALL cells, inhibited their in vitro clonogenic growth, and exhibited potent antileukemic activity in three different SCID mouse models of human B-lineage ALL. Moreover, the plasma concentration versus time plot producing a response in SCID mice is readily achieved in patients. We have previously shown that topotecan systemic clearance in mice after intraperitoneal administration was 20.0 L/h/m², suggesting that the subcutaneous bioavailability was approximately 10% in the present study, accounting for the relatively high dose of topotecan in SCID mice. Despite relatively low bioavailability, cytotoxic plasma concentrations were obtained, as evidenced by the antileukemic responses noted in SCID mice, and these conditions can be achieved in patients.

Apoptosis, also known as programmed cell death, is a common mode of eukaryotic cell death, with distinct ultrastructural features and a ladder-like DNA fragmentation pattern due to endonuclease-mediated cleavage of DNA into oligonucleosome-length fragments. Ionizing radiation and several chemotherapeutic drugs with diverse molecular targets have been reported to induce apoptotic cell death in human cells. The topoisomerase I inhibitor camptothecin was reported to cause apoptosis in acute myelocytic leukemia cells. Detailed studies by Kaufman demonstrated that camptothecin-triggered apoptosis is not dependent on active protein or RNA synthesis and is likely mediated by the action of an extranuclear endonuclease that gains access to the nucleus. Here, we provide evidence that topotecan, a semisynthetic analogue of camptothecin, can induce apoptosis in human B-lineage ALL cells. Remarkably, topotecan was able to induce apoptosis even in highly radiation-resistant leukemia cells expressing high levels of bcl-2 protein, which has been shown to inhibit most types of apoptotic cell death. Importantly, there was no apparent correlation between bcl-2 protein expression and topotecan resistance. Indeed, RS4;11 cells with the highest bcl-2 levels were found to be the most topotecan-sensitive ALL cells in this study. Topotecan-induced apoptosis was not triggered by decrease of bcl-2 in target ALL cells. The observed dose- and time-dependent cytotoxicity by topotecan against ALL cells is likely due to the mechanism of action of topoisomerase I inhibitors (ie, requirement of replication fork arrest and inhib...
bition of DNA synthesis). Our results indicate that maximum
antileukemic activity of topotecan may be achieved with
drug infusions over an extended period of time.

This report extends previous work on topotecan and
amplifies our knowledge of its activity spectrum. Our results
suggest that topotecan may be a useful component in pre-
BMT conditioning regimens for high risk ALL and encour-
age a phase I evaluation of the safety and activity of topo-
tecan in relapsed ALL patients. Alternatively, topotecan
could be used as part of a post-BMT intensification regimen
for eradicating radiation-resistant leukemic cells that escape
pre-BMT conditioning regimens.

REFERENCES

1. Hsiang YH, Liu LF: Identification of mammalian DNA topo-

isomerase I as an intracellular target of the anticancer drug campto-


2. Liu LF: DNA topoisomerase poisons as antimtumor agents. Annu

Rev Biochem 58:351, 1989

3. Slichtenmyer WJ, Rough JK, Donehower RC, Kaufmann


synthesis and DNA fragmentation in stimulated splenocytes by the

concerted action of topoisomerase I and II poisons. Biochem Phar-

cmacol 45:331, 1993

5. Andoh T, Ishii K, Suzuki Y, Ikegami Y, Kusunoki Y, Take-
moto Y, Okada K: Characterization of a mammalian mutant with a

camptothecin-resistant DNA topoisomerase I. Proc Natl Acad Sci

USA 84:5565, 1987

6. Potmesil M: Camptothecins: From bench research to hospital


7. Johnson RK, Hertzberg RP, Kingsbury WD, Boehm JC, Cas-

ranfa MJ, Fausette LF, McCabe FL, Holden KG: Preclinical profile

of SK and F 104864, a water-soluble analog of camptothecin. Pro-

ceedings of the Sixth National Cancer Institute-European Organiza-

tion for Research and Treatment of Cancer Symposium on New

Drugs in Cancer Therapy. Amsterdam, The Netherlands, 1991 (abstr

301)

8. Jansen B, Kersey JH, Jaszcz WB, Gunther R, Nguyen D, Chels-

strom LM, Uckun FM: Effective immunochemotherapy of human

(t; 11) leukemia in mice with severe combined immunodeficiency

(SCID) using B43 (anti-CD19)-pokeweed antiviral protein immuno-

toxin plus cyclophosphamide. Leukemia 7:290, 1993


R, Land VI, Manivel C, Crist W: Human (t; 11)(q23;p13) pre-B

acute lymphoblastic leukemia in mice with severe combined immu-

nodeficiency. Blood 81:3052, 1993


irvin J, Tuel-Ahlgren L, Myers DE, Gunther R: In vivo efficacy of

B43 (anti-CD19)-pokeweed antiviral protein immunotoxin against

human pre-B acute lymphoblastic leukemia in mice with severe

combined immunodeficiency. Blood 79:2201, 1992

11. White JG: The morphology of platelet function, in Harker

LA, Zimmerman TS (eds): Methods in Hematology. Series 8: Mea-

surements of Platelet Function. New York, NY, Churchill-Living-

stone, 1983, p 1

12. White JG: Fine structural alterations induced in platelets by


13. Uckun FM, Tuel-Ahlgren L, Song CW, Waddick KG, Myers

DE, Kirihara J, Leidbetter JA, Schieven G: Ionizing radiation stimu-

lates tyrosine specific protein kinases in human B lymphocyte pre-
cursors triggering apoptosis and clonogenic cell death. Proc Natl

Acad Sci USA 89:9005, 1992


vivo treatment with immunotoxins and mafosfamide: A novel immu-

nochemotherapeutic approach for elimination of neoplastic T cells


15. Beijnen JH, Smith BR, Keijer WJ, Van Gijn R, Huinkin WW,

Viasveld LT, Rodenhuis S, Underberg WJM: High-performance liq-

uid chromatographic analysis of the new antitumour drug SK&F

104864-A (NSC 609699) in plasma. J Pharm Biomed Anal 8:789,

1990


medical Simulations Resource. USC, Los Angeles, CA, USC, 1990

17. Kaufman SH: Induction of endonucloeytotic DNA cleavage

in human acute myelogenous leukemia cells by etoposide, campto-

cerin, and other cytotoxic anticancer drugs: A cautionary note. Can-

cer Res 49:5870, 1989

18. Barry MA, Behnke CA, Eastman A: Activation of pro-

grammed cell death (apoptosis) by cisplatin, other anticancer drugs,


19. Uckun FM, Song CW: Lack of CD24 antigen expression in B-

lineage acute lymphoblastic leukemia is associated with intrinsic

radiation resistance of primary clonogenic blasts. Blood 81:1323,

1993

20. Oberhammer F, Wilson JW, Dive C, Morris JD, Hickman JA,

Wakeling A, Walker PR, Sikorska M: Apoptotic death in epithelial

cells: Cleavage of DNA to 300 and 50 kilobase fragments prior to

internucleosomal fragmentation. EMBO J 12:3679, 1993

21. Tomei LD, Shapiro JP, Cope FO: Apoptosis in C3H/10T

mouse embryonic cells: Evidence for internucleosomal DNA modi-

fication in the absence of double strand cleavage. Proc Natl Acad

Sci USA 90:853, 1993

22. Schwarz LM, Osborne BA: Programmed cell death, apop-

tosis, and killer genes. Immunol Today 14:582, 1993


24. Lockshin RA, Zakeri Z: Programmed cell death and apop-

tosis, in Tomei DL, Cope FO (eds): Apoptosis: The Molecular

Basis of Cell Death, Cold Spring Harbor, NY, Cold Spring Harbor

Laboratory Press, 1991, p 47


D, Purchi T, Lassmann H, Schulte-Hermann R: Condensation of the

chromatin at the membrane of an apoptotic nucleus is not associated


the initial stages of DNA fragmentation in apoptosis. BioTechniques

15:1032, 1993

27. Hockenbery D, Nunez G, Millman C, Schreiber RD, Kors-

meyer SJ: Bel-2 is an inner mitochondrial membrane protein that


29. Poplack DG, Reaman G: Acute lymphoblastic leukemia in


30. Bleyer WA, Sather H, Coccia P, Lukens J, Siegel S, Ham-

mond D: The staging of childhood acute lymphoblastic leukemia:

Strategies of the Childrens Cancer Study Group and a three dimen-

sional technique of multivariate analysis. Med Pediatr Oncol 14:271,

1986

31. Rivera GK, Pinkel D, Simone JV, Hancock ML, Crist WM:


1993


Miller D, Reaman G, Sather H, Hammond D: Modified BFM therapy

for children with previously untreated acute lymphoblastic leukemia


In vitro and in vivo activity of topotecan against human B-lineage acute lymphoblastic leukemia cells

FM Uckun, CF Stewart, G Reaman, LM Chelstrom, J Jin, M Chandan-Langlie, KG Waddick, J White and WE Evans

Updated information and services can be found at:
http://www.bloodjournal.org/content/85/10/2817.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml