Cancer Chemother Pharmacol (1994) 34: 249–256

Cancer Chemotherapy and Pharmacology

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Quantification of topoisomerase-DNA complexes in leukemia cells from patients undergoing therapy with a topoisomerase-directed agent

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Received: 22 September 1993 / Accepted: 27 January 1994

Abstract. Several clinically important drugs utilized in cancer chemotherapy inhibit type I (Topotecan) or type II (amsacrine, etoposide) DNA topoisomerases by stabilizing the formation of DNA-topoisomerase complexes (topoisomerase-DNA cross-links). In various cell lines, the magnitude of drug-induced DNA-protein cross-link production correlates with the magnitude of cytotoxicity induced by the drugs. We developed a simple filter-binding assay that can measure drug-induced DNA-protein cross-links in leukemia cells obtained directly from patients because the assays most widely used for assessment of drug-induced DNA-protein cross-links in cells [sodium dodecyl sulfate (SDS)/KCl precipitation and alkaline elution] are not readily applicable for use on patient material. HL-60 human leukemia cells or freshly isolated patients' leukemia cells were incubated with Topotecan, etoposide, or amsacrine; lysed with SDS; and applied to nitrocellulose filters in a low-salt buffer. DNA is retained on the filter only if it is covalently bound to protein. The amount of DNA retained on the filter is quantified by hybridization to the *alu* sequence of DNA, which is distributed ubiquitously in the human genome. Using radiolabeled cells, we compared the filter-binding assay directly with the SDS/KCl precipitation assay in the detection of etoposide- or amsacrine-induced DNA-protein cross-links in HL-60 cells and amsacrine-resistant HL-60/AMSA cells. Both the SDS/KCl precipitation assay and the filter-binding assay detected etoposide-induced DNA-protein cross-links in HL-60 and HL-60/AMSA cells and detected a greater frequency of amsacrine-induced DNA-protein cross-links in HL-60 cells than in HL-60/AMSA cells. The filter-binding assay detected DNA-protein cross-links in freshly isolated leukemia cells exposed to Topotecan in vitro. The ratios of DNA retention for Topotecan-treated versus untreated cells from leukemia patients ranged from 1.8 to 11.5. The heterogeneity of this detected cross-linking was as might be expected if the assay were predictive of the antileukemic action of Topotecan, which is variable. This new filter-binding technique may be useful for predicting the sensitivity of individual patients' tumors to drugs that inhibit type I or type II DNA topoisomerases.

Key words: Topoisomerase inhibitors – Drug resistance – Leukemia

Introduction

Before initiating a course of chemotherapy in a cancer patient, it would be helpful to know whether the malignant cells are sensitive or resistant to the drugs used to treat the disease. Such knowledge would maximize the chance of attaining a favorable antitumor response while sparing those patients with unresponsive disease the toxicity of the drugs.

To develop and assay useful for determining the drug sensitivity of cancer cells from individual patients, three criteria must be met. First, it is vital to know the cellular target of the drugs utilized for treatment. Second, it is necessary to quantify the drug-target interaction in clinical material. Finally, the quantifiable drug-target interaction must be mechanistically related to the tumoricidal action of that drug.

Several clinically important chemotherapeutic agents inhibit DNA topoisomerases, enzymes that alter DNA three-dimensional structure and are important components of several DNA-dependent cellular functions [23, 26]. Topoisomerase I cleaves a single strand of DNA to permit relaxation of the DNA molecule [23, 26]. Topoisomerase II

This work was supported by a grant from the Physicians Referral Service of The University of Texas M. D. Anderson Cancer Center (to L.A.Z.) and grant CA 32839 from the National Cancer Institute (to W.P.)

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cleaves double-stranded DNA to allow passage of another DNA duplex, a requirement for postsynthetic chromosomal segregation [23, 26, 28].

Topotecan (9-dimethylaminomethyl-10-hydroxycamptothecin), which is currently undergoing clinical trials in both leukemia and solid tumors, targets the type I enzyme [20, 35, 47], and etoposide, amsacrine, and mitoxantrone target the type II enzyme [4, 14, 43, 48]. Inhibitors of either topoisomerase prevent religation of the DNA [27, 40]. This inhibitory effect can be quantified as stabilization of topoisomerase-DNA complexes in which the enzymes remain bound to DNA. Denaturation of this complex leads to the production of covalent DNA-protein cross-links. In several cell lines, the magnitude of topoisomerase-reactive drug-induced DNA-protein complex production correlates with the magnitude of drug-induced cytotoxicity [1, 2, 22, 31]. Thus, the inhibition of topoisomerases by clinically important drugs and the correlation of this inhibition with the drugs' tumoricidal actions fulfill the first and third criteria for individualizing therapy.

The second criterion for individualizing chemotherapy, clinical measurement of the drug-target interaction of topoisomerase inhibitors, has been elusive. Topoisomerase-DNA complexes may be detected as DNA-protein cross-links using either the sodium dodecyl sulfate (SDS)/KCl assay [34, 45] or the alkaline elution assay [21]. The SDS/KCl assay requires that cellular DNA be radiolabeled prior to drug treatment, and this precludes its use on cells obtained directly from patients. Although the DNA of patients' cells can be radiolabeled in culture, material harvested from patients will not always proliferate in culture. In addition, patients' cells that are cultured for several days or weeks may not retain the characteristics (including drug resistance or sensitivity) that they had in vivo [38, 42]. The DNA in cells utilized in the alkaline elution assay is usually radiolabeled as well, although it is possible to perform this assay on unradiolabeled cells if a fluorescence detector is used to quantify eluted DNA [10]. This method, however, is quite labor-intensive and, thus, not readily applicable for large numbers of samples.

Our goal was to develop a simple assay that would allow quantification of drug-induced DNA-topoisomerase complexes in unradiolabeled cells obtained directly from patients, thus fulfilling the second criterion. As filter elution can be used to isolate drug-induced, topoisomerase-mediated DNA-protein complexes on membranes, a similar approach was entertained in developing a clinically useful assay. Several groups of investigators have published methods exploiting ionic conditions that allow protein-bound DNA to be retained on glass-fiber [5, 37, 44] or nitrocellulose [12] filters while DNA that is not associated with protein passes through. Most of these assays have been applied in isolated biochemical systems using purified components [12, 44]. We have modified these procedures for use on SDS lysates of cells treated with inhibitors of type I or type II DNA topoisomerases. Denatured cell lysates are applied to nitrocellulose filters using a dot-blot apparatus, and DNA bound to protein (such as a topoisomerase) is retained. DNA is fixed to the filter by baking and quantified by probing with the *alu* sequence of DNA. Repeated sequences of the *alu* family make up at least 3% of the human genome and appear to be distributed over a minimal range of 30%–60% of the genome interspersed between single copy sequences [15]. Thus, the *alu* sequence appears to be a good probe for quantitatively detecting filter-bound human DNA, as it is not associated with any specific gene.

In the following experiments, we demonstrate that drug-induced stabilization of DNA-topoisomerase complexes enhances filter retention of DNA from cells treated with topoisomerase inhibitors such as Topotecan, amsacrine, or etoposide. Because the cellular DNA does not need to be radiolabeled, the assay was applied not only to cultured cells but also to leukemia cells obtained directly from patients.

Materials and methods

Cells. HL-60 and HL-60/AMSA cells were initially provided by Drs. M. Beran and B. Andersson of M. D. Anderson Cancer Center [3]. The HL-60/AMSA cells are an amsacrine-resistant subline derived from HL-60. These cells are resistant to amsacrine-induced DNA-protein cross-linking as measured by the SDS/KCl or alkaline elution assays [50]. HL-60 and HL-60/AMSA cells were grown in Iscove's modified Dulbecco's medium (JRH Biochemicals, Lenexa, Kan.) supplemented with 10% fetal bovine serum at 37° C in an atmosphere containing 5% CO_2 and doubled in approximately 18 h. L1210 murine leukemia cells grown in RPMI medium (Gibco, Grand Island, N.Y.) were utilized as internal standard cells for alkaline elution experiments [21]. The cell lines were *Mycoplasma*-free (American Type Culture Collection).

Leukemia cells from patients were separated from whole blood using Ficoll-Paque (5.7% Ficoll, 9% diatrizoate sodium; Pharmacia, Piscataway, N.J.). Samples of 10–20 ml of blood were obtained by venipuncture from a patient before drug treatment. Blood cells were separated from plasma by centrifugation at 250 *g* and resuspended in cold phosphate-buffered saline (PBS). Upon resuspension in 40 ml of PBS, cells were layered over 10 ml of Ficoll-Paque and centrifuged for 20 min at 500 *g*. Buoyant mononuclear cells were harvested and diluted in cold PBS for counting. The filter-binding and Western-blot assays performed on these cells are described in detail below. In all, 5×10^5 or 2.5×10^5 cells were suspended in 200 µl of PBS supplemented with 10% fetal bovine serum and deposited on glass slides using a cytocentrifuge. The cells were stained with Wright's stain and examined microscopically to confirm that malignant cells were being assayed.

Drugs. Topotecan, obtained from SmithKline Beecham, was prepared in deionized water at a concentration of 10 m*M* and stored at –20° C. Amsacrine (NSC249992) was obtained from the National Cancer Institute, and etoposide was a gift from Drs. Byron Long and James H. Keller of Bristol-Myers Squibb Company. Amsacrine (1 m*M*) and etoposide (10 m*M*) were dissolved in 100% dimethylsulfoxide (DMSO) and stored at –20° C. When cells were treated with drugs dissolved in DMSO, the final concentration of DMSO in the medium was always 0.1%.

Filter-binding assay. Cells were resuspended to 5×10^5 cells/ml in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and treated with Topotecan, etoposide, amsacrine, or vehicle for 1 h at 37° C in an atmosphere containing 5% CO_2. After drug treatment, 1×10^5 cultured cells or 1×10^6 leukemia cells from patients (in triplicate, when possible) were added to a microfuge tube and spun at 13,000 *g* for 1 min. The medium was aspirated and cells were immediately lysed with 100 µl of 1.25% SDS, 5 m*M* ethylene glycol tetraacetic acid (EGTA, pH 8; lysis solution) at 65° C. In some experiments, 0.5% SDS was used, as indicated. After vigorous vor-

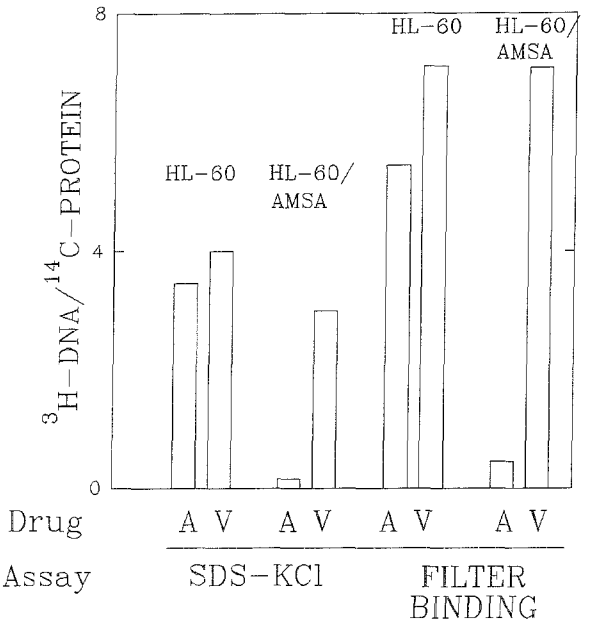

Fig. 1. Comparison of SDS/KCl and filter-binding assays for the assessment of drug-induced DNA-protein cross-links. HL-60 and HL-60/AMSA cells were treated for 1 h with 10 μ*M* amsacrine (*A*) or 100 μ*M* etoposide (*V*) before SDS lysis and quantification of DNA-protein cross-links by the SDS/KCl precipitation or filter-binding assay. Radiolabeled cells ([^{3}H]-thymidine-DNA and [^{14}C]-leucine-protein) were utilized in both techniques for direct comparison of the assays. Nitrocellulose membranes from the filter-binding assay were cut apart and the radioactivity on them was quantified by liquid scintillation spectroscopy. Data from this representative experiment are presented as the ratio of counts per minute of [^{3}H]-DNA/[^{14}C]-protein for drug-treated cells with background (the ratio of [^{3}H]-DNA/[^{14}C]-protein for vehicle-treated cells) subtracted

texing, lysates were incubated for 10 min at 65° C before DNA was sheared by being passed 4–5 times through a 27-gauge needle and a 1-cc syringe that had been coated with lysis solution.

In all, 1 ml of protein-binding buffer [0.4 *M* guanidine HCl, 10 m*M* TRIS (pH 8), 10 m*M* EGTA, 0.01% Sarkosyl, 0.3 *M* NaCl, 10 m*M* $MgCl_2$] heated to 65° C was added to each lysate immediately before its application to a nitrocellulose filter using a dot-blot apparatus (both from Schleicher and Scheull, Keene, N.H.) kept at 65° C to facilitate filtering and to prevent precipitation of detergent. Under these buffer conditions, only protein-bound DNA is retained on the filter. DNA was fixed to the filter by baking for 2 h at 80° C in a vacuum oven.

The amount of DNA fixed to the filter was determined by hybridization [36] to *alu* DNA (derived from the plasmid pB.87HS, provided by Mr. Steven Hewitt and Dr. Grady Saunders, Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center). The *alu* probe was labeled with [^{32}P]-deoxycytidine triphosphate ([^{32}P]-dCTP; Amersham, Arlington Heights, Ill.) using the Amersham multiprime labeling system. The radioactivity associated with the *alu* probe hybridized to DNA on the filter was quantified using a Betascope 603 apparatus (Betagen, Waltham, Mass.). Autoradiography was performed on each blot to obtain a permanent image.

In some early studies, the DNA and protein of HL-60 and HL-60/AMSA cells were radiolabeled as for the SDS/KCl assay (described below) but were lysed and applied to nitrocellulose (as described above) following drug treatment. Instead of probing these filters with the *alu* sequence, we quantified the retention of protein-associated DNA by liquid scintillation spectroscopy. This enabled us to compare the filter-binding assay directly with the SDS/KCl assay.

SDS/KCl assay. The SDS/KCl precipitation assay of DNA-protein complexes was carried out as described by Trask et al. [45] and as modified by Rowe et al. [34]. HL-60 and HL-60/AMSA cells were labeled with 0.6 μCi of [methyl-^{3}H]-thymidine/ml and 0.2 μCi of [^{14}C]-leucine/ml (both from NEN-Dupont, Boston, Mass.) for 24 h prior to treatment with various concentrations of etoposide or amsacrine for 1 h at 37° C. Data are expressed as the ratio of [^{3}H]-DNA to [^{14}C]-protein [50]. The protein serves as an internal measure of the exact number of cells used for any given experimental condition. The [^{3}H]-DNA:[^{14}C]-protein ratio for vehicle-treated cells (background) was subtracted from the ratios calculated for drug-treated cells.

Alkaline elution. Drug-induced DNA cleavage was quantified in HL-60 cells after 1 h treatment with various concentrations of amsacrine or etoposide utilizing alkaline elution [21]. HL-60 cells were incubated with 0.05 μCi of [2-^{14}C]-thymidine/ml for 48 h to radiolabel their DNA and were then incubated for 24 h in fresh, nonradioactive medium to allow joining of DNA synthesis intermediates (Okazaki fragments) and dilute the cellular pool of [2-^{14}C]-deoxythymidine triphosphate ([2-^{14}C]-dTTP) before drug treatment. L1210 cells utilized as an internal standard were labeled with 0.1 μCi of [methyl-^{3}H]-thymidine/ml overnight and incubated in isotope-free medium at least 2 h before use. Cells deposited on elution filters were lysed with either 2% SDS (standard in the alkaline elution assay [21]), 1.25% SDS, or 0.5% SDS to determine whether the lower amounts of detergent were sufficient to denature cellular topoisomerases (see Results).

Table 1. Quantification of drug-induced DNA-protein cross-links in HL-60 and HL-60/AMSA cells using the filter-binding assay with *alu* probe detection

Drug (μ*M*)	Signal (counts)		
	Lysis immediately following drug treatment	Lysis following drug removal, 37° C incubation in fresh medium	Lysis following drug removal, 0° C incubation in fresh medium
HL-60:			
0	864	1,050	656
V 100	2,910	1,596	3,222
A 10	4,574	1,497	4,588
HL-60/AMSA:			
0	794	1,429	1,050
V 100	4,945	2,336	3,075
A 10	1,074	817	1,369

All cells were treated for 1 h at 37° C with the indicated concentrations of drugs. After drug treatment, some cells were lysed immediately and others were washed with fresh medium and incubated at 0° C or 37° C for 1 h before SDS lysis. Numbers are total counts on the filter as quantified by counting on the Betascope for 90 min. 0, 0.1% DMSO; V, etoposide; A, amsacrine

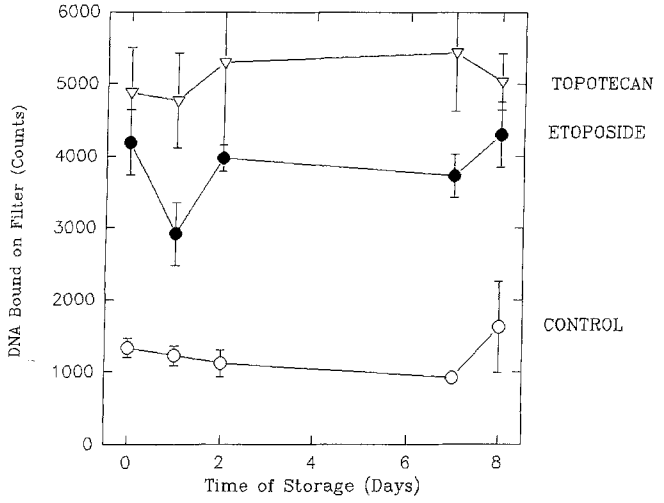

Fig. 2. Storage of lysates from drug-treated HL-60 cells. HL-60 cells were treated with vehicle, 100 μ*M* Topotecan, or 100 μ*M* etoposide for 1 h at 37° C before SDS lysis of 1×10^5 cells in triplicate. Cell lysates were stored at room temperature for the indicated intervals before further processing. Filter-binding buffer (65° C) was added to the lysed samples immediately before application to nitrocellulose filters. Filters were probed with *alu* simultaneously. Values plotted on the graph are the average counts of the triplicate samples ± 1 SD collected in 60 min on the Betascope

Immunoblotting. Circulating mononuclear cells obtained from patients before chemotherapy with Topotecan were isolated from whole blood as described above. The cells were prepared for immunoblotting according to the technique of Kaufmann et al. [17]. Approximately $5-10\times10^7$ cells were solubilized by sonication in 1 ml of alkylation buffer containing 6 *M* guanidine hydrochloride, 250 m*M* TRIS-HCl (pH 8.5), 10 m*M* ethylenediaminetetraacetic acid (EDTA), 1% 2-mercaptoethanol, and 1 m*M* PMSF (phenylmethyl sulfonyl fluoride). Cell solutions were treated with iodoacetamide (27.75 mg/sample) and dialyzed against 4 *M* urea and then against 0.1% SDS before lyophilization. Prior to being loaded onto a 7.5% polyacrylamide gel, lyophilized cells were resuspended in 4 *M* urea, 2% SDS, 62.5 m*M* TRIS-HCl (pH 6.8), and 1 m*M* EDTA. The lysate from 10^7 cells was loaded into each lane of the gel.

Gels were run in TRIS-glycine buffer at 65 V for 17–18 h, until the 46-kDa molecular-weight marker (ovalbumin) had reached the bottom of the gel. The proteins separated on the gel were electrophoretically transferred to nitrocellulose. The nitrocellulose filters were blocked with 3% bovine serum albumin and incubated with topoisomerase I antibody (generously provided by Dr. Leroy Liu, Johns Hopkins University) at a 1:500 dilution. The antibody complex was detected with ^{125}I-labeled protein A (Amersham, Arlington Heights, Ill.). Autoradiographs of the blots were densitometrically scanned (GS300 densitometer, Hoeffer Scientific, San Francisco, Calif.) to determine the amounts of immunoreactive topoisomerase I present in the cells.

Results

Validation of the filter-binding assay

The SDS/KCl and filter-binding techniques were performed in tandem on HL-60 and HL-60/AMSA cells to compare the capacities of the assays in detecting DNA-protein cross-links stabilized by the topoisomerase II inhibitors amsacrine or etoposide (Fig. 1). These studies utilized radiolabeled cells to facilitate direct comparison of the assays. Each assay demonstrated that the frequency of etoposide-induced DNA-protein cross-linking seen in the two cell lines was similar, whereas the frequency of amsacrine-induced DNA-protein cross-linking detected in HL-60 cells was greater than that observed in the amsacrine-resistant HL-60/AMSA cells (Fig. 1).

Other experiments validating the filter-binding assay were performed on nonradiolabeled HL-60 and HL-60/AMSA cells treated with etoposide or amsacrine. DNA retained on the filter (and, thus, cross-linked to protein) was detected by hybridization to the *alu* probe (Table 1). To determine whether drug-induced DNA-protein cross-links would reverse in a temperature-dependent manner (a characteristic of topoisomerase II-reactive drug-induced DNA-protein cross-links [10, 33, 34]), cells were washed free of drug, placed in fresh medium, and incubated for 1 h at 37° C or 0° C before lysis. Filter retention of DNA was enhanced in HL-60 cells after treatment with etoposide or amsacrine (Table 1). In contrast, although treatment of HL-60/AMSA cells with 100 μ*M* etoposide increased filter retention of DNA, no enhancement was observed after treatment with 10 μ*M* amsacrine. This result was predicted from our previous work [50] with HL-60 and HL-60/AMSA cells as well as from Fig. 1. When cells were washed free of drug and incubated for 1 h at 37° C in fresh medium before lysis, the amount of DNA retained on the filter was less than that observed when cells were lysed immediately after exposure to drug. In contrast, when cells were incubated at 0° C after drug removal, filter retention of DNA was not appreciably less than that noted for cells lysed directly following drug treatment (Table 1). This, too, was as expected [24, 48, 49].

Incubation of HL-60 cells with 100 μ*M* Topotecan, an inhibitor of topoisomerase I, also increased filter retention of DNA as detected with the *alu* probe. The DNA-protein complexes stabilized by either etoposide or Topotecan and rendered covalent by SDS appeared to be stable for an extended period. Cell lysates could be stored for at least 8 days before their application to the nitrocellulose filter without a noticeable loss of signal (Fig. 2).

When drugs were added *after* lysis of cells with SDS, filter retention of DNA was not enhanced, indicating that a functional (i.e., nondenatured) topoisomerase was necessary for DNA-protein cross-link formation. In the absence of drug, 840 ± 12 counts (average of triplicate determinations ± 1 SD) were detected by the Betascope after the filter had been probed with *alu*. When 100 μ*M* etoposide or Topotecan was added after lysis of untreated cells, the average count detected on the filter was 939 ± 263 or 644 ± 75, respectively. Consequently, drug-induced filter retention of DNA was not due to drug-DNA interactions that did not involve topoisomerase I or II or to drug-induced adherence of DNA to the filter.

While developing this assay, we noticed that a precipitate would sometimes coat the filters after samples had been applied. We feared that this precipitate could contain protein-bound DNA that was not bound to the filter. We could eliminate the precipitate by reducing the amount of SDS in the lysis buffer from 1.25% (used in the SDS/KCl assay) to 0.5% and by warming the dot-blot apparatus in a 65° C water bath. To demonstrate that this concentration of

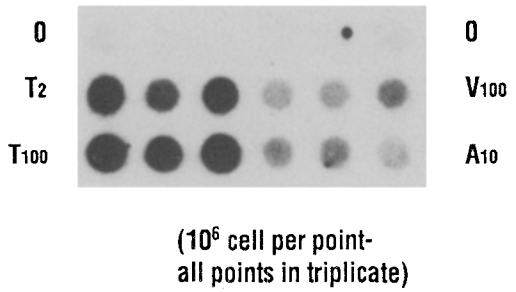

Fig. 3. Application of the filter-binding assay using the *alu* probe detection system to quantify drug-induced, topoisomerase-mediated DNA-protein cross-linking in cells from a patient with CLL. Cells were separated from whole blood obtained prior to chemotherapy and then treated in culture for 1 h with the indicated drugs at 37° C. Cell samples were lysed with SDS and applied to nitrocellulose and blotted in triplicate (10^6 cells/point). See Table 2 for quantification of this blot. *T*, Topotecan; *V*, etoposide; *A*, amsacrine. Subscripts are drug concentrations in μ*M*

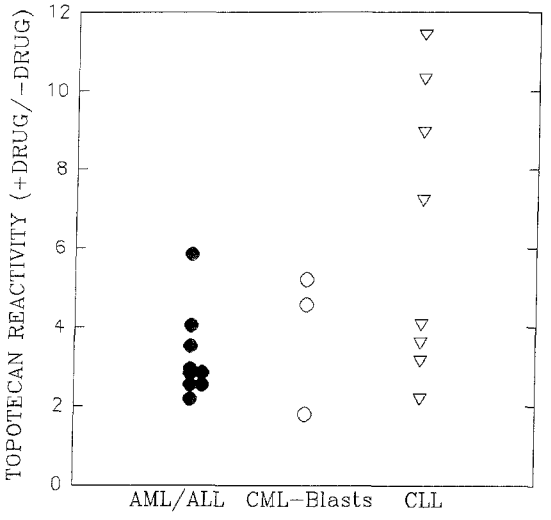

Fig. 4. Reactivity of fresh leukemia cells to Topotecan. Leukemia cells isolated from patients were treated for 1 h in culture with 100 μ*M* Topotecan or vehicle, and DNA-protein cross-links were quantified with the *alu* probe detection system. Points on the graph are the ratios of counts from the Betascope for drug-treated cells divided by counts from vehicle-treated cells. Some of the data in this figure have been presented previously [6]. Cytocentrifuge preparations of isolated cells were available for 18 of 20 patients, and microscopic analysis of these slides demonstrated that 80%–95% of each cell population was malignant. *AML*, Acute myelogenous leukemia; *ALL*, acute lymphocytic leukemia; *CML-Blasts*, chronic myelogenous leukemia in blast crisis; *CLL*, chronic lymphocytic leukemia

SDS is sufficient to denature topoisomerases and, thus, to stabilize DNA-topoisomerase complexes, we performed alkaline elution on HL-60 cells exposed to various concentrations of etoposide and amsacrine. After drug treatment, cells were lysed with 0.5%, 1.25%, or 2% SDS, the latter being the standard concentration used in the alkaline elution assay [21]. No difference was seen in the production of amsacrine- or etoposide-induced single-strand breaks (which correspond to DNA-protein cross-links in the case of topoisomerase-reactive drugs [6, 30] among the concentrations of SDS used to lyse the cells (data not shown).

Table 2. Quantification of a filter-binding blot of cells from a patient with chronic lymphocytic leukemia

Drug (μ*M*)	DNA on filter (counts)
0 (water)	1,549 ± 563
T 2	7,736 ± 2,499*
T 100	11,193 ± 2,814*
0 (DMSO)	2,025 ± 677
V 100	3,562 ± 413*
A 10	3,371 ± 521*

Quantification of the blot shown in Fig. 3. Cells were obtained prior to the initiation of chemotherapy and then treated in culture for 1 h with Topotecan (T), etoposide (V), or amsacrine (A). Samples were applied to the dot blotter in triplicate. Values are average counts ± 1 SD accumulated in 60 min of Betascope exposure
*P < 0.05 vs water or DMSO control (unpaired, two-tailed t-test)

Except where indicated, the studies presented in this paper used 1.25% SDS in the lysis buffer.

To evaluate the retention of DNA on the filter during hybridization, we applied 0.5% or 1.25% SDS lysates of radiolabeled ([^{14}C]-leucine-protein, [^{3}H]-thymidine-DNA) drug-treated cells to duplicate nitrocellulose filters and fixed DNA to the filters by baking. One filter was assayed immediately, whereas the other was washed in the prehybridization solution used for *alu* probing before scintillation counting. The amounts of DNA and protein on the filters were assessed by liquid scintillation spectroscopy. In all, 92.6% ± 19.7% of the DNA and 28.4% ± 4.6% of the protein remained on the filter after the wash when 0.5% SDS lysates were used. When 1.25% lysates were used, 73.3% ± 10.6% of the DNA and 19.4% ± 6.0% of the protein remained on the filter after the wash. This demonstrated that protein-bound DNA fixed to the nitrocellulose filter was not washed off during the hybridization process, although the majority of the protein (which may have consisted of that not covalently bound to DNA) did wash off the filter. The experiment also showed that filter retention of protein-bound DNA was not different when buffer containing either 0.5% or 1.25% SDS was used to lyse the cells. However, we recommend using the 0.5% SDS buffer to facilitate filtration.

Application of the filter-binding assay to leukemia cells from patients

The filter-binding assay was applied not only to cultured cells but also to leukemia cells obtained directly from patients. The representative blot presented in Fig. 3 shows the results obtained using cells from a patient with chronic lymphocytic leukemia (CLL) who had been treated with Topotecan. Blood was drawn from the patient before the initiation of chemotherapy with Topotecan.

Leukemia cells harvested from the patient's blood were treated with Topotecan, etoposide, or amsacrine for 1 h in culture before their lysis and application to nitrocellulose. Table 2 shows the quantification of the blot shown in Fig. 3. In vitro incubation of the patient's cells with 100 μ*M* Topotecan increased the filter retention of DNA by a factor of about 7. In vitro incubation of the leukemia cells with the

Table 3. Comparison between Topotecan reactivity and amount of immunoreactive topoisomerase I in cells obtained from leukemia patients

Patient number	Diagnosis[a]	Count ratio per 10^6 cells[b]	Immunoreactivity per 10^6 cells[c]	Topotecan reactivity index[d]
1	AML	4.0	0.473	0.85
2	CLL	4.0	None detected	–
3	AML	2.8	0.336	0.83
4	CML-blast	4.6	0.442	1.04
5	AML	2.2	0.216	1.02

Mononuclear cells were separated from whole blood drawn from leukemia patients, treated for 1 h with vehicle or 100 μ*M* Topotecan, and lysed with SDS. Lysates were filtered onto nitrocellulose and DNA was quantified by probing with *alu* (as in Fig. 3 and Table 2). Other mononuclear cells from the same patients were treated with alkylation buffer as described above and immunoblotting was performed

[a] AML, Acute myelogenous leukemia; CLL, chronic lymphocytic leukemia; CML-blast, chronic myelogenous leukemia in blast crisis

[b] Ratio of counts accumulated by the Betascope for cells treated with 100 μ*M* Topotecan divided by counts from cells treated with vehicle

[c] Immunoreactivity was measured by densitometrically scanning the autoradiographs of immunoblots, quantifying the area of the curve of the densitometric reading, and normalizing the data for 10^6 cells. To compare patient samples run on two separate immunoblots, densitometric readings for patient cells were divided by the reading for HL-60 cells that had been run on the same immunoblot. The sizes of the bands corresponding to topoisomerase I in the two immunoblots were 108 and 111 kDa, respectively. Both immunoblots were probed and exposed to film simultaneously

[d] The result of dividing the count ratio by the immunoreactivity was divided by 10

topoisomerase II inhibitors etoposide (100 μ*M*) or amsacrine (10 μ*M*) slightly increased the filter retention of DNA (Fig. 3, Table 2), but not to the same extent as either 2 μ*M* or 100 μ*M* Topotecan. This observation is consistent with a previous finding that the level of immunoreactive topoisomerase II is lower than that of topoisomerase I in CLL cells [32].

The heterogeneity of responses observed among cells from 20 patients with various leukemia diagnoses following exposure of the cells in vitro to 100 μ*M* Topotecan is shown in Fig. 4. The cellular response to Topotecan may be mediated by the amount of drug target (topoisomerase I) in the cells. Immunoblotting for topoisomerase I was used to quantify the amount of enzyme present in leukemia cells from several patients (Table 3). In most cases, the amount of immunoreactive topoisomerase I detected in the untreated patients' cells correlated with the drug reactivity of the enzyme as determined by the filter-binding assay (Table 3). This finding suggests that a major determinant of cellular sensitivity to Topotecan in clinical situations may be the *amount* of the drug's target (topoisomerase I) in the cells rather than the presence of a mutant, drug-resistant enzyme.

Discussion

The results of our studies indicate that the filter-binding technique with the *alu* probe detection system can be used to quantify drug-induced topoisomerase I- or II-mediated DNA-protein cross-links in both cultured cells and leukemia cells obtained directly from patients. This newly adapted method for detecting DNA-protein cross-links compared favorably with the standard SDS/KCl precipitation assay. The ability of the filter-binding assay to detect the difference in amsacrine sensitivity between HL-60 and HL-60/AMSA cells strongly suggests that the technique quantifies topoisomerase-mediated events. Further evidence supporting this claim was provided in that the filter-binding assay detected reversal of drug-induced topoisomerase II-mediated DNA-protein cross-links at 37° C but not at 0° C, which is indicative of cellular topoisomerase II activity [48, 49].

The filter-binding technique was successfully applied to patients' leukemia cells treated with drugs in vitro immediately following their separation from whole blood. The amounts of DNA retained on the filter after cells had been treated with 2 and 100 μ*M* Topotecan were 5.0 and 7.2 times higher than the background level, respectively. When leukemia cells freshly obtained from 20 patients were treated with Topotecan in vitro, the responses varied. Several factors may mediate cell sensitivity or resistance to inhibitors of topoisomerase I or II. One is the intracellular level of enzyme. Low levels of topoisomerase I or II have been associated with resistance to drugs that inhibit the enzyme [33, 39, 41] because the amount of complexes formed will be diminished and the magnitude of complex formation has been shown to correlate with the magnitude of drug-induced cytotoxicity [1, 2, 22, 25, 31, 46]. Immunoblotting for topoisomerase I indicated that the level of this enzyme correlated with drug reactivity as quantified by the filter-binding technique in cells from several of the patients studied (Table 3).

Another factor thought to produce drug resistance is the presence of a mutant topoisomerase enzyme not inhibited by drugs, such as the topoisomerase II occurring in HL-60/AMSA cells [13]. Our laboratory has looked for the mutation found in the topoisomerase II gene of HL-60/AMSA cells in DNA from 34 leukemia patients sensitive and resistant to amsacrine. However, this mutation was not found [18]. These data, combined with the correlation found between Topotecan reactivity and levels of immunoreactive topoisomerase I (Table 3) as well as the paucity of clinical observations of mutant topoisomerase [7], suggest that enzyme levels may be the more clinically relevant of these two resistance factors. The relatively large number of drug treatments and high drug concentrations required to produce resistant cell lines containing mutant topoisomerases are not often reproduced in the clinic; thus, the rarity of the event is to be expected.

Another possible means of Topotecan resistance in patients' cells containing an ample level of topoisomerase I is

the presence of the multidrug resistance (*mdr*) phenotype. The *mdr* gene encodes a 170-kDa glycoprotein that resides in the cell membrane and has the capacity to pump xenobiotics actively out of the cell, thus preventing a cytotoxic intracellular concentration from being achieved [8, 9]. Several antagonists of topoisomerase II have been shown to be substrates for the transport protein including Adriamycin, daunorubicin, and, to a lesser extent, etoposide [19, 29]. The topoisomerase I antagonist Topotecan also appears to be a substrate for this pump [11]. High levels of immunoreactive topoisomerase I and low degrees of drug reactivity would also be compatible with this form of drug resistance. The presence of the *mdr* phenotype was not specifically examined in this work; however, the correlation between topoisomerase I levels and Topotecan reactivity would not be expected if *mdr* expression was preventing Topotecan drug molecules from reaching the intracellular target. Although the filter-binding assay cannot distinguish between mechanisms of drug resistance, it would be capable of recognizing drug resistance in cells manifesting any of the phenotypes described above.

The next phase of our studies utilizing the filter-binding technique will be to determine prospectively whether the amount of DNA-protein cross-linking induced in vitro by drug treatment of freshly isolated patiens' cells is predictive of the antineoplastic effect of that drug in individual patients. Thus far, we have applied this technique to leukemia cells isolated from whole blood, but we intend to work toward applying it to specimens of bone marrow from leukemia patients who may not have circulating blast cells and to cells obtained from solid tumors either in readily accessible compartments such as ascites fluid and pulmonary effusions or, eventually, from fine-needle aspirations.

If the filter-binding technique can be used to predict the efficacy of drugs that inhibit topoisomerase I or II, it would provide valuable data for determining a course of chemotherapy in patients who are potential candidates for these drugs. In addition to providing a rapid and readily reproducible method of individualizing anticancer therapy, this assay could be used to study intracellular pharmacokinetics and pharmacodynamics in target cells during therapy and could serve as an intermediate end point for quantifying the success of clinical trials of new topoisomerase-directed drugs.

Acknowledgements. The authors wish to thank Drs. Hagop Kantarjian and Susan O'Brien, Department of Hematology, Division of Medicine, University of Texas M. D. Anderson Cancer Center (UT MDACC), for providing the cells from patients under their care utilized in these studies and Dr. Sanford Stass, Chief, Department of Hematopathology, Division of Laboratory Medicine, UT MDACC, for analyzing cytocentrifuge preparations of cells isolated from these patients. We thank Dr. L. E. Robertson (Department of Hematology, Division of Medicine) for his assistance in performing this work. We also wish to thank Mr. Steven Hewitt and Dr. Grady Saunders, Department of Biochemistry and Molecular Biology, UT MDACC, for the generous gift of the plasmid pB.87HS; Dr. Leroy Liu, Johns Hopkins University, for the topoisomerase I antibody; Ms. Laurie Soares and Ms. Susan Loughlin for their excellent technical assistance; and Ms. Diane Rivera for editing the manuscript.

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