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. Amsacrine-induced DNA-protein cross-links in HL-60 cells were quantitated by both assays, but the filter-binding assay was more sensitive. 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...
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)/KCI assay [34, 45] or the alkaline elution assay [21]. The SDS/KCI 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/KCI 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% CO2 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. Biuyant 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, 5x10⁵ or 2.5x10⁶ 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 mM 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 mM) and etoposide (10 mM) 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 5x10⁶ 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% CO2. After drug treatment, 1x10⁶ cultured cells or 1x10⁶ 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 mM 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-