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High-throughput fluorescence flow-injection topoisomerase II inhibition assay

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Abstract

A high-throughput fluorescence flow-injection assay is described, suitable for determining the catalytic inhibition of DNA topoisomerase II. The method, which separates high molecular mass trypanosome kinetoplast DNA from its decatenated product by centrifugation, should be useful for the rapid and accurate screening of potential anticancer topoisomerase II inhibitors and the determination of their inhibition constants. Advantages of the flow-injection method over agarose gel electrophoresis and radioactive centrifugation assays are that it is faster, more sensitive, highly linear in its response to product formation, and does not require the production of radioactive trypanosome kinetoplast DNA substrate.

Keywords: Flow-injection analysis; Topoisomerase II

1. Introduction

Topoisomerase II (EC 5.99.1.3) is the target for a number of the most important anticancer drugs. Topoisomerase II is an ATP-dependent nuclear and mitochondrial enzyme that alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in the second helix. Thus topoisomerase II relieves torsional stress that occurs during replication and transcription of DNA, and is likely involved in condensation/decondensation, and segregation of chromosomes [1]. A number of anticancer drugs, including the anthracyclines doxorubicin and daunorubicin, the epipodophyllotoxins etoposide and teniposide, mitoxantrone, quinolones, and amsacrine, are thought to be cytotoxic by virtue of their ability to stabilize a covalent topoisomerase II–DNA intermediate (the cleavable complex) [1]. These so-called cleavable complex forming drugs or topoisomerase II poisons [1] also inhibit topoisomerase II catalytic activity. A second class of drugs called the catalytic inhibitors, which include the bisdioxopiperazines [2], mitindomide [3], suramin, fostriecin, merbarone, and aclarubicin, inhibit the catalytic activity of topoisomerase II without inducing cleavable complex formation [1].

Inhibition of topoisomerase II catalytic activity is usually measured in a kinetoplast DNA (kDNA) decatenation assay using either an agarose gel or centrifugation to separate the decatenated kDNA [4]. Kinetoplast DNA (kDNA), a mitochondrial DNA obtained from trypanosomes, is a highly interlocked

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network of double-stranded DNA minicircles (2.5 kb each) that is decatenated into single DNA minicircles and small higher molecular mass catenanes by topoisomerase II [3,4]. In the gel electrophoresis assay the degree of decatenation can be determined after staining the separated minicircles with the DNA-binding dye ethidium bromide. The currently used centrifugation assay requires the use of radiolabeled kDNA isolated from the trypanosomes grown in the presence of tritium-labeled thymidine [2,5].

In this study, we describe a high-throughput method for the determination of topoisomerase II-catalyzed kDNA decatenated products. The decatenated kDNA was separated by centrifugation from the very high molecular mass kDNA substrate and then quantitated by flow-injection fluorimetry. The method should be useful in screening potential topoisomerase II inhibitors and the rapid and accurate determination of enzyme inhibition constants. Advantages of the flow-injection method compared to either the ethidium bromide gel electrophoresis assay [3,4,6] or the centrifugation assay using tritium-labeled kDNA [2,4] are that it requires 10-fold less kDNA substrate, it is much faster, more sensitive, highly linear in its response to product formation, requires smaller quantities of mutagenic DNA intercalating agents (for agarose gel electrophoresis [3,4,7]), and does not require the production of radioactive trypanosome kDNA.

2. Experimental

2.1. Materials

Dexrazoxane (Zinecard, ICRF-187) was a gift from Pharmacia & Upjohn (Columbus, OH, USA). The kDNA and the marker kDNA used in gel electrophoresis was from Topogen (Columbus, OH, USA). 4′,6-Diamidino-2-phenylindole (DAPI), ATP, *N*-lauroylsarcosine, bovine serum albumin (BSA) and ethidium bromide were from Sigma (St Louis, MO, USA) and Ultrapure Agarose was from Gibco (Burlington, Canada). Chinese hamster ovary (CHO) cells (Type AA8) were obtained from the American Type Culture Collection (Rockville, MD, USA).

2.2. Topoisomerase II-containing nuclear extract preparation

Nuclear extracts containing topoisomerase II were prepared by a previously described method [7] from exponentially growing attached CHO cells (10^8 cells) harvested by trypsinization. A modified protease inhibitor content was used in the wash, lysis and salt extraction buffers (protease inhibitors: 2 m*M* phenylmethylsulfonylfluoride, 1 m*M* benzamidine, 2 m*M* dithiothreitol, 1–10 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin). Nuclear extracts were adjusted to 50% (v/v) glycerol and stored at −80°C. Total protein content was determined with the Bradford assay [8]. Dilutions of the topoisomerase II-containing extract were made immediately prior to the decatenation assay in 10 m*M* Tris–HCl, 260 m*M* KCl, 1 m*M* phenylmethylsulfonylfluoride, 2 m*M* dithiothreitol, 50 μg/ml BSA, pH 7.5.

2.3. Preparation of decatenated kDNA standards

Concentrated assay buffer (10×) consisted of 500 m*M* Tris–HCl (pH 8.0), 1200 m*M* KCl, 100 m*M* $MgCl_2$, 5 m*M* ATP, 5 m*M* dithiothreitol, and 300 μg/ml BSA. Reactions were carried out in a total volume of 200 μl containing 20 μl of 10× assay buffer, 2100 ng of catenated kDNA and 20 μl of nuclear extract topoisomerase II. The amount of topoisomerase II was adjusted in preliminary experiments to give complete decatenation of kDNA as determined by agarose gel electrophoresis. The reactions were incubated at 37°C for 1 h and terminated with either 50 μl of Stop Buffer A (5% w/v *N*-laurylsarcosine, 0.125% w/v bromophenol blue, 25% glycerol) [2,5] for gel electrophoresis assays or 50 μl of Stop Buffer B (250 m*M* disodium EDTA, pH 8.0) [6] for flow-injection assays. Standards were prepared by serial dilution of the terminated reactions.

2.4. Topoisomerase II inhibition assay

Dexrazoxane was dissolved in DMSO and added to the reaction mixture so that the final DMSO concentration was 5% (v/v). This amount of DMSO was shown not to affect the assay. Reactions were

carried out in a total volume of 20 μl containing 2 μl of 10× assay buffer, 210 ng of catenated kDNA, 0–500 μ*M* dexrazoxane, and 2 μl of nuclear extract topoisomerase II. The amount of kDNA used in the assay was sufficient for replicate injections with loop flushing. If partial loop or centered loop injection had been used, even smaller amounts of kDNA could have been used in the assay. The nuclear extract topoisomerase II was adjusted in preliminary experiments to decatenate a maximum of approximately 80% of the kDNA under our assay conditions. The reactions were incubated at 37°C for 30 min and terminated with either 5 μl of Stop Buffer A for gel electrophoresis assays or 5 μl of Stop Buffer B for flow-injection assays.

2.5. Fluorescence flow-injection assay

Following reaction termination, the microcentrifuge tubes were centrifuged at 8000 *g* for 15 min at room temperature to separate the reaction products [2,6]. To 20 μl of the supernatant, 140 μl of DAPI solution (in 5 m*M* Tris–HCl, 0.5 m*M* Na_2EDTA, pH 8.0) was added to give a final DAPI concentration of 25 ng/ml, which was sufficient for three separate determinations. This concentration of DAPI was optimal as it was the highest concentration of DAPI that could be used that did not produce significant background fluorescence under our assay conditions. Tubes in which the samples were stored were kept in the dark until analysis. The flow-injection assay was conducted using a Varian (Varian Canada, Mississauga, Canada) 9010 pump, a Rheodyne (Cotati, CA, USA) injector with a 20-μl sample loop, a Shimadzu (Columbia, MD, USA) RF-551 HPLC fluorescence detector, and Varian Star integration software. The excitation wavelength for the DAPI–kDNA complex was 358 nm and the emission wavelength was 461 nm. The flow buffer consisted of 9.5 m*M* Tris–HCl, 14.6 m*M* KCl, 1 m*M* $MgCl_2$, 6.7 m*M* disodium EDTA (pH 8.0). The flow-rate was 1 ml/min. Since there was no chromatographic column in the flow system, samples could be injected as close as 1 min apart. To prepare the HPLC for other uses, the injector was cleaned at the end of a series of runs with 10 m*M* injections of HCl. The entire flow-injection system was also cleaned with 1 m*M* HCl at 1 ml/min for 2 h to elute small amounts of DAPI adsorbed to flow components.

Two other fluorescent DNA intercalating dyes were tried in the assay and found to be unsatisfactory. Ethidium bromide (3 μg/ml, excitation 518 nm, emission 605 nm) produced a high background fluorescence that contributed up to 30% of the DNA signal under our assay conditions. It was also noted that the baseline fluorescence signal increased steadily after each injection, which suggested that ethidium bromide bound to the flow-injection system and then gradually leached out. Hoechst 33242 (5 μg/ml, excitation 352 nm, emission 461 nm) in the absence of added kDNA gave a lower background fluorescence than did ethidium bromide. However, baseline drift and background fluorescence signal increases due to the dye binding to the flow components were still a problem with Hoechst 33242.

2.6. Agarose gel electrophoresis

Following reaction termination, 22-μl samples were loaded on a 1% (w/v) agarose gel (7×15 cm) and run out to about 5 cm at 100 V for about 50 min [3]. The gel and electrophoresis buffers were identical (100 m*M* Tris, 100 m*M* boric acid, 2 m*M* disodium EDTA, 0.2 μg/ml ethidium bromide, pH 8.3). Gels were destained in water for several hours and photographed with Polaroid Type 667 film under UV transillumination. The photographs were scanned with a Hewlett-Packard ScanJet 4B scanner (Hewlett-Packard, Palo Alto, CA, USA) and the 256-grey scale digitized images were analyzed using SigmaGel (Jandel Scientific, San Rafael, CA, USA) [3].

3. Results and discussion

3.1. Comparison of the flow injection and gel electrophoresis assays

As can be seen from Fig. 1A, the fluorescence signals of the decatenated kDNA standards determined by the flow-injection assay method were highly linear in the range of 0.26–42 ng decatenated kDNA. Under typical reaction conditions used in the topoisomerase II inhibition assay (21 ng of kDNA

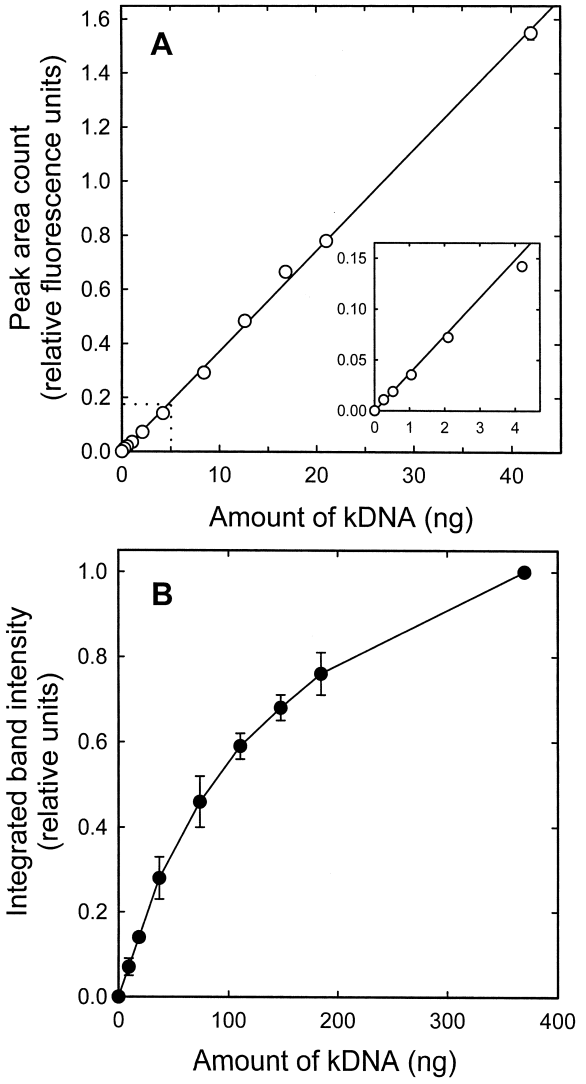

Fig. 1. Comparison of kDNA calibration curves obtained for the totally decatenated products of kDNA using a fluorescence detection flow-injection assay and ethidium bromide gel electrophoresis. (A) Peak area counts of decatenated fluorescent DAPI–kDNA products by flow-injection assay on an HPLC apparatus (○). The results shown were from triplicate determinations. The S.E. values are smaller than the data points. Linear least squares analysis gave a slope of 0.0372 ± 0.0004 ng^{-1} and an intercept of -0.0005 ± 0.0067 with an r^2 of 0.999 ($n=11$). (B) Integrated band intensities of nicked open circular decatenated kDNA by ethidium bromide-stained agarose gel electrophoresis (●). The errors shown are average deviations from two determinations on two different gels that were normalized to a band intensity of 1 at the highest kDNA amount shown.

substrate in the sample loop), as little as 0.26 ng of decatenated kDNA could be accurately quantified, which represented 1.2% decatenation. In contrast, the gel electrophoresis assay yielded a highly non-linear calibration curve (Fig. 1B). In addition, day-to-day variability in the gel electrophoresis calibration curve was high. This was probably due, in part, to differences in background fluorescence of the agarose gels, film exposure, and the analysis of scanned images. Under typical topoisomerase II reaction conditions, which used 185 ng of kDNA in each gel well, 9 ng of decatenated kDNA could be accurately quantitated, which represented 12% decatenation.

3.2. Determination of the optimal topoisomerase II activity in the flow-injection assay

The flow-injection assay was used to determine the amount of nuclear extract topoisomerase II that gave approximately 80% decatenation under typical reaction conditions. At enzyme levels higher than this the measurements of the degree of drug inhibition would be affected. The fluorescence signal as a function of the amount of nuclear extract topoisomerase II protein is shown in Fig. 2. This data also shows that in the absence of added nuclear extract topoisomerase II there is a small background fluorescence signal due to DAPI itself, small amounts of trypanosome genomic DNA, and endogenous decatenated kDNA present in the substrate mixture. The amount of topoisomerase II-catalyzed decatenated products in the supernatant increased linearly with enzyme concentration after an initial lag. This may be due to the complexity of the highly catenated network nature of the substrate. It is likely that at low enzyme concentrations, reaction products consisted mainly of large catenanes which co-sedimented with the catenated kDNA [6]. The smaller catenanes and minicircles would not appear in the supernatant until sufficient enzyme was present to almost completely decatenate the network.

3.3. Inhibition of topoisomerase II decatenation activity by dexrazoxane, other bisdioxopiperazines, etoposide and an etoposide analog

The effect of the catalytic topoisomerase II inhibitor dexrazoxane [2,3] concentration on topoisomer-

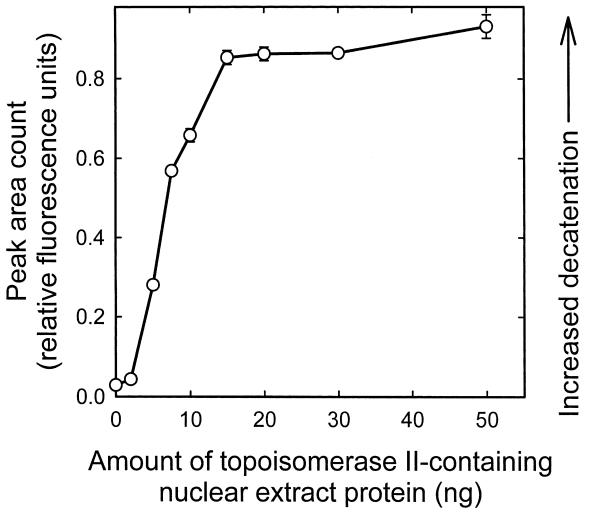

Fig. 2. Peak area counts of decatenated fluorescent DAPI-labeled kDNA products obtained by flow-injection analysis as a function of the amount of topoisomerase-containing nuclear extract II protein. The 20-μl reaction mixture, which contained 210 ng of kDNA, was incubated with increasing levels of nuclear extract topoisomerase II. The S.E. values shown are from triplicate determinations.

ase II decatenation activity was determined by flow-injection assay and by agarose gel electrophoresis. The IC_{50} values for both methods were determined by fitting the data using a non-linear least square fit to a four-parameter logistic equation [2,3]. The flow-injection assay gave an IC_{50} for dexrazoxane of 2.3 ± 0.5 μ*M* (±S.E.) from three independent experiments. A typical plot is shown in Fig. 3A.

The detection of decatenated products at various concentrations of dexrazoxane that were separated by gel electrophoresis are shown in Fig. 4. The two major products of kDNA decatenation were nicked open circular kDNA (NOC) and covalently closed circular kDNA (CC) [3,4,6]. As seen in lane 1, the kDNA control with no nuclear extract topoisomerase II, had a very small amount of endogenous kDNA. The nicked open circular kDNA was the most intense band and was used to determine the extent of decatenation [2,3]. Typical results obtained by agarose gel electrophoresis for the inhibition of topoisomerase II by dexrazoxane are shown in Fig. 3B. Analysis from three independent experiments resulted in a IC_{50} value of 7.1 ± 3.3 μ*M* (±S.E.), which compares well to our previous determinations

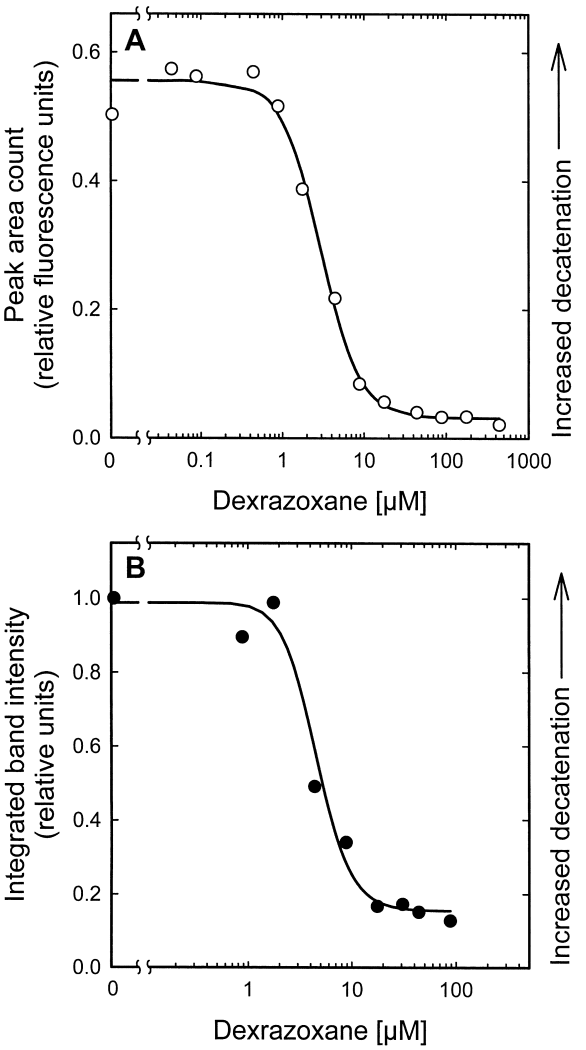

Fig. 3. Dexrazoxane-mediated inhibition of topoisomerase II catalytic activity in CHO cell nuclear extracts. The data shown in (A) were typical of that obtained by fluorescence flow-injection assay (○); and that shown in (B) was typical of that obtained by ethidium bromide agarose gel electrophoresis (●). The solid lines are non-linear least squares fits of the data to a four-parameter logistic equation. The zero values to the left of the axis break were obtained in the absence of dexrazoxane.

of 6.2 μ*M* for CHO nuclear extract topoisomerase II [3] by gel electrophoresis and 13 μ*M* for human nuclear extract topoisomerase II [2] using a radio-labeled kDNA centrifugation assay. The IC_{50} values derived from the gel electrophoresis assay were higher than for the flow-injection assay, which could

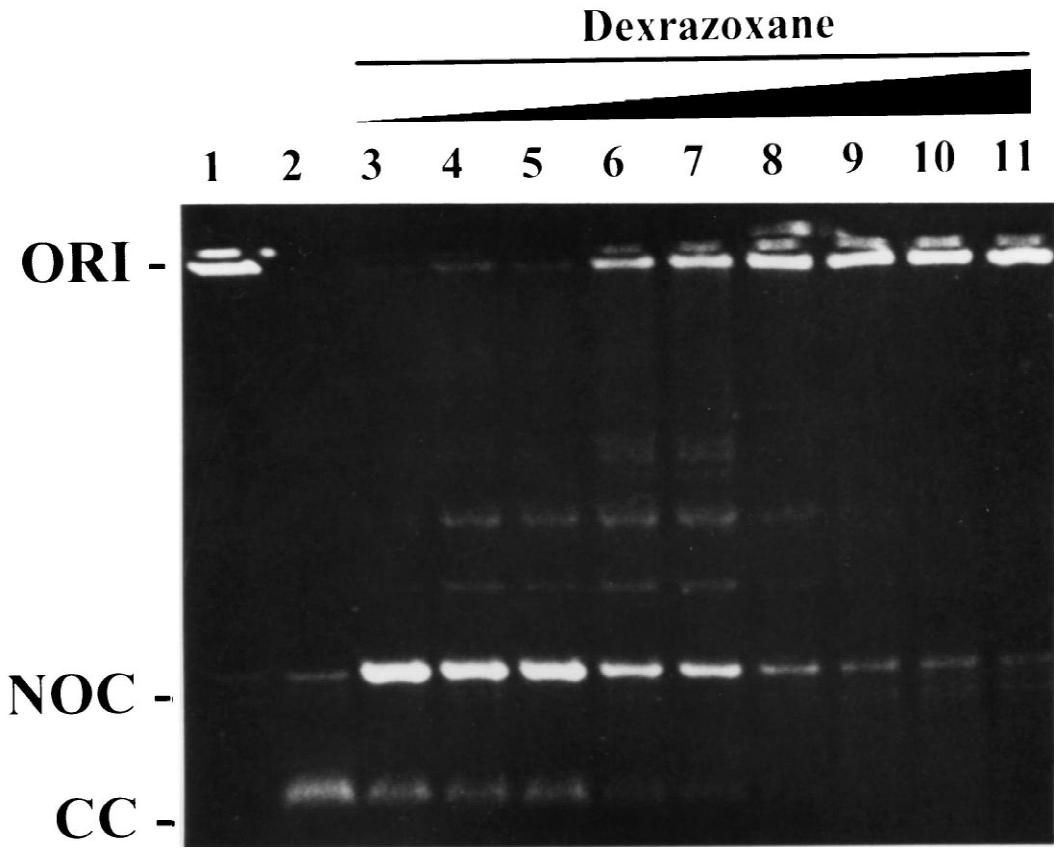

Fig. 4. Inhibition of topoisomerase II decatenation activity by dexrazoxane obtained using agarose gel electrophoresis. Lane 1: catenated kDNA control with no topoisomerase II; lane 2: decatenated kDNA control with no topoisomerase II; lanes 3–11: 0, 1, 2, 5, 10, 20, 35, 50, 100 μ*M* dexrazoxane in the assay buffer. The assay buffer contained 200 ng kDNA and sufficient nuclear extract topoisomerase II to decatenate approximately 80% of the kDNA in the absence of dexrazoxane. ORI, loading well origin; NOC, nicked open circular decatenated kDNA; CC, covalently closed circular decatenated kDNA. Unlabeled slower running bands are intermediate size catenanes (dimers, trimers, and so forth).

be due, in part, to the non-linearity of the gel electrophoresis assay calibration curve. Other bis-dioxopiperazine analogs that were tested using the flow-injection assay included ICRF-186, ICRF-192, and ICRF-219 which gave IC_{50} values of 1.4, 16, and 132 μ*M*, respectively. The flow-injection assay was also used to determine the IC_{50} for the inhibition of topoisomerase II by the covalent cleavable-complex forming inhibitors etoposide and an photoaffinity etoposide probe analog which gave IC_{50} values of 4 and 7 μ*M*, respectively [9]. These results indicate that the flow-injection assay can be used to screen for both cleavable complex-forming and catalytic-type topoisomerase II inhibitors. However, a limitation of the flow-injection assay is that it will not distinguish cleavable complex forming compounds from catalytic inhibitors. However, the assay will give a positive result for cleavable complex-forming compounds as they also inhibit the catalytic activity of topoisomerase II. In order to distinguish between the two types of inhibitors an assay that measures cleavable complex formation would have to be employed [5].

The possibility arises that there are other proteins present in the nuclear extract that have the potential to bind to and modulate the drug activity. Nuclear extract topoisomerase II is a convenient, stable, and inexpensive source of topoisomerase II and has

proved satisfactory in our hands when compared to commercial human topoisomerase II [3]. However, to avoid any possibility of interference from other proteins in the nuclear extract, the use of purified topoisomerase II in the assay would be preferable.

Advantages of the fluorescence flow-injection assay versus the gel electrophoresis assay include: significant increases in sensitivity and linearity of response, detection of 10-fold lower levels of decatenated product, 10-fold lower levels of percentage decatenation, and a requirement for much smaller quantities of mutagenic intercalating agents. The fluorescence flow-injection assay was also much faster and allowed for handling of many more samples in 1 day. The gel electrophoresis assay is limited by the number of wells cast, the lengthy preparation time of agarose gels, and the lengthier photographic method of analysis. Because of the limited number of wells available on a single gel, it is difficult to simultaneously run sufficient numbers of calibration standards and various concentrations of inhibitors. While in principle this type of assay could be carried out with a conventional spectrofluorimeter, much larger volumes of expensive reagents would be required. It would also be much more time consuming and, thus, not suited to high-throughput screening. The novel fluorescence flow-injection method described in this paper using DAPI-labeling of decatenated kDNA should be useful for high-throughput screening of potential topoisomerase II inhibitors and the rapid and accurate determination of their inhibition constants.

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