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Use of anion-exchange chromatography and chromatofocusing to reveal the structural and functional heterogeneity of topoisomerase II in a HL-60 cell line resistant to multi-drug treatment

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ABSTRACT

Fractionation of nuclear extracts from a multi-drug-resistant subclone of the human promyelocytic subline HL-60 by anion-exchange chromatography and chromatofocusing resolves at least two different subtypes of topoisomerase II, which are not identical to the known α- and β-forms of the enzyme because both forms are contained in each subtype. The two subtypes are present in about equal proportions and differ remarkably with respect to the optimum of reaction and sensitivity to *m*-amsacrine and orthovanadate. Both subtypes are highly insensitive to etoposide inhibition *in vitro*.

INTRODUCTION

DNA topoisomerases can relax supercoiled DNA and their action is vital in cell division and gene transcription [1]. Topoisomerase I (Topo I) is independent of 5′-triphosphate (ATP) and acts via transient DNA single-stranded breaks, whereas topoisomerase II (Topo II) is dependent on ATP and acts via tansient DNA double-stranded breaks [2]. Two forms of Topo II with relative molecular masses of 180 000 and 170 000 have been purified from mamalian cell lines by Pharmacia fast protein liquid chromatography (FPLC). The two forms are encoded by separate genes and differ biochemically and pharmacologically [3].

A number of cytostatic agents are inhibitors of Topo II. Some of these substances, for example, anthracyclines, epipodophyllotoxines and *m*-amsacrine (mAMSA) are important compounds in therapeutic protocols used in the treatment leukaemia and malignant lymphoma. These substances inhibit Topo II after binding to the DNA. This results in DNA double-stranded breaks which cannot be repaired by the cell because of the covalently bound topoisomerase subunits ("cleavable complex"). The number of double-stranded breaks correlates to the cytostatic potency of the substances [4]. The resistance of malignant cells to these cytostatic drugs is a major problem in the treatment of these diseases. Several molecular mechanisms of cellular resistance have been described, one of which is an alteration of the drug target Topo II [5,6]. Tumour cells with this form of "atypical" (AT) resistance display a broad cross-resistance to many anti-cancer drugs that interact with Topo II, but are not altered in drug accumulation, do not over-express P-glycoprotein (Pgp), are unaffected by Pgp modulators such as verapamil, and show alterations of Topo II activity [7,8]. It has been shown in one instance that AT resistance can be associated with a point mutation in the Topo II gene [9].

In this work topoisomerases were studied in a subclone of the human promyleocytic cell line HL-60, which exhibits a typical AT resistance. Topoisomerase subtypes were separated by anion-exchange chromatography and chromatofocusing to evaluate the contribution of each subtype to the resistance pattern. This kind of analysis may be a possible route to pre-treatment screening of haematopoietic malignancies.

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EXPERIMENTAL

Cells

Cells were grown in liquid culture medium (RPMI 1640 + 5% fetal calf serum, 1% Pen/Strep, all from Gibco) in a humidified atmosphere containing 5% carbon dioxide. The medium was changed twice weekly. Only cells in the logarithmic growth phase ($1-5 \cdot 10^6$ cells per ml) were harvested. To determine the cellular resistance to various cytostatic drugs the half-lethal concentrations (LD_{50}) of the cytotoxic substances studied were determined for each individual cell line and the factor of resistance between two indicated cell lines was expressed as the LD_{50} quotient. Cell viability was evaluated by determining the cellular trypan blue exclusion rate after growing the cell for 48 h in the presence of various concentrations of the indicated substance. This study was carried out in a subclone of the human promylelocytic cell line HL-60 resistant to multi-drug treatment (American tissue culture collection CCL240, Rockville, MD, USA), which developed by spontaneous mutation. Compared with the parent cell line, the subclone (HL-60 R) exhibited a markedly increased resistance to typical Topo II inhibitors such as etoposide (sixty-fold) and *m*-amsacrine (twenty-fold). The resistance could not be modulated by verapamil, proving an AT-type of resistance, which was expressed in a stable manner without selection and was routinely monitored by LD_{50} (etoposide) measurements.

Preparations

Isolation of nuclei. All steps were performed on ice. A total of 10^6–10^8 cells were suspended in 7.5 ml of isolation buffer (0.3 *M* sucrose, 0.5 m*M* ethyleneglycol tetraacetate (EGTA), 60 m*M* potassium chloride, 15 m*M* potassium phosphate, 15 m*M* sodium chloride, 0.15 m*M* spermine, 0.05 m*M* spermidine, 15 m*M* 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), pH 7.5, and 14 m*M* 2-mercaptoethanol) and 0.5 ml of isolation buffer containing 40 μl of Triton X-100 (mixed at 37°C) was added. After 15 min the nuclei were sedimented (1000 *g*, 10 min, 4°C), resuspended in 100–400 μl of isolation buffer and resedimented through 1 ml of 50% sucrose (3500 U/min, 10 min, 4°C). To avoid the contamination of nuclear proteins with cytosolic proteases it was important to use only preparations which contained more than 95% intact nuclei (the integrity of the nuclear membrane was controlled by trypan blue exclusion). The overall yield (nuclei per cells) was greater than 90%, indicating that this step did not select for the mechanical stability of the nuclei.

Extraction of nuclear proteins. Nuclei were resuspended in extraction buffer (5 m*M* potassium phosphate, pH 7.5, 100 m*M* sodium chloride, 10 m*M* 2-mercaptoethanol and 0.05 m*M* phenylmethylsulphonyl fluoride) at a concentration of $3 \cdot 10^7$ per 780 μl, and 220 μl of 2 *M* sodium chloride were added dropwise to a final concentration of 0.45 *M*. After 10 min the nuclear membranes and debris were sedimented (10 000 U/min, 4°C, 10 min). The supernatant, containing high activities of topoisomerase, but also large amounts of genomic DNA fragments, was bound to a one tenth volume of hydroxyapatite (Bio-Rad Labs.). DNA fragments were removed by washing with twenty bed volumes of 500 m*M* potassium phosphate, pH 7.5, and the DNA-free topoisomerase activity was eluted with two bed volumes of 800 m*M* potassium phosphate, pH 7.8. The nuclear extracts were stable at 4°C for at least 96 h and could be stored at −70°C at least 4 weeks without loss of activity after the addition of 25% glycerol.

Anion-exchange chromatography (Mono Q). Nuclear extracts were desalted using a Pharmacia FPLC fast desalting column equilibrated with Q-buffer (20 m*M* Tris–HCl, pH 7.8, 10% glycerol, 10 m*M* β-mercaptoethanol and 0.5 m*M* EDTA) and loaded onto a Mono Q HR 5/5 column (Pharmacia) from a superloop at a flow-rate of 0.5 ml/min. The column was washed with 8–10 ml of Q-buffer and then eluted with a 20-ml linear gradient from 0 to 400 m*M* sodium chloride. Each 1-ml fraction was collected for the DNA relaxation activity assay.

Chromatofocusing (Mono P). Active fractions from Mono Q runs were diluted three-fold with Q-buffer and loaded onto a Mono P HR 5/5 column (Pharmacia) equilibrated with the same buffer at a flow-rate of 0.5 ml/min. The column was eluted isocratically with 25 ml of a mixture of 1.5% Polybuffer 96, 3.4% Polybuffer 74, pH 5.0 (hydrochloric adid), 10% glycerol, 10 m*M* β-mercaptoethanol and 0.5 m*M* EDTA from a superloop. This created a pH gradient from 8 to 5 as measured by a flow-through pH electrode (Pharmacia). Fractions (1 ml)

were collected and tested for topoisomerase activity.

Assays

Topoisomerase catalytic assays. The relaxation of 500 ng of pBR322 plasmid DNA by 4-μl aliquots of the eluate fractions was assessed in assay buffer [70 m*M* Bis–Tris propane buffer, pH adjusted with hydrochloric acid to 8.0 or as indicated, which contained 140 m*M* potassium chloride, 0.7 m*M* EDTA and 0.2 mg/ml of bovine serum albumin, (BSA). For Topo II activation, 10 m*M* magnesium chloride and 2 m*M* ATP (Boehringer Mannheim, vanadate-free) were freshly added to a final volume of 20 μl. After incubation at 37°C for 30 min, the DNA was electrophoresed overnight at 20 V in a 1% agarose submarine gel. DNA was stained for photography with ethidium bromide. The electrophoretic mobility of native twelve-fold supercoiled pBR322 was typically reduced stepwise by topoisomerase action. The activity was quantified from the disappearance of supercoiled pBR322 substrate by densitometric scanning of the photographic negatives [10] and expressed as units/μl (one unit being the amount of topoisomerase that will give a 90% relaxation of 500 ng of pBR322 plasmid DNA under these conditions). For a fast control of the assay results, 0.1 μl (25 ng of pBR322) was applied to 0.7% agarose gels (0.5 mm) cast on GelBond films (pharmacia) and electrophoresed in a PhastSystem at 100 V, 24 mA for 20 min at 13°C. Topo II specific catalytic activity was assayed by the unknotting of bacteriophage P4 DNA [11]. Briefly, 500 ng of P4 DNA (Biotechnology Centre, University of Oslo, Oslo, Norway) were incubated with 4-μl Topo II fractions in a final volume of 20 μl of assay buffer containing 10 m*M* magnesium chloride and 2 m*M* ATP (freshly added) at 37°C for 30 min. P4 DNA was electrophoresed in 0.7% agarose submarine gels (100 V, 2 h) and detected with ethidium bromide. The enzyme converts the knotted P4 DNA, which forms a smear to its unknotted closed circular form which migrates as a band.

Immunoblot analysis of Topo II. Active Mono Q eluate fractions were concentrated (Centricon, Amicon Grace, incubated with a one tenth volume of ten-fold concentrated sample buffer (1.25 *M* Tris–HCl, pH 6.8, 10% sodium dodecyl sulphate, 10 mg/ml bromophenol blue) for 1 h at room temperature and the proteins were separated in 12.5% homogeneous PhastGels (Pharmacia) and transferred to nitrocellulose sheets using a PhastSystem and standard conditions. Immunostaining of Topo II was carried out at room temperature. Nitrocellulose sheets were blocked with phosphate-buffered saline (PBS) containing 5% fat-free dried milk (Toepfer) for 1 h, washed with PBS containing 0.1% BSA (PBSA) and incubated overnight with a 1:500 dilution (PBSA) of rabbit anti-human Topo II antiserum (a kind gift of Dr. E. Schneider from Professor L. F. Liu's Laboratory), which recognizes both forms of human Topo II. After washing again with PBSA, the sheets were incubated with gold-labelled goat anti-rabbit IgG (Auroprobe, Amersham, 1:100 dilution in PBSA containing 5% gelatin) for 4 h, again washed with PBSA and finally developed with InteseBL (Amersham) silver enhancement for 30–120 min. Marker proteins (Pharmacia) included in each run contained rabbit muscle myosin, α_2-macroglobulin, β-galactosidase, transferrin and glutamic dehydrogenase and were stained with gold after electrophoretic transfer using Aurodye (Amersham).

RESULTS AND DISCUSSION

As shown in Fig. 1, the pBR322 DNA relaxation activities present in nuclear extracts of HL-60 R cells eluted from a Mono Q column over a relatively wide concentration range (50–250 m*M* sodium chloride). Three distinctive maxima of activity showing complete pBR322 relaxation (marked activity peaks 1–3) are separated by less active fractions showing only partial pBR322 relaxation (fractions 6, 7 and 9). The relaxation activity in the first peak (eluting at 50–120 m*M* sodium chloride) was independent of ATP. This is typical of Topo I. Activity peaks eluting at 170 and 200 m*M* sodium chloride showed ATP-dependent relaxation activity, typical for the type II enzyme. Titrating the activity in each peak by serial dilution gave only 3% of the total acitvity in peak 1 (independent of ATP), 53% in peak 2 and 44% in peak 3 (both dependent on ATP). The activity maxima could not clearly and reproducibly be related to UV-absorption peaks.

A further biochemical characterization of the three activity peaks is summarized in Table I. In

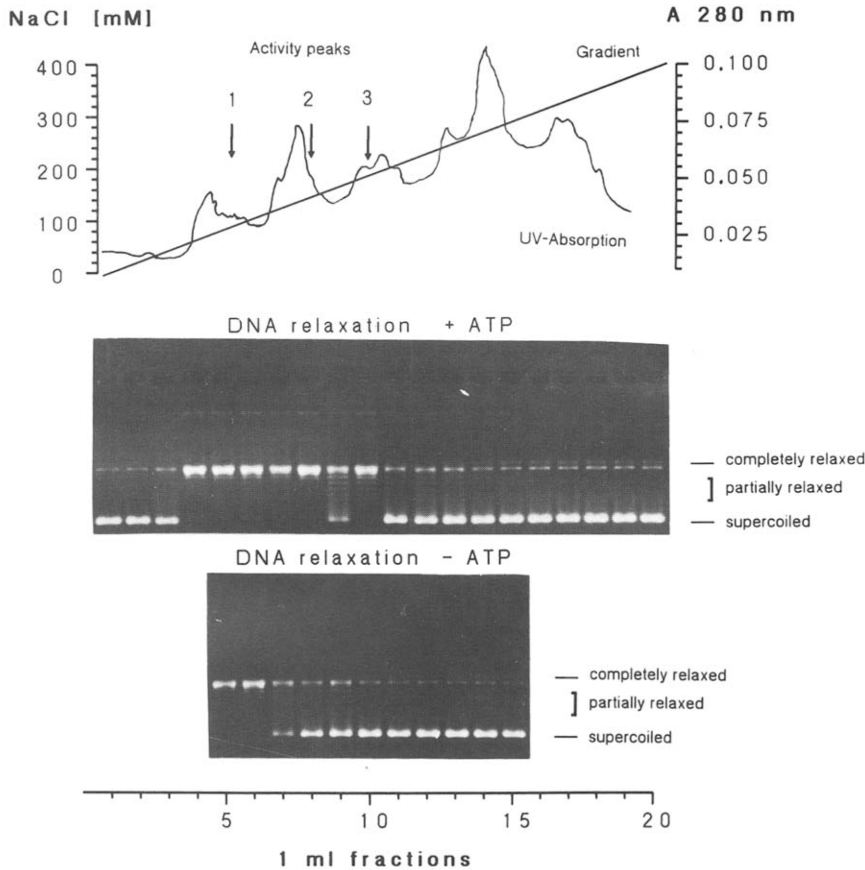

Fig. 1. Separation of topoisomerase subforms by anion-exchange chromatography using Mono Q. A 10-ml volume of nuclear extract (hydroxyapatite eluate) was desalted, loaded onto a Mono Q HR 5/5 column at pH 7.9 and eluted with a 20-ml linear gradient from 0 to 400 m*M* sodium chloride. Fractions (1 ml) were assayed for pBR322 plasmid DNA relaxation activity in the presence and absence of ATP (2 m*M*). This is one of at least five similar experiments.

Western blots a double band of relative molecular mass 170 000/180 000, typical of human Topo II [3], could be immunostained with anti-human Topo II antibodies in both peaks 2 and 3 but not in peak 1, indicating that the ATP-dependent pBR322 relaxation observed in peaks 2 and 3 was due to the presence of both α- and β-forms of the human type II enzyme. This is further confirmed by the observation that Topo II-specific P4-DNA unknotting activity [11] could only be measured in these two peaks.

Studies of the *in vitro* inhibition of pBR322 DNA relaxation by Topo II inhibitors is also summarized in Table I. Both activity peaks containing Topo II (peaks 2 and 3) and peak 1 were completely resistant to *in vitro* inhibition by etoposide (up to 100 μg/ml), which was to be expected as the cells are 100-fold resistant to etoposide. For *m*-amsacrine marked differences were found. Peak 1 (presumably Topo I) and peak 2 activities were both relatively insensitive. The IC_{50} (concentration of drug at which a 50% inhibition of enzyme activity was achieved *in vitro*) of 3 mg/ml observed most probably reflects non-specific steric inhibition of DNA binding, due to the DNA-intercalating properties of the drug. In contrast, peak 3 was inhibited at 300-fold lower doses, the IC_{50} (0.01 mg/ml) observed being in accordance with Topo II-specific inhibition [4].

Orthovanadate, an inhibitor of certain types of

TABLE I

BIOCHEMICAL CHARACTERISTICS OF HL-60-R TOPOISOMERASE ISO-ACTIVITIES

Parameter	Mono-Q fractions		
	Peak 1	Peak 2	Peak 3
Eluate concentration (mM)	50–100	170	200
Nuclear extract[a] (units/ml)	25	400	333
IC_{50}			
Etoposide	–	–	–
m-Amsacrine (mg/l)	3	3	0.01
Orthovanadate (μM)	–	0.2	30
Anti-Topo II immunoblot (relative molecular mass)	–	170 000/180 000	170 000/180 000
ATP/Mg^{2+} dependency	–	+	+
P4 unknotting activity	–	+	+
Iso-form	I	IIα,β	IIα,β
Optimum pH	7.9	8.3	7.9/9.0

[a] One unit is defined as the amount of topoisomerase that catalyses 90% relaxation of 500 ng of supercoiled pBR322 plasmid DNA at 37°C, pH 8.3, in 30 min.

ATPases, most notably ion-translocating enzymes such as the NA/K ATPase [12] has also been shown to be a potent inhibitor [IC_{50} 2 μM] of both forms of ATP-dependent human Topo II [3]. The dose-dependent inhibition of pBR322 relaxation activity by orthovanadate was studied in all three activity peaks of HL-60 R topoisomerase (Table I). As expected, the ATP-independent enzyme present in peak 1 was completely insensitive to orthovanadate (tested up to 100 μM), whereas the two ATP-dependent peaks differed in sensitivity: the peak 3 activity (IC_{50} 30 μM) was 150-fold more resistant to orthovanadate than the peak 2 activity (IC_{50} 0.2 μM). This is of particular interest as recent findings [7,9] suggest that changes in the Topo II ATPase activity are involved in AT resistance.

It was recently observed [13] that the distinct iso-activities of topoisomerases can be discriminated on the basis of pH profiles in nuclear extracts of wild-type HL-60 cells and that a reduced topoisomerase activity at pH 7.9 is particularly involved in etoposide cytotoxity in these cells. In this work the pH profile of fractionated topoisomerase from etoposide resistant HL-60 R cells was studied. In peak fractions 1 and 2 the pBR322 relaxation activity had a single narrow maximum at pH 7.9 and 8.3, respectively, whereas peak 3 showed two narrow and distinctive maxima at pH 7.9 and 9.0 (Table I). This finding suggested that the activity of peak 3 might contain two functionally different enzyme types. To subfractionate peak 3, high-resolution chromatofocusing was performed. As shown in Fig. 2, this technique allowed the resolution of at least three distinct activity peaks: the activity maxima in fracitons 6/7, 11/12 and 19 showed complete pBR322 relaxation and were separated by fractions of lesser activity, which only partially relaxed supercoiled pBR322. The three activity peaks were not reproducibly related to UV-absorption peaks, which are caused by major contaminant proteins.

Taken together, the data presented here suggest that 0.45 M sodium chloride nuclear extracts from HL-60 R cells contain mainly Topo II in addition to minor contaminations of Topo I (about 3% of the total activity). Anion-exchange chromatography allows the separation of at least two (a further resolution into three or four might be obtained by chromatofocusing) functionally different subtypes of Topo II which are not identical to the two known molecular forms α and β, as both are contained in each subtype in about equal proportions. The Topo II subtypes, which are present in about equal proportions, differ with respect to the optimum-pH of reaction and sensitivity to orthovanadate and *m*-amsacrine. Neither subtype can be inhibited by etoposide *in vitro* and therefore they most probably both contribute to the high etoposide resistance of HL-60 R cells.

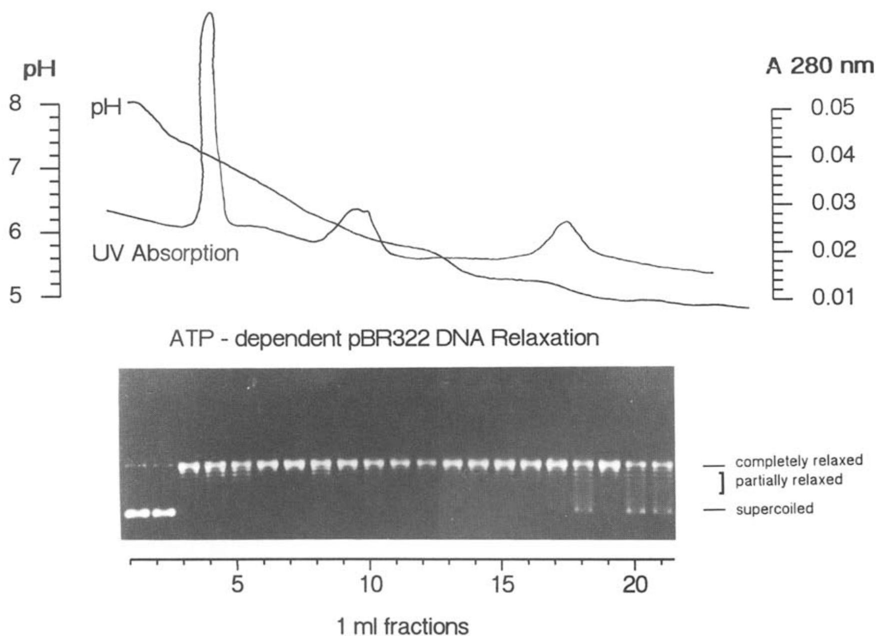

Fig. 2. Separation of topoisomerase isoactivities by chromatofocusing using Mono P. Fraction 10 from the Mono-Q run shown in Fig. 1 was diluted and loaded onto a Mono P HR 5/5 column at pH 7.9. The column was isocratically eluted with 25 ml of Polybuffer, pH 5.0 (for details, see text). Fractions (1 ml) were assayed for pBR322 plasmid DNA relaxation activity in the presence of ATP (2 m*M*). This is one of three similar experiments.

It is temping to speculate about the structural feature that causes the observed differences in function and protein net charge. Whereas isoenzymes in the classical sense can be virtually excluded on the basis of the immunoblot results, epigenetic modifications such as phosphorylation, acylation and isoprenylation are the most probable structures. Little is known about the *in vivo* regulation of Topo II, but it has been reported that purified mammalian Topo II is a substrate for protein kinase C [14,15] and that *in vitro* phosphorylation by protein kinase C alters the DNA catalytic activity in addition to the ATPase activity of the enzyme [14]. This is of particular interest as the induction of cell differentiation by dimethylsulphoxide involves both the stimulation of protein kinase C and alterations of Topo II activity and drug sensitivity [13,14,16]. It may therefore be speculated that phosphorylation–dephosphorylation could be a possible regulatory mechanism to modulate Topo II activity and as by-product to produce target protein resistance to Topo II inhibitors.

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