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SHORT COMMUNICATION

Differential Sensitivities of Recombinant Human Topoisomerase IIα and β to Various Classes of Topoisomerase II-interacting Agents

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ABSTRACT. A series of topoisomerase-interacting antitumour agents were tested for their ability to differentially inhibit the catalytic activity of either topoisomerase (TOPO) IIα or β, as judged by a DNA decatenation assay. The α form, relative to the β isoform, proved 1 to 3 times more sensitive to nonintercalating complex-stabilizing TOPO II-interacting agents (etoposide and derivatives) and up to 18 times more sensitive to non-complex-stabilizing inhibitors of TOPO II ((±)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane [ICRF 159] and *meso*-2,3-bis(3,5-dioxopiperazine-1-yl)butane [ICRF 193]). However, the β form of the enzyme appeared 1 to 3 times more sensitive to intercalating TOPO II-interacting agents (daunorubicin, aclarubicin and mitoxantrone). A possible implication of these data are that tumours preferentially expressing either the α or the β isoform may be differentially responsive to various classes of TOPO II-interacting agents. BIOCHEM PHARMACOL **56**;4:503–507, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. topoisomerase IIα; topoisomerase IIβ; catalytic activity; antitumour agents; DNA-decatenation; topoisomerase II-interacting agents

TOPO II† is an important nuclear enzyme controlling DNA topology through catalysis of a transient breakage of double-stranded DNA in an ATP-dependent fashion, allowing for the passage of double-stranded DNA followed by resealing of the DNA [1]. Relaxation of DNA supercoils by TOPO II plays a major role in DNA replication and transcription, and TOPO II plays a critical role in chromosome condensation and separation during mitosis and in the attachment of DNA loops to the nuclear matrix and chromosomal scaffold [1]. Two isoforms of TOPO II, namely α and β, have been characterised [2], and the corresponding human genes cloned [3–6] and expressed [7, 8] in yeast. Both isoforms seem to be functionally equivalent and both complement defective TOPO II mutants in yeast [9, 10]. However, whilst the β isoform is expressed throughout the cell cycle, expression of the α isoform is strictly cellular proliferation-dependent [11]. During mitosis, TOPO IIα appears completely bound to the mitotic chromatin, while isoform β diffuses into the cytosol [12], suggesting a role for TOPO IIα in chromosome disentanglement. However, the specific localisation of the isoforms during interphase seems controversial [12, 13]. Nevertheless, both isoforms can be overexpressed in human tumours [13], with a higher proportion of cells in individual tumours showing an elevation in TOPO IIβ than in α. These data, combined with the fact that exposure of cells to etoposide [12, 14] or to ICRF 187 [14] resulted in a comparable diminution of salt-extractable TOPO IIα and β, suggest that either isoform could be targeted by TOPO-interacting drugs.

TOPO II is the target of clinically useful anticancer drugs which either stabilize the cleavable complex, such as the nonintercalating agent etoposide and its derivatives and the DNA intercalating anthracyclines, or those which do not stabilize this complex, such as the catalytic inhibitor ICRF 159 and derivatives [15]. In spite of preliminary reports [2, 8, 10, 16], the question of the relative sensitivity of each isoform of the human enzyme toward TOPO-interacting agents remains open. If, indeed, there is any preferential inhibition of either isoform by specific antitumour agents, this information could be of value in designing optimal drug combinations for different tumours. Therefore, we systematically compared the effects of a series of TOPO-interacting agents on the DNA decatenation activity of the α and β forms of recombinant human TOPO II.

MATERIALS AND METHODS

Materials

Genistein, actinomycin D and distamycin A, amsacrine, doxorubicin, aclarubicin and daunorubicin as hydrochlorides were purchased from Sigma, netropsin from Boehr-

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† *Abbreviations*: ICRF 159, (±)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane (razoxane); ICRF 187, (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane (dexrazoxane); ICRF 193, *meso*-2,3-bis(3,5-dioxopiperazine-1-yl)butane; and TOPO II, topoisomerase II.

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inger Mannheim, mitoxantrone and bisantrene from Lederle, suramin from RBI, ICRF 187 hydrochloride (dexrazoxane) from Chiron and NPF (4′-demethyl-4p.fluoro-aniline-4-desoxypodophyllotoxin) from Duphar. Teniposide, Top-53 (4′-demethyl-4β-[2-[*N*-(2-(*N*′*N*′-dimethylamino)ethyl)-*N*-methylamino]ethyl]-4-desoxypodophyllotoxin) dihydrochloride, GL-331 (4′-demethyl-4-p.nitro-aniline-4-desoxypodophyllotoxin), etopofos, etoposide and ICRF 159 (razoxane) were provided by Pierre Fabre Medicament. Intoplicine mesylate was obtained from Rhone-Poulenc Rorer and ICRF 193 was a gift from Dr. A. Creighton, St. Bartholomew's Hospital Medical College (London).

The plasmid YEpWob6 encoding human TOPO IIα under the control of the GAL1 promoter was purchased from Prof. J. C. Wang (Harvard University) and YEph-Top2β expressed in the yeast JEL1 encoding human topoisomerase IIβ under the control of the GAL1 promoter was purchased from Dr. C. Austin (University of Newcastle).

Production and Purification of Human Recombinant TOPO IIa and β in Yeast

Production was according to published methods [8, 17]. In brief, TOPO expression was induced by switching growth from 2% glucose to 2% galactose for 24 hr. The yeast pellet was weighed, resuspended in 1 to 2 volumes of buffer B_0 and used for purification or stored at −70°. Buffer B: 50 mM Tris pH 7.7, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 20 mM KF, 1 mM dithiothreitol, 0.1 mM paranitrophenylphosphate, 0.1 mM β-glycerophosphate, 1 mM benzamidine, 200 μg/mL of aminoethylbenzenesulfonyl fluoride, 20 μg/mL of aprotinin, 5 μg/mL of leupeptin and pepstatin. The suffix to B indicates the KCl concentration added; e.g. B_{200} signifies B plus 200 mM KCl.

Published methods were used in purification [7, 8, 17], although an additional step was introduced for TOPO II α. Briefly, the phosphocellulose eluate containing TOPO IIα was adjusted to 200 mM KCl with B_0 and applied to a 1-mL heparin column (HiTrap, Pharmacia) preequilibrated with buffer B_{200}. Following elution with increasing concentrations of KCl, TOPO IIα eluted with 500 mM KCl. Fractions of interest were stored in 50% glycerol at −70°. SDS PAGE was performed according to Laemmli [18], and gels were stained with a Silver Stain Plus kit purchased from Bio-Rad.

Assays for kDNA Decatenation Activity of TOPO II

Eighteen microliters of buffer A (50 mM Tris pH 8.0, 120 mM KCl, 0.5 mM DTT, 0.5 mM ATP, 10 mM $MgCl_2$) containing 200 ng of kDNA (TopoGen) and 1 U of TOPO II (the amount of enzyme resulting in complete decatenation of 200 ng of kDNA) were added to an Eppendorf tube containing 2 μL of either vehicle (DMSO) alone or the test drug in vehicle [19]. After a 30-min incubation at 37°, the reaction mixture was analysed on a 1% agarose gel, run at 35 mA for 2 hr in Tris-borate-EDTA buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3). Gels stained with ethidium bromide were photographed under UV. Assays to determine inhibition of decatenation were carried out on at least three separate occasions when definite activity was detected, using a range of concentrations lower than the highest tested of 100 μM.

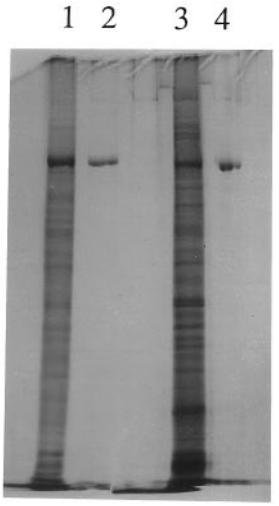

FIG. 1. Assessment of the purity of isolated TOPO IIα and β by separation of the proteins on SDS PAGE followed by silver staining. Lane 1: fraction containing TOPO IIβ before application to the phosphocellulose column; lane 2: TOPO IIβ eluted from the phosphocellulose column; lane 3: fraction containing TOPO IIα before application to the phosphocellulose column; and lane 4: TOPO IIα eluted from the heparin column.

RESULTS AND DISCUSSION

TOPO IIα and IIβ were purified to homogeneity (Fig. 1). Whilst the purity of the β isoform seemed satisfactory after chromatography on a phosphocellulose column (Fig. 1, lane 2), the α form, though apparently pure on a Coomassie-stained gel, was contaminated by proteins of lower molecular mass detected by silver staining (data not shown). Therefore, a second chromatographic step on a heparin column was added, leading to purification to apparent homogeneity (Fig. 1, lane 4).

To compare the pharmacological profiles of the two enzymes, the minimal amount of each necessary to achieve full decatenation of 200 ng of kDNA was determined, from three independent experiments, as 16 ng (0.1 pmol) and 8 ng (0.05 pmol) per assay for the α and β isoforms respectively.

The first experiments were performed with both the minimal defined enzyme concentration and the slightly lower one, so as to rule out any possible excess of enzyme which might affect the results. As shown in Fig. 2, lowering the minimal amount of enzyme needed for full decatenation from 16 to 14 ng per assay led to incomplete decatenation of the control (lane T3) and to an increased sensitivity to the inhibitor (lanes d and e, shown here for aclarubicin). The consistency of the results obtained led to our using

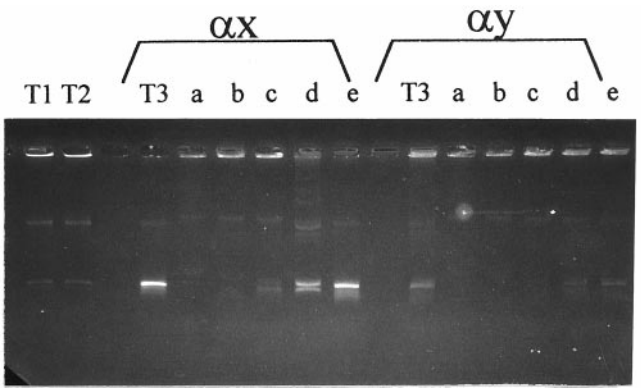

FIG. 2. Effects of aclarubicin on the catalytic activity of TOPO IIα. T1: no enzyme; T2: no enzyme + 10^{-4} M aclarubicin; T3: enzyme without drug; a–e: enzyme plus aclarubicin (a: 1.0 × 10^{-4} M; b: 3.2 × 10^{-4} M; c: 1.0 × 10^{-5} M; d: 3.2 × 10^{-6} M; e: 1.0 × 10^{-6} M); αx: 16 ng of enzyme (minimal amount required to obtain the complete decatenation of 200 ng of kDNA/assay); αy: 14 ng of enzyme/assay. Note in each of the T3 lanes that while decatenation is complete with the concentration × (16 ng) of enzyme, this is not the case with the lower concentration y (14 ng) of enzyme. Moreover, this slight change in amount of enzyme led to a dramatic shift in drug sensitivity (compare lanes d and e in gels αx and αy, respectively). In gel αx, the EC_{50} value derived from this experiment is 5.6 × 10^{-6} M, which is the log mean between the last active (1.0 × 10^{-5} M) and the first inactive (3.2 × 10^{-6} M) concentration.

only the minimal enzyme concentration in the assays detailed below.

The ratio of the EC_{50} values obtained against TOPO IIβ as opposed to α, abbreviated as the β/α ratio, was used as an indicator of the "preference" of a test product for either the TOPO IIα or β isozyme. Therefore, values greater than 1 reflect a preference for the α isozyme.

Epipodophyllotoxin derivatives inhibited DNA decatenation catalysed by TOPO II and, as a class, showed an apparent preference for isoform α versus β with values for the β/α ratio ranging from 1 to 3 (Table 1). Both GL-331, the most potent inhibitor of both TOPO IIα and β, with EC_{50} values of 5.6 and 18 μM respectively, and etoposide showed a stronger inhibition of α over β, with a β/α ratio of 3. Teniposide and Top-53 also showed a preference for TOPO IIα with a β/α ratio >1 and >2, respectively. On the other hand, NPF appeared equipotent against both isozymes. In our hands, the prodrug etopofos was inactive against both isoforms at concentrations up to 100 μM.

Genistein, an inhibitor of TOPO II which stabilizes the cleavable complex [15], showed equivalent effects on both enzymes. Within the class of DNA-intercalating TOPO II inhibitors [15], notably daunorubicin, aclarubicin and mitoxantrone (Table 1), a preference for β over α is apparent, with the β/α ratio ranging from 0.3 to 1. Amongst those inhibitors of TOPO II which do not stabilize the cleavable complex [15], suramin showed equivalent effects on both enzymes, whereass ICRF 159 and ICRF 193 displayed preferential inhibition of TOPO IIα over β, with values of >18 for the β/α ratio. ICRF 187, however, exhibited a more modest value for this ratio (Table 1).

Amongst the dual TOPO I/II inhibitors, the DNA-intercalator intoplicine [15] (like the TOPO II specific intercalators) favored the β form, with a β/α ratio of 0.3, while actinomycin D, another intercalator, and distamycin A, a minor groove-binder, appeared neutral with ratios of 1 (Table 1).

In summary, TOPO IIα appears 1 to 3 times more sensitive to the nonintercalating TOPO II-interacting agents tested (epipodophyllotoxin derivatives) and up to 18 times more sensitive to the specific catalytic inhibitors of TOPO II (ICRF 159 and derivatives), as compared to the β form. However, the β form of the enzyme appears 1 to 3 times more sensitive to intercalating agents than the α isoform. This latter fact seems to rule out any potentially unrecognized bias in our assays which might favor activity against the α as compared to the β forms of the enzyme. Moreover, data presented here are consistent with those reported both for the mouse enzyme (pointing to a greater effectiveness of teniposide toward the TOPO IIα form [2]) and for the human enzyme using a DNA-cleavage assay: the α form was described as either slightly more sensitive than β to epirubicin and etoposide [16] or as sensitive as the β form to teniposide and etoposide [8]. Amsacrine has been reported to induce more cleavage with TOPO IIβ [8] and mitoxantrone has displayed a fourfold preference for β [10], in line with the generally higher sensitivity of the β form to intercalating agents that we observed in this study. In a DNA relaxation assay, suramin proved equipotent against both isoforms, as described in our decatenation assays, whilst merbarone showed a 2- to 3-fold selectivity for TOPO IIα [10].

These data identify an apparently clear-cut differential sensitivity between TOPO IIα and β in terms of decatenation activity from a purely biochemical point of view. However, whether they can be considered in any way predictive of the *in vitro* or *in vivo* relative effectiveness of these drugs against either form of TOPO II remains to be established. Indeed, results at the cellular level appear rather contradictory. For example, Brown *et al.* [20] pointed to a negative correlation between levels of TOPO IIβ and resistance to etoposide in six lymphoma lines (which proved insignificant), while Houlbrook *et al.* [21] described a correlation among seven breast cancer lines between levels of TOPO IIβ and sensitivities to etoposide. However, Withoff *et al.* [22] showed in one tumour cell line that resistance to teniposide was associated with a decrease in TOPO IIα, but not the IIβ protein. Interestingly, in yeast strains where either human TOPO IIα or β was expressed instead of the native yeast enzyme, cell killing with etoposide (as with doxorubicin and mitoxantrone) was greater in strains expressing the α form, whereas amsacrine produced comparable levels of killing in both strains [10]. Therefore, evidence at the cellular level of any definite specific targeting of TOPO II-interacting drugs is still awaited. Such data may result from studies of "knock-out" cell lines expressing only a single enzyme isoform.

TABLE 1. Effects of a series of TOPO II-interacting agents on the catalytic activity of recombinant TOPO IIα and β

	Inhibition of decatenation							
	For TOPO IIα Concentrations (μM)			For TOPO IIβ Concentrations (μM)				
Test compound	Range of first active concentration	Range of last inactive concentration	EC_{50}	Range of first active concentration	Range of last inactive concentration	EC_{50}	(N)	EC_{50} Ratio IIβ/IIα
A. TOPO II cleavable complex stabilizing agents								
Nonintercalating agents								
Etoposide	32–100	10–32	**32**	100–>100	32–100	**100**	(4)	**3**
Teniposide	100–>100	32–100	**100**	100–>100	32–100	**>100**	(4)	**>1**
Etopofos	>100	>100	**>100**	>100	≥100	**>100**	(2)	—
GL-331	3.2–10	1–3.2	**5.6**	32	10	**18**	(3)	**3**
Top-53	100–>100	32–100	**56**	>100	≥100	**>100**	(3)	**>2**
NPF	32–100	10–32	**56**	32–100	10–32	**56**	(3)	**1**
Genistein	100–>100	32–100	**100**	100–>100	32–100	**100**	(2)	**1**
Intercalating agents								
Doxorubicin	0.32–1	0.1–0.32	**0.56**	1.0	0.32	**0.56**	(4)	**1**
Daunorubicin	1–3.2	0.32–1	**1.8**	1.0	0.32	**0.56**	(3)	**0.3**
Amsacrine	100–>100	32–100	**100**	100–>100	32–100	**100**	(4)	**1**
Aclarubicin	3.2–10	1–3.2	**5.6**	3.2–10	1–3.2	**1.8**	(3)	**0.3**
Mitoxantrone	1–3.2	0.32–1	**1.0**	0.32–1	0.1–0.32	**0.32**	(4)	**0.3**
Bisantrene	3.2–10	1–3.2	**1.8**	1–3.2	0.32–1	**1.0**	(4)	**0.6**
B. Catalytic Inhibitors								
ICRF 187 (Dexrazoxane)	100	32	**56**	>100	≥100	**>100**	(3)	**>2**
ICRF 159 (Razoxane)	10	3.2	**5.6**	>100	≥100	**>100**	(3)	**>18**
ICRF 193	1–3.2	0.32–1	**1.0**	32–100	10–32	**18**	(4)	**18**
Suramin	10–32	3.2–10	**5.6**	10–32	3.2–10	**5.6**	(3)	**1**
C. Dual Inhibitors of TOPO I & II								
Intoplicine	3.2	1	**1.8**	1	0.32	**0.56**	(3)	**0.3**
Actinomycin	3.2–10	1–3.2	**5.6**	3.2–10	1–3.2	**5.6**	(3)	**1**
Distamycin A	1–3.2	0.32–1	**0.56**	1–3.2	0.32–1	**0.56**	(3)	**1**

EC_{50}: the effective concentration at which 50% of the assays are positive for some visible inhibitory activity. Generally, this was the intermediate concentration between the last inactive concentration and the first active one, calculated as the log mean of these two concentrations. The last inactive concentration is defined as the concentration at which 1/3 or 0/3 assays (i.e. under 50%) are positive for inhibitory activity, and the first active concentration is that concentration at which 2/3 assays (i.e. over 50%) are active in terms of inhibition. The solvent used was DMSO to a final concentration of 1%. N = number of experiments.

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