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Complementation of temperature-sensitive topoisomerase II mutations in *Saccharomyces cerevisiae* by a human TOP2β construct allows the study of topoisomerase IIβ inhibitors in yeast.

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Abstract We show herein that human DNA topoisomerase IIβ is functional in yeast. It can complement a yeast temperature-sensitive mutation in topoisomerase II. The effect on human topoisomerase IIβ of a number of topoisomerase II inhibitors was analysed in a yeast in vivo system and compared with that of human topoisomerase IIα and wild-type yeast topoisomerase II. A drug permeable yeast strain (JN394 *top2–4*) was used to analyse the in vivo effects of known anti-topoisomerase II agents on human topoisomerase IIβ transformants. A parallel analysis on human topoisomerase IIα transformants provides the first in vivo analysis of the responses of yeast bearing the individual isoforms to these drugs. The strain was analysed at 35 °C, a non-permissive temperature at which only plasmid-borne topoisomerase II is active. A shuttle vector with either human topoisomerase IIβ, human topoisomerase IIα or yeast topoisomerase II under the control of a GAL1 promoter was used. The key findings were that amsacrine produced comparable levels of cell killing with both α and β, whilst etoposide, doxorubicin and mitoxantrone produced higher degrees of cell killing with α than with β or yeast topoisomerase II. Merbarone had the greatest effect on the yeast strain bearing plasmid-borne yeast topoisomerase II. Suramin, quercetin and genistein showed little cell killing in this system. This yeast in vivo system provides a powerful way to analyse the effects of anti-topoisomerase II agents on transformants bearing the individual human isoforms. This system also provides a means of analysing putative drug-resistance mutations in human topoisomerase IIβ or to select for drug-resistance mutations in human topoisomerase IIβ.

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Introduction

Type II DNA topoisomerases are essential cellular enzymes. They catalyse the interconversion of topological isomers of DNA by passing one DNA helix through a transient enzyme-bridged double-stranded DNA break in another [34]. Two genetically different topoisomerase II isoforms, α and β, are produced by mammalian cells, unlike lower eukaryotes such as *Drosophila melanogaster* and *Saccharomyces cerevisiae*, which appear to have only a single enzyme form [1, 2, 7, 32]. The two isoforms display different patterns of temporal and spatial expression. Topoisomerase IIβ is expressed in a wide range of tissues, whilst topoisomerase IIα is expressed predominantly in tissues containing proliferating cells, [5, 10, 26]. The two human isoforms thus appear to be regulated independently and may have different roles in the cell.

Topoisomerase II is the intracellular target for a number of structurally diverse clinically important antitumour agents, including doxorubicin, epipodophyllotoxins, doxorubicin and mAMSA (amsacrine). These anti-topoisomerase II agents interfere with DNA breakage-reunion by the enzyme, stabilising an enzyme-DNA intermediate or 'cleavable complex' in which the two DNA strands are broken and covalently attached at their 5′ termini, one to each subunit of the enzyme dimer [29, 30]. Cleavable complexes are the principal lesion by which topoisomerase II inhibitors exert their cytotoxic effects. During transcription and replication, cleavable complexes are processed into DNA strand breaks, which trigger cell death. Hence, the cytotoxicity of these drugs is not only dependent upon the intrinsic properties of the target enzyme but is also a function of the cellular events involved in processing the drug-DNA-enzyme ternary complexes [27]. Both human α and β topoisomerase II isoforms are inhibited in vitro by cleavable-complex-forming agents [3, 10].

A second class of topoisomerase II inhibitors, including merbarone, suramin and ICRF193, act by mechanisms that do not involve stabilisation of the cleavable complex [4, 6, 9, 17, 18, 31]. ICRF 193 acts by stabilising the closed-clamp form of DNA topoisomerase II and prevents its conversion to the open-clamp form [28]. The precise mode of action of suramin and merbarone is not yet known.

Resistance to anti-topoisomerase II agents develops by two routes: 'classical' multidrug resistance (MDR) involving P-glycoprotein and atypical MDR, a form of resistance specific to topoisomerase II-targeting agents. Atypical MDR involves a decrease in topoisomerase II protein levels or alterations within the protein. Cells with diminished enzyme levels display resistance to a variety of cleavable-complex-forming drugs. Conversely, overexpression causes hypersensitivity to anti-topoisomerase II agents [11]. Both human topoisomerase II isoforms have been implicated in drug resistance [8, 12–14]. Human topoisomerase IIβ protein levels have been shown to be down-regulated in two drug-resistant cell lines [8, 12–14]. However, the relative importance of the two isoforms as in vivo targets for anti-topoisomerase II agents remains to be clarified.

A yeast model system for in vivo analysis of the interaction of yeast topoisomerase II with its anticancer inhibitors has been developed by Nitiss and Wang [23]. This system has been used extensively to analyse the effects of anti-cancer agents on yeast topoisomerase II. Overexpression of yeast topoisomerase II rendered yeast hypersensitive to mAMSA and etoposide [24]. This system has also been used to test putative drug-resistance mutations [15, 16, 19, 25] and to select for drug-resistance mutations in yeast topoisomerase II [14, 20, 22, 23, 35]. This approach is very powerful and has provided a wealth of useful information about drug interactions with yeast topoisomerase II. However, the more relevant targets for cancer chemotherapy are human topoisomerase IIα and β. We have previously utilized an in vivo yeast system to study human topoisomerase IIα [36]. In this paper, we extend this work to human topoisomerase IIβ, describing for the first time the complementation of a yeast *top2 ts* mutation with a plasmid-borne human TOP2β gene. The effects of anti-cancer agents on human topoisomerase IIβ in this yeast in vivo system are presented, and compared with the results of parallel experiments involving yeast topoisomerase II or the human α form.

Materials and methods

Materials

Chemicals and drugs

Merbarone, suramin, mAMSA and oAMSA were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, (Bethesda, Md.) Mitoxantrone, doxorubicin, quercetin and genistein were obtained from Sigma. Etoposide was kindly provided by Prof. Herbie Newell. Drugs were dissolved in appropriate solvents as follows: dimethylsulfoxide (DMSO) for mAMSA, oAMSA, genistein, quercetin and merbarone; methanol for etoposide;, and water for suramin, mitoxantrone and doxorubicin. Recombinant human topoisomerases α and β were purified as described previously [3, 36]. All other reagents were obtained from commercial sources.

Yeast strains:

Saccharomyces cerevisiae strains JN362A *a ura3–52 leu2 trp1 his7 ade1–2 ISE2*; JN394t2-4 *Matα ISE2 ura3–52 top2–4 rad52::LEU2* [23]; SD117 *MATα ade2 leu2 his7 top2–1 trp1 ura3–52*; SD119 *MATα top1–1 top2–1 trp1 ura3–52*; JCW26 *MATα, leu2, trp1–63, ura3–52, his3–200, top2–4* and JCW28 *MATα, leu2, trp1–63, ura3–52, his3–200, top2–4, Δtop1::URA3* [36] were kindly provided by Professors J.C. Wang and R. Sternglanz.

Plasmids

YEphTOP2βKLM was made by mutagenesis of plasmid YEphTOP2β. Base 495T in the TOP2β cDNA was mutated to an A using the Stratagene Chameleon mutagenesis system according to the manufacturer's instructions. Plasmids YEpWOB6, YCpDEDWOB10, YEphTOP2β, YCp50 and YEpTOP2-PGAL1(G1T2) have been described elsewhere [3, 36].

Methods

Protein concentrations were measured by the method of Bradford using the Biorad protein assay kit. DNA topoisomerase II activity was determined by relaxation of a supercoiled pBluescript-derived plasmid and by decatenation of kinetoplast DNA (kDNA) [36].

Complementation analysis

The viability of yeast strains transformed with various plasmids was tested by growth at 25 °, 30 ° and 35 °C. This was done either qualitatively by streaking single colonies onto the test plate or quantitatively by plating cultures of known optical density (OD) from microtitre trays using a replicator consisting of an aluminium block with an array of cylindrically shaped prongs. Yeast strains were grown in selective liquid culture at 25 °C to an OD_{600} of 1 and then serially diluted in sterile microtitre trays. These cultures were transferred to the plates using the replicator. All experiments were carried out at least twice.

Determination of drug sensitivity in vivo

Two methods have previously been reported for the analysis of drug sensitivity in this in vivo yeast system; we have utilised both. The first method involved continuous drug exposure by plating the yeast on agar plates containing drugs at varying concentrations and incubation at 25 ° or 35 °C for 3–4 days [36]. The second method involves short-term drug exposure in liquid culture followed by growth in drug-free plates [23].

For short-term drug exposure, logarithmically growing yeast strain JN394 *top2–4* transformed with a human TOP2α, human TOP2β or yeast TOP2 plasmid was cultured in selective media at 25 °C; diluted to a titre of 5×10^6cells/ml in complete medium (YPDA) containing yeast extract, bacto-peptone, glucose (dextrose) and adenine sulfate and grown for 1 h at 35 °C. Pregrowth at 35 °C inactivates yeast topoisomerase II derived from the host *top2–4* allele, so the only active topoisomerase II is produced by the plasmid- borne TOP2 gene. Drug or solvent was added and growth was continued for a further 16 hours. The yeast were then diluted appropriately and plated in triplicate in molten agar. Plates were incubated for 3–4 days at 35 °C, and the number of colonies were counted. The percentage survival was determined by comparison of the number of colonies counted in the no-drug control culture with those in the drug-treated culture. The IC_{50} is the drug concentration that reduces the number of colonies by 50% as

compared with cells grown in the absence of drug. All experiments were repeated at least three times and the means and population standard deviations were calculated for each set of data. The Student's *t*-test (two tailed paired) was performed to determine the statistical significance of the differences between dose-response curves.

Cleavage of supercoiled plasmid DNA

Cleavage reactions were carried out in 50 m*M* TRIS-HCl buffer, (pH 7.5), 100 m*M* KCl, 10 m*M* $MgCl_2$, 0.1 m*M* dithiothreitol (DTT), 0.5 m*M* ethylenediaminetetraacetic acid (EDTA), and bovine serum albumin (BSA) at 30 μg/ml, containing 0.4 μg of supercoiled pBluescript-derived plasmid and 4 pmoles of human topoisomerase IIα or β in the presence or absence of the drugs. After incubation for 1 hour at 37 °C, sodium dodecyl sulfate (SDS) was added to a final concentration of 1% followed by proteinase K to a final concentration of 0.5 mg/ml, and incubation was continued for 1 hour at 37 °C. Loading buffer was added (0.5% SDS, 25% glycerol, 0.1% bromophenol blue) and samples were analysed by electrophoresis in 0.8% agarose in TBE buffer (89 m*M* TRIS, 89 m*M* boric acid and 2.5 m*M* EDTA). Gels were stained with ethidium bromide and photographed under UV transillumination.

Inhibition of DNA relaxation

DNA-relaxation reactions containing 0.4 μg of supercoiled pBluescript-derived plasmid DNA were carried out in the absence or presence of drugs in 50 m*M* TRIS-HCl (pH 7.5), 100 m*M* KCl, 10 m*M* $MgCl_2$, 0.1 m*M* DTT, 0.5 m*M* EDTA, 1 m*M* ATP and 30 μg/ml BSA, (R buffer), 250 ng of recombinant human DNA topoisomerase II and solvent (DMSO or methanol) to a maximum of 5% (vol/vol). Following incubation at 37 °C for 1 h the reaction was stopped by the addition of loading buffer. The products were analysed by gel electrophoresis in 0.8% agarose gels in 1X TBE. Gels were stained with ethidium bromide and photographed under UV transillumination.

Results

Human TOP2β construct complements the *top2−1* and *top2−4 ts* mutations in *Saccharomyces cerevisiae*

We have previously described plasmids YEpWOB6 and YEphTOP2β which carry the respective human TOP2α and TOP2β cDNA sequences downstream of the GAL1 promoter [3, 36]. Except for their TOP2 inserts, the plasmids were identical, bearing *URA* and *amp* genes, and the replication origins of pBR322 and the 2 μm plasmid allowing growth and selection in *Escherichia coli* or yeast. Unlike YEpWOB6, construct YEphTOP2β was not capable of rescuing growth at 35 °C of *S. cerevisiae* strains carrying *top2−1* or *top2−4* temperature-sensitive mutations in their single chromosomal TOP2 gene [36, unpublished results). The TOP2β gene in YEpTOP2β produces a recombinant protein in which residues 46−1621 of the human β isoform are linked to the first 5 residues of *S. cerevisiae* topoisomerase II. Although the recombinant β protein was catalytically active [3], it evidently lacks complementing activity in yeast, possibly due to alteration of its N-terminal end. The construction of YEpTOP2β involved the introduction of two base changes into the TOP2β cDNA that concomitantly altered codon 165 to specify serine instead of the conserved arginine found in the wild-type protein. Given that the N-terminally truncated α protein produced by YEpWOB6 can complement in yeast, we decided to revert the serine codon to arginine in YEphTOP2β and test its effect on complementation. This change was achieved by site-directed mutagenesis, generating the new construct YEphTOP2βKLM. The ability of this plasmid to foster growth of *S. cerevisiae top2* mutants was then tested under a variety of conditions (Fig. 1, and Table 1).

S. cerevisiae ura3 strains SD117 and JCW26 bearing *top2−1 ts* or *top2−4 ts* mutations, respectively, and double mutants SD119 and JCW28 bearing *top1−1, top2−1 ts* and Δtop1, top2−4 ts mutations, respectively, were transformed to *URA+* with plasmids encoding human α (YEpWOB6), human β (YEphTOP2β KLM) or yeast topoisomerase II (YCpDEDWOB10). (YCpDEDWOB10 is a single-copy plasmid constructed for the expression of yeast TOP2 gene from a constitutive yeast DED1 gene promoter [24]). Liquid cultures were grown to an OD_{600} of 1 and then serially diluted in microtitre trays prior to transfer onto a range of plates. The strains were tested for growth at both 25 ° and 35 °C on either URA-selective media or complete media (YPA), using three different carbon sources: glucose, raffinose and galactose. As the plasmid-encoded human TOP2 cDNAs are under the control of the GAL1 promoter, the carbon source affects their level of expression. On glucose plates, the GAL1 promoter is repressed such that only a low level of topoisomerase II protein is produced. Galactose activates the GAL1 promoter, inducing high levels of topoisomerase II, whilst raffinose produces intermediate effects as it neither represses nor induces expression from the GAL promoter [33]. Figure 1 shows the growth of the various transformants on complete medium with glucose as the carbon source (YPDA). Untransformed or transformed SD117 *top2−1* or JCW26 *top2−4* strains grew normally at 25 °C, a permissive temperature for these temperature-sensitive mutants. As expected, at 35 °C no growth was observed for the untransformed strains or transformants containing the control vector plasmid YCp50 (Fig. 1, columns a and b). However, introduction of YCpDEDWOB10 bearing yeast TOP2 allowed the growth of both strains under all conditions, demonstrating that expression of a functional topoisomerase II could complement the *ts* mutation (Fig. 1, column c). Similarly, transformation of JCW 26 *top 2−4* and SD117 *top 2−1* with either YEpWOB6 or YEphTOP2βKLM, plasmids encoding human topoisomerase IIα or β, was also capable of rescuing the lethal phenotype of the parent strains at 35 °C (Fig. 1, columns d−f). The human α and β TOP2 plasmids facilitated similar levels of growth in these *ts* strains. Thus, the human TOP2β cDNA could rescue the growth of *top2−1* and *top2−4* strains, indicating that the recombinant human β enzyme expressed from YEphTOP2βKLM is functional in this heterologous yeast system.

Topoisomerase I can perform some of the functions of topoisomerase II. Therefore, double mutants JCW28 (Δ*top1, top2−4)* and SD119 *(top1−1, top2−1),* whose topoisomerases I and II are non-functional at the non-permissive temperature, were also studied on glucose-

containing medium (Fig. 1). At 25 °C, both of the strains and their transformants grew as expected. Neither non-transformed strain or YCp50 transformants grew at 35 °C. Strain JCW28 (Δ*top 1, top2−4)* transformed with a plasmid encoding yeast topoisomerase II, human topoisomerase IIα or β survived at the non-permissive temperature (Fig. 1, columns c−f). With strain SD119 *(top1−1, top2−1)*, complementation was seen with human topoisomerase IIα or β but growth was less vigorous than that obtained with JCW28. The double mutants grew less vigorously than the single mutants, but both TOP2α and TOP2β supported growth, though less well than the yeast TOP2.

The ability of human TOP2β to complement yeast *ts* mutants was also analysed on media containing raffinose or galactose. A summary of at least two independent experiments to analyse yeast growth at 35 °C on both selective and complete media with either glucose, raffinose or galactose as carbon sources is shown in Table 1. Strains carrying a single *ts* mutation (JCW26 and SD117) transformed with either TOP2α, TOP2β or yeast TOP2 were capable of growing on both media with any of the three sugars. However, growth was significantly reduced for TOP2α or TOP2β transformants on media containing galactose, suggesting that high-level expression of topoisomerase II is toxic. The double mutants JCW28 and SD119 transformed with YCpDEDWOB10, a low-copy-number plasmid bearing yeast TOP2 under the control of the constitutive pDED promoter, grew on all media. Double mutant JCW28 transformed with human TOP2α grew on all three sugars, but with human TOP2β, very poor growth, was observed on galactose-containing media (Table 1). Conversely, SD119 transformed with TOP2β grew on all media, whilst TOP2α growth was media dependent (Table 1). Thus, a human TOP2 β cDNA can complement temperature-sensitive yeast *top2* mutations, in both the presence and the absence of a functional topoisomerase I.

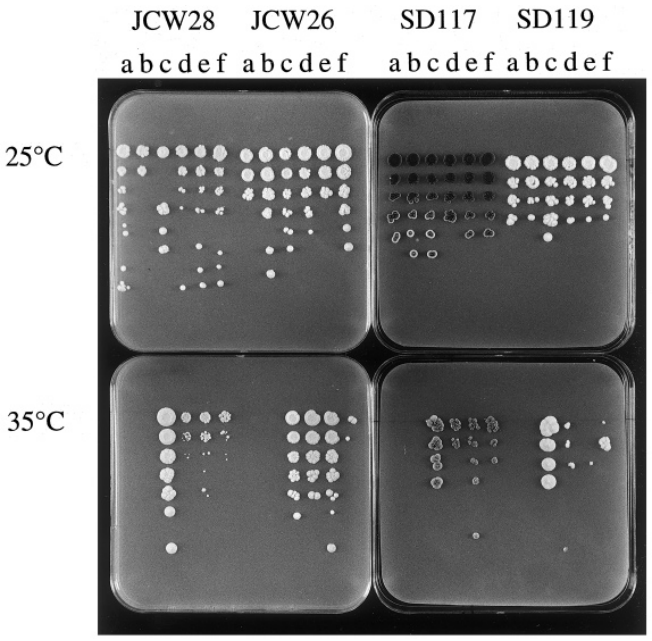

Fig. 1 Human TOP2β construct rescues the growth of *Saccharomyces cerevisiae top2ts* strains. Strains SD117, JCW26, SD119 or, JCW28 (*a*); were transformed with vector YCp50 (*b*); YCpDEDWOB10 bearing yeast TOP2 (*c*); YEpWOB6 bearing human TOP2α (*d*); and YEpTOP2βKLM bearing human TOP2β (*e, f*). The strains or their transformants were grown to an OD_{600} of 1 and serially diluted into a multiwell plate prior to transfer by a replicator onto YPDA agar plates. Plates were grown at 25 ° or 35 °C as indicated

Drug sensitivity of yeast strains expressing human DNA topoisomerase IIβ

To test whether the recombinant human topoisomerase IIβ expressed in yeast could serve as a target for anticancer drugs in vivo, yeast strain JN394 *top2−4 Matα ISE2 ura3−52 top2−4 rad52::LEU2* [23] was employed. This strain has the *top2−4* mutation allowing growth at 25 °C but not at 35 °C. The *ISE2* permeability mutation facilitates drug uptake and the *rad52* mutation renders the yeast defective in double-stranded DNA break repair, thereby sensitising the strain to the effects of topoisomerase inhibitors [23]. At 25 °C, JN394*top2−4* was drug-sensitive, indicating that the endogenous yeast *top2−4* enzyme is sensitive to drugs at the permissive temperature. In previous work it has been shown that the growth of JN394*top2−4* at 35 °C is strictly dependent on transformation with a plasmid-borne TOP2 gene, e.g. yeast TOP2 plasmid YEpTOP2-PGAL1 or human TOP2α plasmid YEpWOB6, each of which has TOP2 under GAL1 regulation. We therefore tested the drug sensitivity of JN394*top2−4* transformed with YEphTOP2βKLM in comparison with YEpTOP2-PGAL1 and YEpWOB6 transformants at 35 °C. As additional controls, JN394*top2−4* transformed with YCp50, YCpDEDWOB10 and YEpTOP2β and the parental strain JN362A *ISE2* were also examined (data not shown).

The drug sensitivities of yeast transformants were tested initially by plating on agar plates containing drugs at varying concentrations and incubating at 25 ° or 35 °C for 3−4 days [36]. This method ensures continuous exposure to drug for an extended period and allows a rapid and direct comparison of relative sensitivites under identical conditions. Doxorubicin, mitoxantrone and mAMSA were the most toxic agents in this assay. Quercetin, genistein and oAMSA were the least toxic and were not studied further. However, this procedure is only semi-quantitative. For quantitative analysis of drug killing a second method was used involving short-term exposure to drug in liquid culture followed by growth in drug-free agar for 3−4 days [23]. Yeast were grown at 25 °C until they reached mid-log phase, were then diluted to 10^7 cells/ml and were then incubated at 35 °C for 1 h to inactivate the temperature-sensitive host topoisomerase II. Either drug or the relevant solvent control (DMSO or methanol) was then added and the cells were grown for a further 16 h at 35 °C. The yeast were then diluted appropriately, plated in molten YPDA agar (48 °C) without drug and incubated for 3−4 days at 35 °C. The yeast cells were plated in agar as this produced a more even spread of colonies than surface spreading,

Table 1 Summary of the complementation analysis of temperature-sensitive yeast topoisomerase II by human topoisomerase IIα or β at 35 °C. Four yeast strains – SD117, SD119, JCW26 and JCW28 – were transformed with control plasmid (YCp50) or plasmids expressing yeast topoisomerase II (YCpDedWOB10) or human topoisomerase IIα (YEpWOB6) or β (YEphTOP2βKLM). Liquid cultures of each transformant were grown in glucose containing minimal media at 25 °C to an OD_{600} of 1 prior to serial dilution and imprinting onto agar plates containing the medium indicated. Plates were incubated at 25 °, 30 ° and 35 °C and the results obtained at 35 °C are shown. No growth is indicated by *minus symbols* and poor to very good growth are indicated by *plus/minus* and *plus* or *multiple plus symbols*. All experiments were repeated at least twice. For YEphTOP2βKLM a number of independent transformants of each strain were tested and representive results are shown

Strain	Plasmid	Media					
		-Uracil + glucose	-Uracil + raffinose	-Uracil + galactose	YPA + glucose	YPA + raffinose	YPA + galactose
top2 ts							
SD117	None	–	–	–	–	–	–
(*top2–1*)	YCp50	–	–	–	–	–	–
	YCpDEDWOB10	++	+++	++	+++	+++	++
	YEpWOB6	++	+++	+/–	++	++	+/–
	YEpTOP2βKLM	++	++	+/–	++	++	+/–
JCW26	None	–	–	–	–	–	–
(*top2–4*)	YCp50	–	–	–	–	–	–
	YCpDEDWOB10	++	++	+++	+++	++	++
	YEpWOB6	++	++	+	+++	++	+/–
	YEpTOP2βKLM	++	++	+/–	+++	++	+
top1 top2 ts							
SD119	None	–	–	–	–	–	–
(*top1–1,*	YCp50	–	–	–	–	–	–
top2–1)	YCpDEDWOB10	+++	++	+++	++	++	+++
	YEpWOB6	–	+/–	+/–	+	+/–	+/–
	YEpTOP2βKLM	+	+	+	++	+	+
JCW28	None	–	–	–	–	–	–
(Δ*top1,*	YCp50	–	–	–	–	–	–
top2–4)	YCpDEDWOB10	++	+++	+++	+++	+++	++
	YEpWOB6	+	+	++	++	+	+
	YEpTOP2βKLM	++	+	+/–	++	+	–

making counting easier. Individual colonies were counted and the percentage of survival was calculated by comparison to the cell number in the control cultures treated with solvent alone. The percentage survival versus drug concentration were plotted and are shown in Fig. 2a–f ; each point is the average of at least three experiments. Kill curves were determined for mAMSA, etoposide, doxorubicin, mitoxantrone, suramin and merbarone. The drugs fell into two categories: agents to which human TOP2α and TOP2β yeast transformants showed similar sensitivity, i.e. mAMSA, suramin and merbarone, and those to which the TOP2α transformant was more sensitive than that bearing TOP2β i.e. etoposide, doxorubicin and mitoxantrone.

For mAMSA a concentration range of 1–50 μg/ml was tested (Fig. 2a). At 1 μg/ml an initial 20–30% drop in survival was observed for yeast transformants containing any one of the three TOP2 plasmids. Similar survival profiles were observed for TOP2α and TOP2β transformants, yielding IC_{50} values of 7.75 and 9.75 μg/ml, respectively (Table 2). The yeast transformant bearing a plasmid encoding yeast topoisomerase II was about 4 times less sensitive than TOP2α or TOP2β transformants, having an IC_{50} of 38 μg/ml. The effects of short-term exposure to etoposide (5–200 μg/ml) are shown in Fig. 2b. At 100 and 200 μg/ml the yeast strain expressing human α was significantly more sensitive than the yeast expressing human β or wild-type yeast topoisomerase II as reflected in the respective IC_{50} values of 23, 77 and 79 μg/ml (Table 2).

Of all the drugs tested, doxorubicin exhibited the most dramatic cell killing (Fig. 2c); at 50 μg/ml there was little survival, if any, of yeast bearing any of the three plasmids. Yeast expressing human topoisomerase IIα exhibited significantly greater sensitivity to doxorubicin, with only 50% survival being observed at 2.25 μg/ml and very little survival being seen at concentrations above 10 μg/ml. Yeast expressing human β or yeast topoisomerase II shared a similar survival pattern, both being 3–4 times less sensitive to doxorubicin than the TOP2α transformant. Similar responses were seen for mitoxantrone survival curves (Fig. 2d).

Two non-cleavable complex-forming drugs, suramin and merbarone, were also analysed. These two agents were the least effective drugs tested. Thus, yeast expressing human α, β or yeast topoisomerase II were capable of surviving a maximal suramin dose of 1 mg/ml (Fig. 2e). Merbarone was tested between 50 and 500 μg/ml; TOP2α and TOP2β transformants showed comparable levels of survival (Fig. 2f). Somewhat surprisingly, the yeast TOP2 transformant was significantly more sensitive to merbarone (Fig. 2f, Table 2).

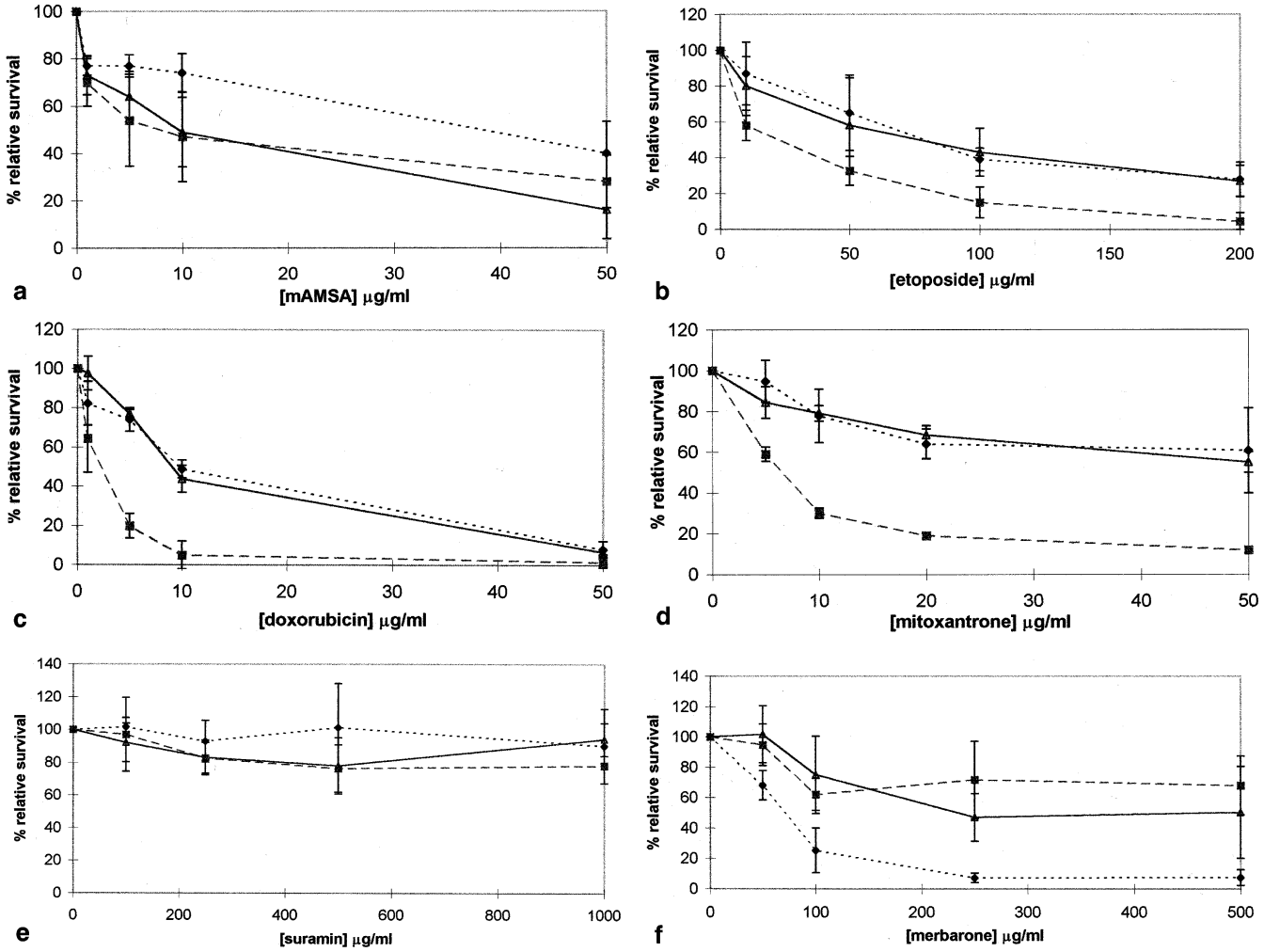

Table 2 IC_{50} values recorded for topoisomerase II-targeting agents on yeast JN394*top2–4*transformants

Drug	JN394*t2–4* + plasmid	Protein	IC_{50} (μg/ml)
Etoposide	YEpTOP2-PGAL1	Yeast Topo II	79
	YEpWOB6	Human Topo IIα	23
	YEphTOP2βKLM	Human Topo IIβ	77
mAMSA	YEpTOP2-PGAL1	Yeast Topo II	38
	YEpWOB6	Human Topo IIα	8
	YEphTOP2βKLM	Human Topo IIβ	10
Doxorubicin	YEpTOP2-PGAL1	Yeast Topo II	10
	YEpWOB6	Human Topo IIα	2
	YEphTOP2βKLM	Human Topo IIβ	9
Mitoxantrone	YEpTOP2-PGAL1	Yeast Topo II	>50
	YEpWOB6	Human Topo IIα	7
	YEphTOP2βKLM	Human Topo IIβ	>50
Merbarone	YEpTOP2-PGAL1	Yeast Topo II	70
	YEpWOB6	Human Topo IIα	>500
	YEphTOP2βKLM	Human Topo IIβ	235
Suramin	YEpTOP2-PGAL1	Yeast Topo II	>1000
	YEpWOB6	Human Topo IIα	>1000
	YEphTOP2βKLM	Human Topo IIβ	>1000

Fig. 2a–f Viability of yeast transformants following exposure to topoisomerase II inhibition, Strain JN394*t2–4* bearing either plasmid-borne wild-type yeast topoisomerase II (YEpTOP2-PGAL1, ...◆.....), plasmid-borne human DNA topoisomerase IIα (YEpWOB6, ---■---) or plasmid-borne human DNA topoisomerase IIβ (YEphTOP2βKLM, ——Δ——) were treated for 16 h with increasing concentrations of the indicated drug at 35 °C. After 16 h three aliquots of cells were taken, plated in YPDA and grown for 3–4 days to determine the titre. The percentage survival was determined by comparison with the no-drug control. Plots represent an average of three independent experiments. Error bars indicate ±1SD from the mean. **a** mAMSA. **b** Etoposide (VP16). **c** Doxorubicin. **d** Mitoxantrone. **e** Suramin. **f** Merbarone

Comparison of topoisomerase IIα and β interactions with drugs in vitro

The in vitro effect of the anti-topoisomerase II agents on purified human topoisomerase IIα and the human topoisomerase IIβ (produced from plasmid YEpTOP2βKLM) were analysed in one of two ways: promotion of DNA cleavage or inhibition of relaxation. In vitro cleavage of supercoiled plasmid DNA was used to assess the relative in vitro inhibition of human topoisomerases. Recombinant human topoisomerase IIα and human topoisomerase IIβ were analysed. Etoposide, mAMSA and oAMSA cleavage

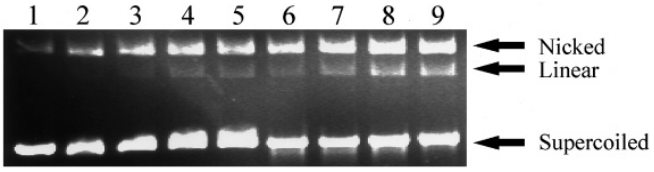

Fig. 3 Mitoxantrone-stimulated DNA cleavage of supercoiled DNA by topoisomerase IIα and β in vitro. *Lane 1* Supercoiled DNA; *lane 2* human topoisomerase IIα with no drug; *lanes 3–5* human topoisomerase IIα with mitoxantrone at 0.125, 0.25 and 0.5 μg/ml, respectively; *lane 6* human topoisomerase IIβ with no drug; *lanes 7–9* human topoisomerase IIβ with mitoxantrone at 0.125, 0.25 and 0.5 μg/ml, respectively

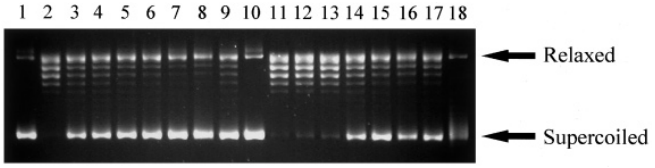

Fig. 4 Inhibition by merbarone of DNA relaxation by human topoisomerase IIα and β. Purified enzymes (250 ng) were incubated with 0.4 μg of supercoiled plasmid DNA with and without merbarone and incubated for 1 h at 37 °C. *Lane 1* Supercoiled plasmid alone; *lane 2* human topoisomerase IIα with no drug; *lanes 3–8* human topoisomerase IIα with merbarone at 31.25, 62.5, 125, 250, 500 and 1000 μg/ml, respectively; *lane 9* human topoisomerase IIα with no ATP; *lane 10* supercoiled plasmid alone; *lane 11* human topoisomerase IIβ with no drug; *lanes 12–17* human topoisomerase IIβ with merbarone at 31.25, 62.5, 125, 250, 500 and 1000 μg/ml, respectively; *lane 18* human topoisomerase IIβ with no ATP

has been reported elsewhere [3]. Mitoxantrone cleavage was significantly enhanced with the β isoform as compared with the α isoform. The amount of cleavage was determined in several experiments by scanning the gels using a Biorad gel documentation system and Molecular Analyst software. A sample gel is shown in Fig. 3. Human topoisomerase IIβ promoted cleavage at 0.125 μg/ml, whilst the α isoform needed at least 4 times more drug (>0.5 μg/ml) to promote similar levels of cleavage. The non-cleavable complex formers suramin and merbarone were analysed by their inhibition of relaxation. Suramin inhibited both α and β isoforms to similar extents (data not shown). Merbarone inhibited α at 2- to 3-fold lower concentrations as compared with β; as shown in Fig. 4, yeast topoisomerase II required 8 times more merbarone than did human topoisomerase IIα to inhibit relaxation (data not shown).

Discussion

The present study demonstrates that expression of human topoisomerase IIβ from plasmid YEphTOP2βKLM rescues growth of *Saccharomyces cerevisiae* mutants bearing either *top2–1* or *top2–4 ts* mutations affecting the host topoisomerase II (Fig. 1). In previous work (confirmed herein), we showed the complementing activity in yeast of human topoisomerase IIα expressed from plasmid YEpWOB6 [36], but this is the first report of a yeast system in which growth is dependent on the human β isoform. It appears that either human isoform can substitute for yeast topoisomerase II and discharge its essential functions in chromosome condensation and segregation. The construction of YEphTOP2βKLM and YEpWOB6 involved cloning of the appropriate TOP2 cDNA in the same vector downstream of a GAL1 promoter, allowing different levels of induction, depending on the carbon source used for growth (Table1) [3, 36]. These constructs and the corresponding yeast TOP2 plasmid, YEpTOP2-PGAL1, allowed us to compare under identical conditions the drug sensitivities of yeast JN394 *top2-4ts* transformants growing at 35 °C by virtue of human topoisomerase IIβ, α or yeast topoisomerase II.

The survival of JN394 *top2–4* transformants was examined after both continuous and short-term exposure to topoisomerase II-targeting drugs, including the cleavable-complex-forming agents mAMSA, etoposide, doxorubicin and mitoxantrone and two inhibitors, suramin and merbarone, that act on topoisomerase II by a non-cleavable-complex mechanism (Fig. 2). The IC_{50} values (Table 2) illustrate the three main conclusions. First, irrespective of whether human α, β or yeast topoisomerase II was expressed in JN394*top2–4*, the DNA intercalator doxorubicin was the most lethal drug tested. The other intercalators mAMSA and mitoxantrone were somewhat less potent than doxorubicin, followed by etoposide, merbarone and suramin. Second, in terms of their differential inhibition of TOP2α, TOP2β and yeast TOP2 transformants, the drugs could be assigned to two classes: those such as mAMSA, merbarone and suramin, which displayed similar killing abilities toward TOP2α and TOP2β transformants, and those including doxorubicin, mitoxantrone and etoposide, to which JN394top2–4 expressing the human α isoform was the most sensitive. None of the agents tested preferentially inhibited yeast expressing human topoisomerase IIβ. Third, merbarone was unique in that the strain expressing yeast TOP2 was the most sensitive (Fig. 2f). These results can be considered in the context of what is known about topoisomerase II inhibitor action in vivo and in vitro.

Doxorubicin, mAMSA and mitoxantrone are highly cyotoxic to a variety of human cell lines, with lower toxicities being displayed by etoposide, merbarone and suramin. In principle, both α and β isoforms may contribute to drug sensitivity. Studies of drug-resistant cell lines suggest that both α and β isoforms play a role. A drug-resistant cell line selected for resistance to ellipticine is down-regulated for β [8]. A mitoxantrone resistant cell line has been reported that has human topoisomerase IIβ down-regulated and alterations in topoisomerase IIα [12–14]. These cell lines provide circumstantial evidence that both α and β forms can indeed be in vivo drug targets. However, the complexity of the genetic background in human cells combined with the expression of two topoisomerase isoforms has hindered an understanding of the relative contributions of the α and β as drug targets. The importance of α and/or β isoforms as killing targets for these drugs in human cells and tumours will depend on the nature and

levels of their respective cleavable complexes and how these lesions are processed. Factors other than intrinsic drug interactions with the target, such as the levels of cleavable-complex formation, the intracellular α and β levels, target accessibility, or differential cellular responses to α- and β-induced DNA damage could modulate the anti-cancer drug action. Previous studies have identified yeast topoisomerase II as the target in *S. cerevisae* for several anticancer drugs, including mAMSA. The work reported herein suggests that either human topoisomerase IIα or, indeed, β can act as a drug target in the *in vivo* yeast system.

Irrespective of whether the human α or β isoform was being expressed, the yeast JN394*top2–4* system showed the same general ranking of drug sensitivities as human cells, notably the high-level cytotoxicity of the DNA intercalators doxorubicin, mitoxantrone and mAMSA (Fig. 2). Using purified human α and β topoisomerase II, we have shown that both enzymes are inhibited and undergo comparably efficient cleavable-complex formation with mAMSA in vitro [3, 21]. These results are in accord with the comparable sensitivities of the appropriate yeast transformants (Fig. 2). However, in two cases, in vitro and in vivo results were not in accord. Interestingly, mitoxantrone promoted significantly more DNA cleavage *in vitro* with human topoisomerase IIβ than with α (Fig. 3), in contrast to the observation in vivo that the drug is more effective in killing yeast cells expressing the α isoform (Fig. 2d). Similarly, inhibition of topoisomerase II isoforms in vitro by the non-cleavable complex-forming drug merbarone did not mirror the observed effects in yeast. Although merbarone was least effective in inhibiting DNA relaxation by yeast topoisomerase II (Fig. 4), yeast transformed with a yeast TOP2 plasmid were the most sensitive to killing by the drug (Fig. 2f). It is conceivable that merbarone is metabolised in vivo to a more active compound that has a greater inhibitory effect on the yeast enzyme than on the α or β isoform. Alternatively, differences between in vitro and in vivo results may arise simply because the enzymatic assay measures purely the interaction with the target, whereas cell killing arises from a complex process involving not only topoisomerase II inhibition but also DNA damage or repair and induction of cell death. These complexities have not been examined in detail for the yeast system but should be borne in mind as potential modulators of the drug response *in vivo*.

In summary, we have established a yeast system in which cell growth is dependent on the expression of human topoisomerase IIβ. We show that either the human α or β isoform can act as a drug target. The system can be used to test both existing drugs and novel compounds against one or the other human isoform independently. Moreover, the dominant phenotype of YEphTOP2βKLM in yeast JN394*top2–4* should prove useful in allowing the selection of drug-resistance mutations in human topoisomerase IIβ or in screening the phenotypes of putative TOP2β resistance mutations identified in tumour cell lines or biopsy material.

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