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# Yeast cells expressing differential levels of human or yeast DNA topoisomerase II: a potent tool for identification and characterization of topoisomerase II-targeting antitumour agents

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**Abstract** *Purpose*: To identify and characterize the specificity and potency of topoisomerase II-interacting antitumour drugs in an in vivo model utilizing the yeast *Saccharomyces cerevisiae*. *Methods*: Four yeast transformants were selected for the expression of either human or yeast DNA topoisomerase II at different, biologically relevant, levels under the tight control of promoters of various strengths. *Results*: Analyses of 24 drugs permitted their classification into three distinct groups, depending on whether they induced topoisomerase II-related cytotoxicity (etoposide), showed nonspecific cytotoxicity (camptothecin), or exerted no cytotoxicity at all (vinorelbine). Within the first group different patterns of action were distinguishable: (1) classical topoisomerase II expression-dependent cytotoxicity for both species of enzyme (e.g. etoposide, amsacrine, doxorubicin, actinomycin D), although amsacrine and TOP 53 were more active, respectively, on human and yeast topoisomerase II; and (2) compounds that appeared to poison only one species of topoisomerase II with, for example, genistein and the bisdioxopiperazine ICRF-193 lethally targeting only the human type, and mitoxantrone only the yeast isozyme. Three of the 16 known topoisomerase II inhibitors tested were not correctly identified with this assay, possibly owing to restricted cell wall permeability or to the absence of correct processing pathways such as, for example, in the case of the prodrug etopophos. *Conclusion*: This methodology, in vivo in yeast, selected for a large range of potent topoisomerase II-targeting anticancer agents. Of particular interest in this yeast model, and in contrast to yeast topoisomerase II, human topoisomerase II was shown to confer dominant sensitivity in the presence of the series of bisdioxopiperazine derivatives tested. This assay therefore has the potential easily to identify and characterize the potency and specificity of synthesized anticancer drugs with modified original chemical structures or those present, for example, in natural plant extracts or marine organisms.

**Key words** Topoisomerase II · Human · Yeast · Antitumour drugs · Bisdioxopiperazine compounds · ICRF-193

**Abbreviations** *hTOP2* human topoisomerase II · *hTOP2*α human topoisomerase IIα · *kDNA* kinetoplast DNA · *TOP1* topoisomerase I · *TOP2* topoisomerase II · *yTOP2* yeast topoisomerase II

## Introduction

Topoisomerase-targeting antitumour agents exert their cytotoxic effects by altering well-defined steps of the catalytic cycle of topoisomerase II (TOP2). It has been proposed that the stabilization of cleavable complexes formed between DNA and TOP2 acts as a type of unrepaired DNA damage and, therefore, that cells are killed by antitopoisomerase agents, since the enzyme is converted into a poison, rather than by any general lack of essential enzyme activity [19, 28, 29].

Yeast has already proved of definite value in demonstrating the specificity of agents that target topoisomerase I (TOP1). Indeed, several groups have demonstrated that camptothecin specifically targets TOP1 by showing that yeast *top1* null mutants are completely resistant to the cytotoxic action of the drug and that restoration of the expression of yeast TOP1 by plasmid transformation is accompanied by a resensitization to camptothecin [8, 25]. Furthermore, the level of TOP2 activity has been shown to influence drug sensitivities in tumours [16, 33] and in cell lines [4, 7, 11], and Nitiss has shown that overexpression of TOP2 in yeast confers drug hypersensitivity, whereas its downregulation leads to drug resistance [23]. These studies therefore confirm that the cellular content of TOP2 can be an

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important determinant of cellular sensitivity towards TOP2-targeting compounds.

There are several advantages in using yeast to study antitopoisomerase drugs, compared with standard mammalian cell culture: (1) as a single-cell eukaryotic organism, *Saccharomyces cerevisiae* (the budding yeast) can be considered the simplest eukaryotic in vivo model for drug screening because of its rapid growth and nonpathogenic nature; (2) its genetics are well-defined, including a fully sequenced genome, notably demonstrating only one isotype of both the TOP1 and TOP2 genes; (3) it shows a highly versatile DNA transformation ability, along with many markers for selection of recombinants, allowing stable transformation and expression of mammalian proteins; (4) the ease of carrying out targeted gene disruption and gene replacement renders it possible to replace normal wild-type yeast genes with heterologous, mutated, or disrupted alleles [3]. Overall, these features should permit the use of this model for dissecting mechanisms of action of drugs at the molecular level, through the assessment of their effects on single mammalian elements/enzymes/protein targets/molecular pathways in yeast. It is significant also that some of the major shortcomings associated with the use of yeast in such studies have been overcome recently. For example, the ISE2 phenotype has rendered the yeast more permeable to drugs [25], and inactivation of the *RAD52* gene which is required for repair of double-stranded breaks in DNA, has greatly increased sensitivity to drugs which trap DNA/TOP2 cleavable complexes [10, 24].

However, although yeast topoisomerase genes are highly homologous with their mammalian counterparts, it is still possible that differences between the yeast and mammalian proteins result in some differences in their drug sensitivity and/or enzyme activity [15]. This drawback of screening with the yeast enzyme can nevertheless be alleviated since the genes encoding the human enzymes, TOP1 and topoisomerases IIα (hTOP2α) and IIβ have now been cloned and functionally expressed in yeast [2, 5, 21, 34].

We decided to utilize the yeast *Saccharomyces cerevisiae* to probe further the mechanism of action of TOP2-targeting drugs by establishing the cytotoxic effects of potential anticancer drugs on yeast expressing different levels of hTOP2α. Such a model system would provide evidence as to whether drug-induced cytotoxicity was truly associated with a variation in level of TOP2 and permit discrimination between a TOP2-targeting drug and a totally non-TOP2-specific cytotoxic agent, for example an antifungal agent. For this purpose, we used a plasmid construct developed by Hsiung et al. [13], the pMJ1 plasmid that constitutively overexpresses hTOP2α under the control of the yeast TOP1 (yTOP1) promoter, and then designed a plasmid that allowed for a promoter-controlled lower level of hTOP2α expression. Additionally, the recipient yeast strain used (JN394top2-4) was conditionally deficient in yTOP2 so allowing the study of yeast expressing various levels of TOP2 enzyme of human origin only, when grown at the restrictive temperature of 35 °C. The two resulting yeast transformants, differing only by their levels of constitutive hTOP2α, low or high, could then be grown simultaneously on the same medium and at the same temperature. In this way, their specific sensitivities to anticancer drugs could be compared directly.

This is in contrast to earlier studies [14, 23], in which variations in TOP2 levels relied on thermosensitive yeast strains grown at different temperatures, 25 or 30 °C, which provided the yeast with differing active metabolisms that led to differing growth rates. Furthermore, the yeast transformants developed in the present study supported moderate levels of hTOP2 expression that did not markedly alter their growth characteristics, contrasting with earlier studies in which overexpression of TOP2 under the strong galactose-inducible GAL1 promoter has been shown severely to impair yeast growth or even to lead to cell death [21, 22, 26]. Our reasoning was that yeast expressing nondeleterious levels of hTOP2α might be considered more closely to mimic clinically observed variations in TOP2 levels which have been shown to influence the drug sensitivities of human tumour cells [16, 33].

To complement this study with hTOP2 and to permit an evaluation of species specificity of TOP2 inhibitors, we also set up a similar model with two yeast transformants varying by only one trait of their phenotype, namely differing levels of yTOP2, although again with only a moderate overexpression associated with non deleterious effects on yeast growth, as detailed above for the expression of hTOP2.

In this study, therefore, we built on and extended the range of previously reported studies [12, 23, 26] using yeast as an in vivo model to identify TOP2-interacting antitumour drugs, with the aim of better characterizing their mode of action and specificity at the molecular level. Our methodology was based on analyses of the differential cytotoxicities of yeast transformants expressing varied moderate, biologically relevant, levels of either hTOP2α or yTOP2. We assayed a series of 24 standard antitumour agents, including known TOP2-targeting compounds, and characterized the potency and species specificity of these agents. Additionally, this study permitted the identification of agents that appear specifically to target hTOP2.

## Materials and methods

### Chemicals and drugs

Actinomycin D, amsacrine, cisplatin, colchicine, cytosine arabinoside, daunorubicin, distamycin A, doxorubicin, ellipticine and genistein were purchased from Sigma (Saint-Quentin Fallavier, France), camptothecin from Cipla (Bombay, India), ICRF-187 from Chiron (Suresnes, France), irinotecan from Roger Bellon (Neuilly-sur-Seine, France), suramin from RBI (Illkirch, France), mitoxantrone from Lederle (Paris-La Défense, France), and podophyllotoxin from Aldrich (Saint-Quentin Fallavier, France). The other test compounds, namely, azatoxin, epipodophyllotoxin,

etopophos, etoposide, ICRF-159, TOP 53 and vinorelbine were provided by Pierre-Fabre Médicament (Castres, France). ICRF-193 was kindly donated by Dr. A.M. Creighton (St. Bartholomew's Hospital Medical College, London, UK).

### Yeast Strains and Plasmids

The strains JEL1 (*MAT*α *leu2 trp1 ura3-52 prb1-1122 pep4 Δhis*::*PGAL1GAL4*), JN394 (*MATa ade1 ura3-52 his7 trp1 tyr1 ISE2 rad52::LEU2*) and JN394top2-4 (*MATa ade1 ura3-52 his7 trp1 tyr1 ISE2 top2-4 rad52*::*LEU2*) [23] were provided by Prof. J.C. Wang (Harvard University, Cambridge, Ma.).

YEpWOB6 allows expression from the h*TOP2*α gene under the control of the galactose-inducible GAL1 promoter [11]. pBShTOP2 contains the entire coding sequence of h*TOP2*α cDNA [32]. YCpDEDWOB10 allows expression from the y*TOP2* gene under the control of a constitutive yeast *DED* promoter [34]. pMJ1 carries the h*TOP2*α gene under the control of a constitutive y*TOP1* promoter [13]. The first three plasmids were obtained from Prof. J.C. Wang and the fourth was kindly provided by Dr. J. Nitiss, (St. Jude Children's Research Hospital, Memphis, Tenn.). pYX111 was available commercially (R&D System, Minneapolis, Minn.). All the plasmids designed for expression of TOP2 in yeast were centromeric.

### Construction of a yeast transformant that expresses hTOP2

The ATG site at position 10 of the pYX111 plasmid was removed by dual digestion with *Eco*RI and *Bam*HI. This open plasmid was recircularized by the introduction of a synthetic linker containing the restriction sites for *Mlu*I and *Pst*I between sites for *Eco*RI and *Bam*HI. The resultant p127 plasmid was then digested sequentially by *Sal*I and *Mlu*I, and then ligated with the entire coding sequence of the h*TOP2*α previously excised from pBShTOP2 plasmid with endonucleases *Mlu*I and *Xho*I. This plasmid construct, p134, thereby permitted the expression of the h*TOP2*α gene under the control of the promoter 786. Yeast were transformed with an electroporator GHT 1287B from Jouan (Toulouse, France) at 625 V for one pulse of 16 ms. Yeast were stored, propagated and grown in selective minimal media, as described elsewhere [3].

### In vivo drug screening assays

Yeast in an exponentially growing phase in liquid selective media at 35 °C were adjusted to $10^7$ cells/ml. Thereafter, serial tenfold dilutions of each culture were performed in sterile water, and 2-µl aliquots of each dilution were seeded onto Petri dishes containing selective agar medium including test compound at various concentrations or solvent alone, i.e. 1% dimethyl sulphoxide, that proved nondeleterious to yeast growth. Plates were incubated at 35 °C for 3 days to allow growth of the yeast. The surface of all the plates was then digitally processed using a Geldoc 1000 gel documentation system (Bio-Rad, Hercules, Calif.), and the density of yeast growth for each inoculum was quantitated with the associated Molecular Analyst software provided (Bio-Rad).

### Measurement of drug sensitivity

For each yeast transformant, inhibition of growth by a given concentration of drug was measured as a percentage of growth density on the control plate with solvent alone. The GraphPad Prism software was used to calculate and draw nonlinear regression curves of cytotoxicity, using the "sigmoidal dose-response (variable slope)" mode. The top plateau corresponded to yeast growth unaffected by the presence of the test compound, and the bottom one to total inhibition of growth. Drug concentrations that reduced the density of growth by 50% ($IC_{50}$ values) were also calculated. Furthermore, differential sensitivities observed between two yeast transformants challenged by any test compound were assessed in terms of the ratio of the two respective $IC_{50}$ values. A series of independent experiments indicated that this ratio had a variability not exceeding 20%. Therefore, ratios of 0.8 to 1.2 were judged indicative of an absence of any significant differential sensitivity. Additionally, results from the serial dilutions of yeast on the plates were used visually to confirm the effect of the drug on the viability of the yeast. Each test compound was evaluated at least twice in independent experiments.

### Decatenation assays

The protocol used to determine TOP2 activity through decatenation of 100 ng kinetoplast DNA (kDNA) from *Crithidia fasciculata* as a substrate was based on the TOP2 drug screening kit from Topogen (Colombus, Ohio) [22], except that a crude cell extract of proteins, prepared by glass bead lysis as described previously [20, 35] from each yeast transformant, was used in place of purified hTOP2α. DNA in the reaction was separated by electrophoresis on a 0.9% agarose-TBE gel for 2 h at 170 V and then stained with a 0.5 µ*M* ethidium bromide solution for 20 min, and excess ethidium bromide was washed out for 5 min. The gel was then digitized on a Geldoc 1000 gel documentation system (Biorad) and signals quantitated with the Molecular Analyst software provided (Biorad). One unit of activity of TOP2 was defined as the minimal amount of TOP2/crude extract required to decatenate 100% of the 100 ng kDNA present in the reaction. In addition, starting from a JEL1 yeast transformant overexpressing hTOP2α following transformation with plasmid YEpWOB6 [34], hTOP2α was purified as described previously [35], and used as a reference.

## Results

### Expression of differential levels of TOP2 in yeast

Yeast transformants Y136, Y141 and Y122 resulted from transformation of the JN394top2-4 strain with, respectively, plasmids p134, pMJ1 and YCpDEDWOB10. Strain Y116 resulted from transformation of the JN394 strain with a control plasmid pYX111 containing the URA3 marker gene similar to the three other above-mentioned plasmids. The characteristics of these transformants are summarized in Table 1. The yeast JN394top2-4 was not viable at 35 °C owing to a mutation that inactivates yTOP2 [24]. In contrast, recombinant yeast transformants Y141 and Y136 grew at 35 °C, indicating that their ectopically expressed hTOP2α proteins, through the plasmid constructs pMJ1 and p134, respectively, were able to complement for this lack of functional yTOP2 at this temperature. In all the assays described here, the four transformants were always grown at 35 °C. Furthermore, these yeast transformants were viable with no alteration of their doubling times or phenotypic appearance from that of the wild-type JN394 (data not shown), indicating that the various levels of expression were moderate, i.e. neither too low to allow for complementation nor too high to avoid poor growth or lethality.

In order to define the topoisomerase status of each of the yeast transformants Y116, Y122, Y136 and Y141, we set out to quantitate TOP2 expression through their levels of enzymatic activity. Crude cell extracts were prepared and the amount of total protein necessary to

**Table 1** Characteristics of the yeast transformants used in these studies (*ND* not determined)

| Strain | Genotype | TOP2 status at 35 °C | |
|---|---|---|---|
| | | Type | TOP2 activity[a] |
| JN394 | *MATa ade1 ura3-52 his 7 trpl ISE2 rad 52*::LEU2 | yTOP2 | ND |
| JN394top2-4 | As JN394, but top2-4 | Not viable | |
| Y136 | As JN394top2-4, but introduction of 134 | hTOP2α | 0.5 |
| Y141 | As JN394top2-4, but introduction of pMJ1 | hTOP2α | 1.7 |
| Y116 | As JN394, but introduction of pYX111 | yTOP2 | $<0.17$ |
| Y122 | As JN394top2-4, but introduction of YCpDEDWOB10 | yTOP2 | 0.5 |

[a] TOP2 activity was defined as the units of TOP2 activity per microgram of crude cell extract, one unit being the amount of enzyme necessary to decatenate 100% of 100 ng kDNA under standard reaction conditions

fully decatenate 100 ng kDNA was assessed (Fig. 1). Increasing quantities of purified hTOP2α were associated with increasing decatenation of kDNA (lanes 1–6). One unit of activity was defined as the minimal amount of protein required to decatenate 100% of the kDNA added to the reaction (lane 2). Decatenation of DNA was observed to various extents in crude extracts from all four transformants developed in this study, depending on the protein loaded/lane from yeast transformants Y141 (lanes 8–11), Y136 (lanes 12–15), Y122 (lanes 16–19) and Y116 (lanes 20–23). One unit of TOP2 activity, corresponding to 100% decatenation was observed with 0.6 μg of crude extract from Y141 (lane 10), whereas 2 μg of extract from Y136 were necessary for the same level of activity (lane 13), indicating that yeast Y141 exhibited approximately 3.3-fold more TOP2 activity than yeast Y136 (Table 1).

The modest levels of expression of TOP2 activity were more elusive to detect in crude extracts prepared from yeast Y122 and Y116 expressing differing levels of yTOP2. However one unit of TOP2 activity was estimated to result from 2 μg of extract from Y122 (lane 17), whilst only marginal activity was detectable with 2 μg of extract from Y116 (lane 21), providing evidence of at least a lower TOP2 activity in transformants Y116 than in yeast Y122. The use of excess protein extract (6 μg) was found to distort the normal migration pattern of the DNA fragment, with retardation of the electrophoretic band including decatenated DNA (lanes 8, 12, 16 and 20). It could therefore be considered that yeast Y122 exhibited at least threefold more TOP2 activity than yeast Y116 (Table 1). Intermediate electrophoretic bands were also observed in all the reactions including crude cell extracts, probably associated with the presence of a number of other DNA-modifying enzymes in these preparations. The characteristics of the four yeast transformants studied in terms of the type of TOP2 enzyme expressed together with estimations of their TOP2 activities are summarized in Table 1. Ribonuclease protection assays revealed that all four yeast transformants expressed yTOP1 RNA at similar levels, compared to the level of transcription from the y*ACT* housekeeping gene (data not shown).

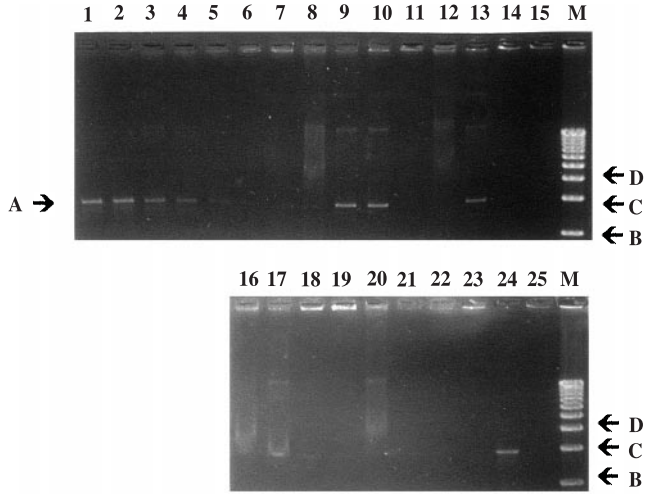


**Fig. 1** Decatenation assays. Reactions were performed with various amounts [6 μg (*lanes 8, 12, 16, 20*), 2 μg (*lanes 9, 13, 17, 21*), 0.6 μg (*lanes 10, 14, 18, 22*), or 0.2 μg (*lanes 11, 15, 19, 23*)] of crude cell extract from yeast transformants Y141 (*lanes 8–11*), Y136 (*lanes 12–15*), Y122 (*lanes 16–19*) and Y116 (*lanes 20–23*). Control reactions included various amount of purified hTOP2α expressed in units: 3 (*lane 1*), 1 (*lanes 2 and 24*), 0.8 (*lane 3*), 0.6 (*lane 4*), 0.4 (*lane 5*), 0.2 (*lane 6*) and 0 (*lanes 7 and 25*). The position of decatenated DNA is indicated by arrow *A*. A molecular DNA weight marker was included to the right of the gels (*M*) with indication of sizes of linear DNA fragments: 2036 bp (*B*), 3054 bp (*C*) and 4072 bp (*D*)

## A screening assay in yeast transformants expressing differential levels of hTOP2 for TOP2-inhibiting agents

The in vivo screening assay was validated initially with etoposide, a clinically useful inhibitor of TOP2 [27]. Following digitization and quantitation of the density of growth of each inoculum after exposure to a range of etoposide concentrations, cytotoxicity curves were drawn (Fig. 2A). Both yeast transformants Y141 and Y136 were found to be sensitive to etoposide. However, those expressing the higher level of hTOP2, Y141, were hypersensitive to etoposide as compared to the Y136 transformants expressing the lower level of this enzyme.

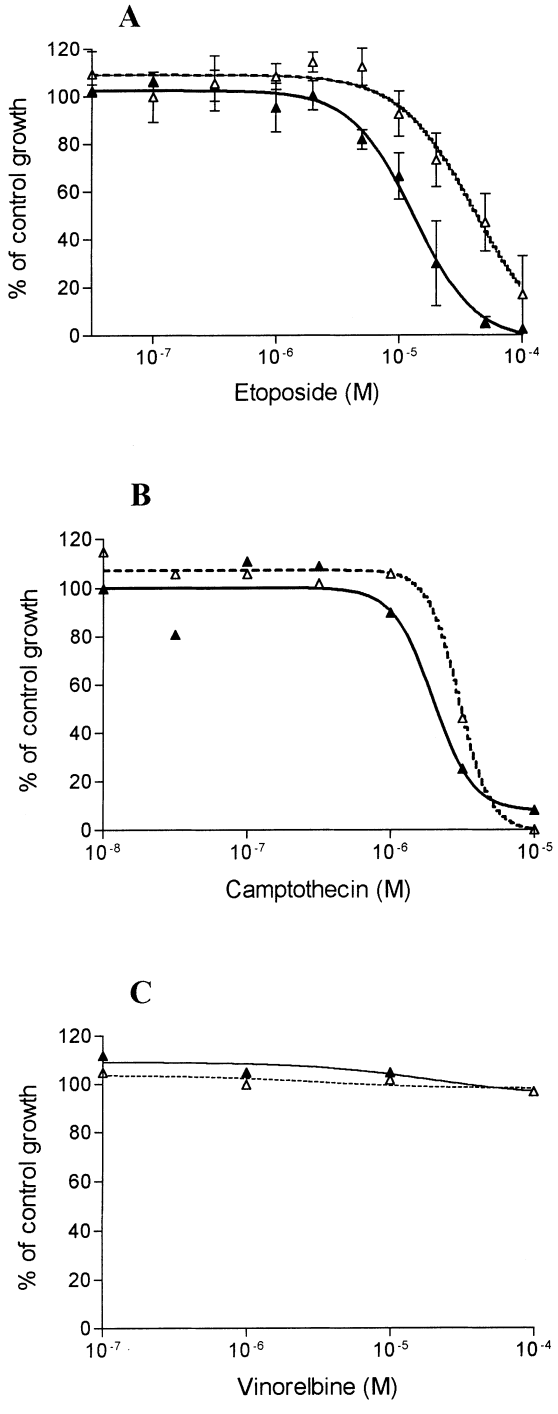


**Fig. 2A–C** Cytotoxicity curves for test drugs on hTOP2-expressing yeast transformants: **A** etoposide (mean of four experiments, *bars* represent standard deviation); **B** camptothecin; **C** vinorelbine). The yeast transformants studied were Y136 (----△) and Y141 —▲). Data were plotted using a Windows-based Prism program

As shown by the standard deviation values from the mean of four independent experiments, represented as bars in Fig. 2A, the two cytotoxicity curves were clearly separable and distinct from one other. $IC_{50}$ values were calculated for each yeast transformant. Then, the ratio [$IC_{50}$ from Y136]/[$IC_{50}$ from Y141] was determined and

**Table 2** Differential cytotoxic effects of a series of 24 antitumour agents against yeast transformants expressing different, biologically relevant, levels of hTOP2. $IC_{50}$ values corresponded to drug concentrations that reduced the density of growth by 50%. Drug-induced differential cytotoxicity between two yeast recombinants was assessed as the ratio [$IC_{50}$ from Y136]/[$IC_{50}$ from Y141]. Depending on the ratio values, three groups of drugs were: those with ratios above 1.2 (group A), those with ratios between 0.8 and 1.2 (group B), and those resulting in no toxicity (group C)

| Test compound | $IC_{50}$ ($\mu M$) | | Ratio[a] |
|---|---|---|---|
| | Y136 | Y141 | |
| Group A | | | |
| Daunorubicin | 6.8 | 4.0 | 1.7 |
| Doxorubicin | 2.9 | 1.6 | 1.8 |
| Genistein | 100 | 47 | 2.1 |
| ICRF-187 | 23 | 9.2 | 2.5 |
| ICRF-193 | 3.4 | 1.3 | 2.6 |
| Actinomycin D | 13.5 | 5.1 | 2.7 |
| Etoposide | 60 | 19 | 3.2 |
| Ellipticine | 21 | 6.0 | 3.5 |
| Distamycin A | 2.5 | 0.7 | 3.6 |
| ICRF-159 | 80 | 21 | 3.8 |
| TOP 53 | 87 | 18 | 4.8 |
| Amsacrine | 95 | 14 | 6.9 |
| Groupe B | | | |
| Cisplatin | 25 | 20 | 0.8 |
| Camptothecin | 3.0 | 2.6 | 1.1 |
| Group C | | | |
| Vinorelbine | | Suramin | |
| Cytosine arabinoside | | Irinotecan | |
| Podophyllotoxin | | Azatoxin | |
| Epipodophyllotoxin | | Etopophos | |
| Colchicine | | Mitoxantrone | |

[a][$IC_{50}$ fron Y136]/[$IC_{50}$ from Y141]

used to assess the differential drug sensitivities of the two transformants. The ratio of 3.2 obtained with etoposide clearly shows that cytotoxicity increased with the level of expression of hTOP2. In contrast, the cytotoxicity curves for Y141 and Y136 challenged with camptothecin, a specific inhibitor of TOP1 used as a negative control for the assay [12, 17], were superimposable with similar $IC_{50}$ values (Fig. 2B) and a ratio close to unity, indicative of an absence of any differential sensitivity. Next, we screened for activity with vinorelbine, as an example of a drug known to have no interaction with the nuclear topoisomerases, and found no growth inhibition at concentrations up to and including $10^{-4}$ $M$ (Fig. 2C).

## Sensitivity of hTOP2-expressing yeast transformants to a range of anticancer drugs

The growth inhibitory properties of 24 antitumour compounds were then tested against these transformants using concentrations ranging from $10^{-4}$ to $10^{-7}$ $M$. Growth inhibition induced by each drug towards the pair of yeast transformants Y136 and Y141 differentially expressing hTOP2 was defined in terms of an $IC_{50}$ value and then the ratio [$IC_{50}$ from Y136]/[$IC_{50}$ from Y141]

was calculated. Overall the $IC_{50}$ values recorded for the different compounds varied over approximately two logs ranging from 0.7 to 100 $\mu M$ (Table 2), although it is difficult to attribute any definite significance to these figures. Indeed, these $IC_{50}$ values merely reflect the level of growth inhibition induced by each bioavailable test compound whose amount may in fact be severely limited by the permeability barrier at the cell wall of the yeast; this permeability appears to vary from drug to drug, as previously described [25]. Therefore $IC_{50}$ values measured for yeast transformants cannot readily be applied or compared with data obtained using mammalian cells that are devoid of this cell wall. However, based on their induced patterns of sensitivities as depicted in Fig. 2A–C and the resulting ratios of $IC_{50}$ values, the drugs tested could be divided into three main groups, and these are listed in Table 2.

Drugs included in group A, as exemplified by etoposide (Fig. 2A), had ratios above 1.2, indicating hypersensitivity of the yeast transformant Y141, so paralleling the levels of hTOP2 expression in the two transformants. Daunorubicin, doxorubicin, genistein, ICRF-187, ICRF-193, actinomycin D, etoposide, ellipticine, distamycin A, ICRF-159, TOP 53 and amsacrine are included in this class and are arranged in order of increasing ratio (Table 2, group A). Within group A, ratios ranged from 1.7 to 6.9, being lowest for the two closely related anthracyclines, doxorubicin and daunorubicin (1.7–1.8), and highest (6.9) for amsacrine. The other compounds in this group, however, had ratios with values between 2.1 and 4.8. This narrow range of differential sensitivities could suggest that, for the same increase in hTOP2 expression, the corresponding test compounds affected growth to a relatively similar extent. In this context, only amsacrine and anthracycline derivatives might be, respectively, more or less effective.

In contrast, cisplatin was characterized by a ratio of 1.0 (Table 2, group B), indicating that the yeast transformants Y141 and Y136 were equally sensitive to this drug, with growth inhibition curves similar to those of camptothecin (Fig. 2B). Thus cisplatin, and camptothecin are placed in group B in Table 2. All the test compounds which failed to exhibit any growth-inhibitory effects against either of the transformants Y141 or Y136, such as vinorelbine (Fig. 2C), are listed in Table 2 as group C. These include cytosine arabinoside, podophyllotoxin, epipodophyllotoxin, colchicine, suramin, irinotecan, azatoxin, etopophos and mitoxantrone. Finally, it is notable that none of the drugs tested led to a ratio of less than 0.8.

Therefore, from this survey, the assay method used, based on the differential expression of hTOP2, correctly identified all the known inhibitors of TOP2 from the 24 compounds tested, except mitoxantrone, azatoxin and etopophos, and these are listed in Table 2 as group C compounds. With mitoxantrone, azatoxin and etopophos, a total lack of growth inhibition was noted against both yeast transformants, under the assay conditions used, at concentrations of $\leq 10^{-4}$ $M$.

## A screening assay in yeast transformants expressing differential levels of yTOP2 for TOP2-inhibiting agents

The differential sensitivities to etoposide of the yeast transformants expressing either the higher (Y122) or the lower level (Y116) of moderate overexpression of yTOP2 were first evaluated and the results are illustrated in Fig. 3A. Transformants Y122 proved most sensitive to

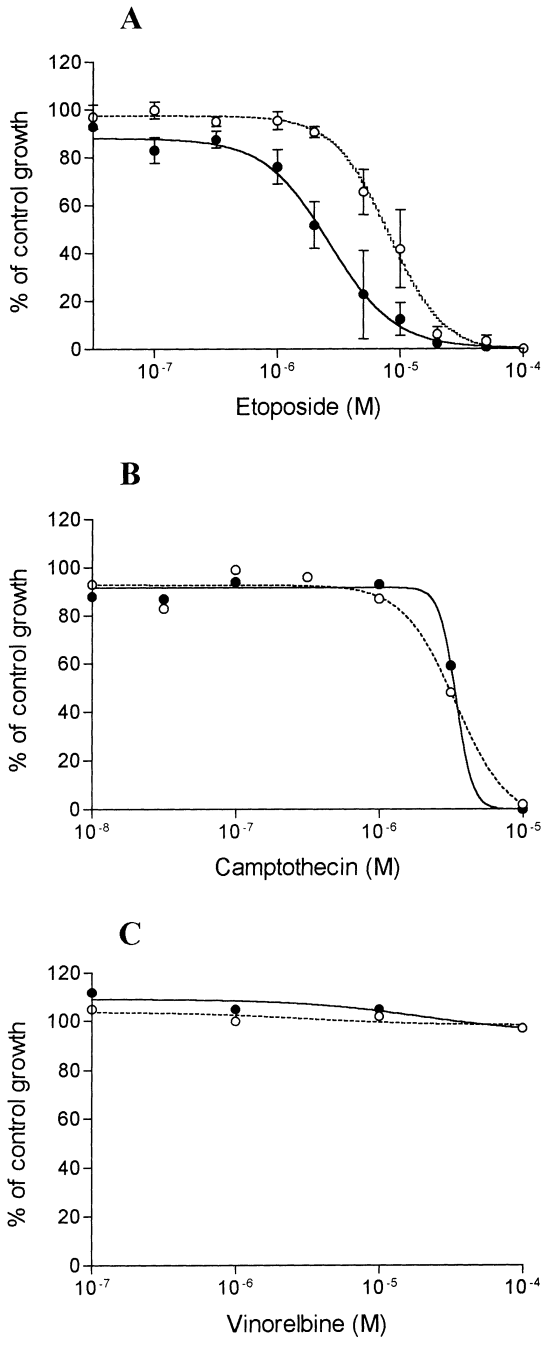


**Fig. 3A–C** Cytotoxicity curves for test drugs on yTOP2-expressing yeast transformants. For detailed explanation, see the legend for Fig. 2. The yeast transformants studied were Y116 (- - -○) and Y122 (—●)

the growth-inhibitory properties of etoposide, with the ratio [$IC_{50}$ from Y116]/[$IC_{50}$ from Y122] being 3.8. This result provides evidence that within this assay system sensitivity to etoposide increased with the level of yTOP2 expression. When evaluating camptothecin, these two yeast recombinants proved equally sensitive, with a ratio of 1.0, as illustrated in Fig. 3B, whilst vinorelbine showed no growth inhibitory properties at all (Fig. 3C). These data therefore clearly complement the studies with the transformants Y136 and Y141 overexpressing the human enzyme.

## Sensitivity of yTOP2-expressing yeast transformants to a range of anticancer drugs

The sensitivities of yeast Y122 and Y116, expressing the differential yet moderate levels of overexpression of yTOP2, were next determined for the 24 antitumour agents evaluated earlier using the hTOP2-overexpressing transformants. Each drug was then characterized by the ratio of the $IC_{50}$ for yeast Y116 versus that for yeast Y122. Again the drugs tested induced patterns of sensitivity that allowed them to be placed in three major groups (Table 3). The archetype of group A was etoposide which generated a hypersensitivity paralleling the level of yTOP2 expression (Fig. 3A). Amsacrine, doxorubicin, daunorubicin, mitoxantrone, ellipticine, actinomycin D and TOP 53 also belonged to this group, but with ratios varying widely from 1.9 to 136. Amsacrine, etoposide, doxorubicin and daunorubicin had moderate ratios between 1.9 and 3.4, with the two anthracyclines again having similar values. In contrast, these yeast transformants seemed to be more sensitive to variations in yTOP2 levels when challenged with mitoxantrone, ellipticine and actinomycin D providing ratios of $> 5$ to 26, and this effect was even more marked with TOP 53 with a ratio of 136. With regard to mitoxantrone, while the low level of yTOP2 expressed by the yeast transformant Y116 was insufficient to result in any detectable growth inhibition by mitoxantrone, growth inhibition was observed in the Y122 transformants with their increased level of expression of yTOP2. Cisplatin and distamycin A were characterized by ratios approximating 1.0 and so were categorized as group B agents (Table 3), exemplified by camptothecin (Fig. 3B). In these cases this ratio indicates that transformants Y122 and Y116 were equally sensitive to these drugs. The third class of drug, termed group C and exemplified by vinorelbine, failed to induce any cytotoxicity against either Y122 or Y116 within the range of concentrations tested, i.e. $\leq 10^{-4}\ M$ (Table 3). Group C included cytosine arabinoside, vinorelbine, podophyllotoxin, epipodophyllotoxin, colchicine, irinotecan, azatoxin, genistein, etopophos, bisdioxopiperazine compounds (ICRF-159, ICRF-187, ICRF-193) and suramin. Again, it was apparent that none of the drugs tested led to ratios below 0.8.

Therefore, if only the compounds classified in group A are identified as inhibitors of TOP2, the differential expression of yTOP2 in these transformants does not permit the correct selection of all the known inhibitors of TOP2 since both yeast tranformants Y116 and Y122 either had similar sensitivities, for example, to distamycin A with a ratio of 1.0, or showed total insensitivity, for example to genistein, etopophos and the various ICRF bisdioxopiperazine compounds (Table 3, groups B and C).

**Table 3** Differential cytotoxic effects of a series of 24 antitumour agents against yeast transformants expressing different, biologically relevant, levels of yTOP2. $IC_{50}$ values, the ratio [$IC_{50}$ from Y116]/[$IC_{50}$ from Y122] and the drug groups are as described in the legend to Table 2

| Test compound | $IC_{50}$ ($\mu M$) | | Ratio[a] |
|---|---|---|---|
| | Y136 | Y141 | |
| Group A | | | |
| Amsacrine | 112 | 60 | 1.9 |
| Doxorubicin | 3.2 | 1.0 | 3.0 |
| Daunorubicin | 9.3 | 2.8 | 3.4 |
| Etoposide | 7.5 | 2.0 | 3.8 |
| Mitoxantrone | $>100$ | 20 | $>5$ |
| Ellipticine | 14 | 1.6 | 8.8 |
| Actinomycin D | 5.2 | 0.21 | 24 |
| TOP 53 | 0.64 | 0.0047 | 136 |
| Groupe B | | | |
| Cisplatin | 32 | 27 | 0.8 |
| Camptothecin | 3.3 | 3.4 | 1.0 |
| Distamycin A | 2.1 | 2.1 | 1.0 |
| Group C | | | |
| Vinorelbine | Azatoxin | | |
| Cytosine arabinoside | Etopophos | | |
| Podophyllotoxin | ICRF-159 | | |
| Epipodophyllotoxin | ICRF-187 | | |
| Colchicine | ICRF-193 | | |
| Suramin | Genistein | | |
| Irinotecan | | | |

[a][$IC_{50}$ from Y116]/[$IC_{50}$ from Y122]

## Species-specific cytotoxicity exerted by TOP2 inhibitors

In an attempt to assess whether these drugs differentially inhibited TOP2 of human or yeast origin, ratios of the $IC_{50}$ values from yeast transformant Y136 versus the $IC_{50}$ values from yeast Y122 were calculated. Given the expression levels of either hTOP2 in Y136 or yTOP2 in Y122, previously shown to yield similar activites of TOP2, the drugs tested appeared to fall into one of four classes according to their ratio of relative species specificity. Thus, a ratio around one (between 0.8 and 1.2), reflecting a similar affinity for TOP2 of both human and yeast origin, under our conditions of moderate TOP2 overexpression, was considered representative of class I compounds (Table 4). These included cisplatin, camptothecin and distamycin A. Drugs showing a ratio below 0.8, indicating a relative preference for hTOP2 over yTOP2, were considered as class II compounds. These included genistein, ICRF-159, ICRF-187 and ICRF-193.

**Table 4** Classification of a series of 24 antitumour test compounds according to their apparent preferential species-specific TOP2-related cytotoxicities. $IC_{50}$ values corresponded to drug concentrations that reduced the density of growth by 50%. Preferential species-specific TOP2 cytotoxicities were assessed from the ratio [$IC_{50}$ from Y136]/[$IC_{50}$ from Y122]. Depending on the ratio values, four classes of drugs were identified: those with ratios between 0.8 and 1.2 (class I), those with ratios below 0.8 (class II), those with ratios above 1.2 (class III), and those resulting in no toxicity (class IV)

| Test compound | $IC_{50}$ ($\mu M$) | | Ratio[a] |
|---|---|---|---|
| | Y136 | Y141 | |
| Class I | | | |
| Cisplatin | 25 | 27 | 0.9 |
| Camptothecin | 3.0 | 3.4 | 0.9 |
| Distamycin A | 2.5 | 2.1 | 1.2 |
| Class II | | | |
| ICRF-159 | 80 | > 100 | < 0.8 |
| Genistein | 50 | > 200 | < 0.5 |
| ICRF-187 | 23 | > 100 | < 0.23 |
| ICRF-193 | 3.4 | > 100 | < 0.03 |
| Class III | | | |
| Amsacrine | 95 | 60 | 1.6 |
| Daunorubicin | 6.8 | 2.8 | 2.4 |
| Doxorubicin | 2.9 | 1.0 | 2.9 |
| Ellipticin | 21 | 1.6 | 13 |
| Etoposide | 60 | 2.0 | 30 |
| Actinomycin D | 13.5 | 0.21 | 64 |
| TOP 53 | 87 | 0.0047 | 18500 |
| Class IV | | | |
| Vinorelbine | Suramin | | |
| Cytosine arabinoside | Irinotecan | | |
| Podophyllotoxin | Azatoxin | | |
| Epipodophyllotoxin | Etopophos | | |
| Colchicine | Mitoxantrone | | |

[a][$IC_{50}$ from Y136]/[$IC_{50}$ from Y141]

Interestingly, in this class, ratios represented only a minimal value since yeast expressing yTOP2 were not growth inhibited by this array of drugs, whilst ICRF-193 seemed to show an exceedingly high preference for hTOP2. In contrast, drugs showing a ratio above 1.2, indicating a preference for yTOP2 over hTOP2, were considered as class III compounds. This preference was moderate for amsacrine, doxorubicin, daunorubicin, intermediate for ellipticine, etoposide and actinomycin D, and dramatic for TOP 53. The drugs that were nontoxic for the yeast Y136 and Y122 used were allocated to class IV and included cytosine arabinoside, vinorelbine, podophyllotoxin, epipodophyllotoxin, colchicine, suramin, irinotecan, azatoxin, etopophos and mitoxantrone.

## Discussion

We adapted an existing basic technology and devised a functional assay in yeast based on differential moderate expression levels of TOP2 under the control of constitutive promoters of varying strengths complementing for the absence of native yTOP2 expression. This strategy allowed comparisons of growth inhibition of yeast transformants using the same media and temperature conditions and therefore avoided the need for any addition of potential sensitivity-modifying factors such as the source of carbohydrate or temperature conditions [14, 23]. The levels of TOP2 activity were also assessed and found to increase by approximately 3.3-fold for hTOP2, and more than 3-fold for yTOP2, reflecting moderate levels of TOP2 overexpression above wild-type levels. The level of TOP2 activity detectable in yeast transformant Y141 bearing the plasmid pMJ1 was comparable with that reported previously in another recipient yeast [13], whereas we are not aware of any such data on yeast expressing wild-type levels of yTOP2.

This functional assay in yeast involved assessment of growth inhibition over 3 days in culture, not just the use of short time periods of several hours [21, 26], and permitted the correct identification of all known inhibitors of TOP2 from the series tested (Table 2, group A). The sole exception was mitoxantrone which proved nontoxic (Table 2, group C), and a possible interpretation of this result is that the level of hTOP2 expression in yeast Y136 and Y141 may not be as high as that attained under the experimental conditions used by Meczes et al. [21]. Drugs exhibiting comparable sensitivities against the lower versus the higher TOP2-expressing transformants, i.e. with ratios approximating 1.0, validated the specificity of our assay since they are not TOP2-interacting compounds (cisplatin and camptothecin). The group of nontoxic compounds identified (Table 2, group C) included the known non-TOP2-interacting compounds, the prodrugs irinotecan and etopophos [6], as well as mitoxantrone (discussed above), suramin (confirming an earlier report [21]), and azatoxin. It is probable that the cell wall and the plasma membrane of yeast could at least be partly responsible for this lack of discrimination.

Amongst the TOP2-interacting compounds identified were, first those that stabilize cleavable complexes, such as etoposide, amsacrine, doxorubicin, and also genistein, although at a high concentration of $10^{-4}$ $M$. It has previously been stated that genistein shows low cytotoxicity against TOP2-overexpressing yeast, but no concentration was specified [21]. The second group of TOP2-interacting compounds identified were those, such as the ICRF bisdioxopiperazine compounds, known not to stabilize cleavable complexes between DNA and hTOP2, as shown by others [31] and confirmed in this study (data not shown). To our knowledge, this is the first report of differential levels of hTOP2 influencing sensitivities of yeast to the ICRF bisdioxopiperazine compounds.

This study was then extended to the use of yeast transformants expressing differential levels of yTOP2. In this respect, major TOP2-interacting compounds were similarly identified (Table 3, group A), as previously shown for, for example, etoposide and amsacrine [26]. In these assays mitoxantrone was also identified, although only the yeast transformant with the increased level of expression, Y122, was sensitized to any extent by this

compound, which in this respect is consistent with the observed sensitivity to mitoxantrone of yeast overexpressing yTOP2 reported by Meczes et al. [21]. These test compounds responded to these differences in yTOP2 expression with a wide range of hypersensitization, which was especially striking with TOP 53 (ratio 136). This result warrants further mechanistically orientated studies for elucidation. Compounds that did not induce any shift in sensitivity included camptothecin (Table 3, group A), consistent with our demonstration that yTOP1 expression levels were unchanged amongst the four yeast transformants. However, a yeast with decreased expression of yTOP2 has previously been reported to be hypersensitive to camptothecin [23], although it was not stated whether this downregulation results with any associated overexpression of yTOP1, as noted and reported in a mammalian cell line [18].

Distamycin A (also Table 3, group B) provided an intriguing result since it interacted with hTOP2 but not with yTOP2. Furthermore, no cytotoxicity was induced by either genistein or ICRF bisdioxopiperazine compounds, in contrast to that observed against yeast expressing hTOP2. This lack of cytotoxicity with ICRF bisdioxopiperazine compounds is consistent with the report of Ishida et al. [14] since the same yeast strain, JN394top2-4, expressing yTOP2 at the wild-type level, was shown in this study and by Ishida et al. to be insensitive to ICRF bisdioxopiperazine compounds. In addition, Ishida et al. showed that a lowering of the level of yTOP2 is associated with hypersensitization [14], in good agreement with our data indicating that transformants with moderate overexpression of yTOP2 maintained resistance to this chemical agent.

Therefore, under the carefully controlled conditions developed in our assay, based on a difference in cytotoxicity associated with a difference in TOP2 expression, any nonspecific cytotoxic effects inherent to the test compound were excluded, which is not possible when only one level of TOP2 expression is studied [21]. Nevertheless, when comparing our results with previously reported results, it appears that this TOP2-specific activity of compounds was detected at much lower concentrations with the functional assay developed for this study, as for example in the case of doxorubicin [21] and etoposide [21, 26].

In order to assess whether the compounds tested would inhibit the TOP2 of human or yeast origin with different efficiencies, ratios of $IC_{50}$ values from the two yeast transformants expressing the lower levels of each enzyme were calculated. However, the interpretation of the results of this comparative method should be approached with caution taking into account a number of caveats concerning data. The TOP2 activities, as measured by the decatenation assays, from the two yeast transformants Y136 and Y141 were directly comparable since they originated from the same type of enzyme, hTOP2, and similar validity can be claimed when comparing TOP2 expression from yeast Y116 and Y122 expressing yTOP2. However, one cannot assume that the activity of these two species of TOP2 can be recovered and preserved with similar efficiencies from whole cell extracts, or can necessarily decatenate kDNA with similar kinetics. Indeed, neither can one exclude the possibility that exogenous hTOP2 could be more readily degraded by yeast proteases than native yTOP2. Therefore, although the relative effects of drugs on growth inhibition cannot be strictly correlated with actual enzyme activities, they may, however, be compared. Our results showed that each drug studied appeared to fall into one of four classes as listed in Table 4 according to its ratio of relative species specificity. Class I compounds with $IC_{50}$ ratios of 0.8 to 1.2 included drugs known not to interact with TOP2, for example cisplatin and camptothecin, as well as a known DNA interactor, distamycin A. Drugs allocated to class II were characterized by $IC_{50}$ ratios $< 0.8$ and included genistein and all three bisdioxopiperazines tested, therefore indicating a strong preference for hTOP2. On the other hand, drugs included in class III, with ratios above 1.2, expressed a clear preference for yTOP2 with ratios as high as 30 for etoposide and 18 500 for TOP 53. Class IV included all the drugs exerting no cytotoxicity against these Y116 and Y136 transformants expressing low levels of yTOP2 and hTOP2, respectively.

In these studies the extent of hypersensitization associated with an increase in TOP2 expression (see Tables 2 and 3) did not increase in a linear fashion, and was dependent on the test compound, as well as on the type and level of expression of the topoisomerase. Thus, absolute species preferences cannot be categorically stated for any of these test compounds, although they can be assigned relative preferences for given levels of expression. Taking into account all these caveats, therefore, comparison of these data with those of other groups using different conditions of overexpression of TOP2 should be approached with caution. Such comparisons are further complicated if one cannot either rule out any TOP2 nonspecific toxicity which may account for the observed cytotoxicity, or compare the enzyme activities in the yeast transformants studied. These reservations are exemplified by the varied results described in previous publications. Thus, Hsiung et al. [13], showed that yeast transformants expressing hTOP2 or yTOP2 have similar sensitivities to etoposide whereas under the same conditions of expression, amsacrine preferentially hypersensitizes hTOP2 versus yTOP2. In apparent contrast, Meczes et al. [21], studying yeast transformants expressing hTOP2 or yTOP2 under the strong GAL1 promoter, demonstrated that etoposide, doxorubicin, amsacrine and mitoxantrone all produce higher degrees of cytotoxicity against transformants overexpressing hTOP2 as opposed to yTOP2.

Another point of interest of this study relates to the fact that no drug was identified with a ratio below 0.8 which would have indicated that an increase in either hTOP2 or yTOP2 expression were associated with hyposensitivity of the recipient yeast. This finding appears relevant to the testing of the ICRF bisdioxopiperazine

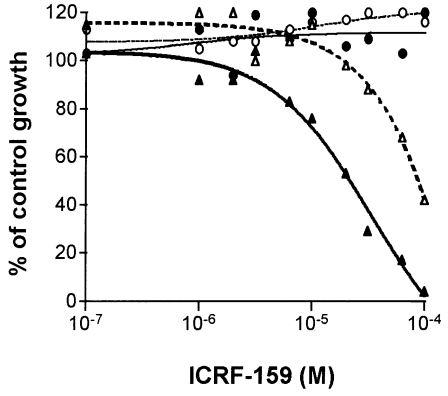


**Fig. 4** Cytotoxicity curves for ICRF-159 on various TOP2-expressing yeast transformants. The yeast transformants studied were Y116 (----○), Y122 (—●), Y136 (----△) and Y141 (—▲). Data were plotted using a Windows-based Prism program

compounds, which are described as inhibitors of the catalytic activity of TOP2 through trapping of the cleavable complexes after the religation of cleaved DNA ends [29, 30]. A decrease in yTOP2 expression in yeast JN394 has been shown to result in a higher susceptibility to the cytotoxic action of ICRF compounds [14]. In this present study, yeast expressing wild-type levels of yTOP2 were similarly resistant to the bisdioxopiperazines at comparable concentrations, whilst a higher level of yTOP2 expression did not sensitize the yeast recipients. In contrast, the sensitivity of the yeast to ICRF compounds increased according to the level of expression of hTOP2 (Table 2), as shown by the detailed curves of growth inhibition for ICRF-159 shown in Fig. 4.

Furthermore, assays performed in parallel at 35 °C, with the sole expression of hTOP2, and at 25 °C, resulting in coexpression of active hTOP2 and yTOP2, yielded similar patterns of sensitivities to these bisdioxopiperazines for all four transformants, indicating that the additional expression of yTOP2 in yeast grown at 25 °C could not overcome the growth inhibition caused by hTOP2 (data not shown). Therefore, the effects of ICRF compounds on hTOP2 appear to confer dominant sensitivity to yeast recipients. This observation is consistent with the interpretation that bisdioxopiperazine compounds might in fact specifically convert hTOP2 into a cellular toxin, in a similar manner to etoposide and amsacrine [26], although not as DNA-damaging agents since ICRF compounds have been shown not to enhance TOP2-induced DNA cleavage [29, 30].

Therefore, overall, these results suggest that the mode of action of bisdioxopiperazine compounds differs between yTOP2 and hTOP2 in this in vivo yeast model. This finding is the subject of further study. Furthermore, our results appear to be supported by the observation that the acquisition of resistance to ICRF-187 by a mammalian cell line, CHO, is associated with a significant decrease in TOP2 expression [9]. Furthermore, Adachi et al. [1] recently demonstrated that the knockout of one of the two alleles for the TOP2 gene leads to an approximate 50% decrease in TOP2 protein, and an increased resistance to ICRF-187.

In conclusion, we adapted and optimized an assay in yeast suitable for rapid, widescale identification and analysis of TOP2-targeting anticancer drugs. Starting from four yeast strains differing in terms of their moderate levels of expression of either hTOP2 or yTOP2, 13 TOP2- and 8 non-TOP2-targeting drugs were readily identified from a panel of 24 anticancer drugs, and the relative potency and species-specificity of the TOP2-targeting compounds were characterized. This assay also revealed that the interaction of drugs, such as the ICRF bisdioxopiperazine compounds, with their target, TOP2, could depend on the species of enzyme used, thereby stressing the importance of the type of substrate, preferably of human origin, used for the screening of potential chemotherapeutic drugs. This panel of four yeast transformants differing only in their varied, biologically relevant levels of TOP2 expression, may thus mimic the clinical situation in which tumours are resistant to chemotherapeutic treatment through the acquisition of the TOP2-related multidrug-resistance phenotype [24, 33]. This primary screening assay in yeast can therefore readily be used to characterize novel anticancer drugs with modified original chemical structures or those present in plant extracts or marine organisms, and aid in the selection of new candidate drugs with different mechanisms of interaction with TOP2, which may be of potential clinical value.

*Addendum:* This provides further evidence in support of our hypothesis that the mode of action of bisdioxopiperazine compounds differs vis-à-vis the hTOP2 and yTOP2 enzymes in the in vivo yeast model used. A second set of yeast recombinants similar to that analysed initially, in terms of TOP2 enzyme expression, except that they lack yeast TOP1 expression, has been established. Briefly, the yeast strain JN394t1 (*MATa ura3-52 leu2 trp1 ade1-2 his7 ISE2 top1*::*TRP rad52*::*LEU2*) derived from yeast JN394 by knocking out the yeast TOP1

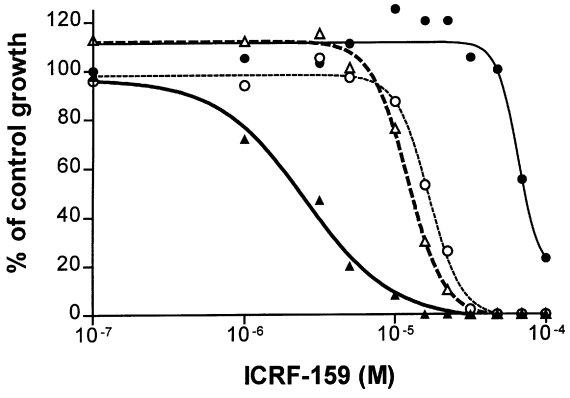


**Fig. 5** Cytotoxicity curves for ICRF-159 on JN394t1 yeast carrying either the plasmid p134 (△----), pMJ1 (▲—), pXY111 (○----), or YCpDEDWOB10 (●—). Data were plotted using a Windows-based Prism program

gene was kindly provided by Dr. J.L. Nitiss. Using standard methods (Ausubel et al. 1995), this strain was transformed with either of the four plasmidic constructs described in Table 1 which allowed for the expression of either hTOP2 or yTOP2 at different, biologically-relevant, levels.

The growth of these four yeast transformants was challenged with the bisdioxopiperazine compound ICRF-159. Data in Fig. 5 revealed an hypersensitivity to ICRF159 of the yeast transformant carrying plasmid pMJ1 versus that carrying plasmid p134, so paralleing the levels of hTOP2 expression in the two transformants driven by these plasmidic constructs (Table 1). In contrast, an hypersensitivity of the yeast transformant carrying plasmid pXY111, i.e. expressing wild-type level of yeast, versus that carrying plasmid YCpDEDWOB10, allowing for overexpression of yTOP2 enzyme was identified (Fig. 5). This result provided further evidence that an increased amount of yTOP2 activity was associated with a higher degree of resistance to ICRF159. Indeed, for the first time in a single model, this set of four yeast recombinants clearly confirmed apparently conflicting results that have been published independently, although using yeast cells of differing genotypic backgrounds, i.e., i) the sensitivity of yeast to this bisdioxopiperazine paralleled the level of hTOP2 expression (in the present study), whereas ii) overexpression of yTOP2 resulted in a lower susceptibility of recipient cells to this ICRF compound (Ishida et al. 1995).

These data therefore further strengten our hypothesis and add to our elucidation of the mechanism of action of these bisdioxopiperazine compounds since the yTOP1 enzyme appears to partially or totally overcome their TOP2-related lethal action on yeast (compare Figs. 4 and 5). This new development is now under further investigation.

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