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Evidence that DNA Topoisomerase I Is Necessary for the Cytotoxic Effects of Camptothecin

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SUMMARY

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are both sensitive to camptothecin, an inhibitor of DNA topoisomerase I. An *S. cerevisiae* DNA repair mutant, *rad52*, is hypersensitive to the drug. In both species, topoisomerase I mutants totally lacking the enzyme are completely resistant to the drug. A strain with a mutation leading to a temperature-sensitive topoisomerase I exhibits temperature dependence in its in vivo response to camptothecin. A strain carrying a plasmid that overproduces topoisomerase I is hypersensitive to the drug. The *rad52* mutant is killed by overproduction of the enzyme, even in the absence of the drug. The response of several of these strains to camptothecin analogs, to DNA topoisomerase II inhibitors, and to other drugs is reported. The cytotoxic effects of camptothecin are discussed in terms of the drug extending the lifetime of a topoisomerase I-DNA covalent intermediate, which is recognized as DNA damage by a DNA repair system.

Cpt is a cytotoxic alkaloid with strong antitumor activity. It inhibits both DNA and RNA synthesis in mammalian cells and also causes reversible fragmentation of cellular DNA (1–3). Cpt has been shown to inhibit purified mammalian DNA topoisomerase I (4). It acts by blocking the rejoining step of the DNA breakage-reunion reaction of the enzyme, leaving the enzyme covalently bound to DNA. It has been proposed that the *in vivo* effects of Cpt on mammalian cells may be explained entirely by the ability of the drug to inhibit topoisomerase I in this manner (4, 5). The finding that Cpt produces protein-concealed DNA single strand breaks in L1210 cells (6) suggests that inhibition of DNA topoisomerase I occurs in whole cells. There is also recent evidence that DNA topoisomerase I from Cpt-resistant human lymphoid cells is itself resistant to the drug (7, 8).

Here we show that two different yeasts, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, are also sensitive to Cpt. Because mutants totally lacking DNA topoisomerase I exist and are viable in both species (9–11), it was possible to test whether such mutants are also sensitive to Cpt. We found that yeast strains lacking DNA topoisomerase I are totally resistant to Cpt, and a strain that carries a plasmid that overproduces the enzyme is hypersensitive. These results demonstrate conclusively that, at least in lower eukaryotes, Cpt exerts its cytotoxic effects solely through DNA topoisomerase I.

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Materials and Methods

Strains and plasmids. The *S. cerevisiae* strain W303-1a *MAT*a *ade2*-1 *trp1*-1 *can1*-100 *leu2*-3,112 *his3*-11,15 *ura3*-1, a gift from R. Rothstein, Columbia University, was the wild-type strain used in these experiments. RS190 is W303-1a plus *top1*-8::*LEU2* (9); it was constructed by gene transplacement (12). RS322 is W303-1a plus *rad52*-8::*TRP1* and RS321 is RS190 plus *rad52*-8::*TRP1*. These two strains were constructed by screening the progeny of a cross of W625-18b with RS190. W625-18b, a gift from B. Thomas and R. Rothstein, is isogenic with W303-1a except that it is *MAT*α and *rad52*-8::*TRP1*. Thus, W303-1a, RS190, RS322, and RS321 are an isogenic set, differing only at *TOP1* and *RAD52*. Other *rad* mutants used were CL167-11D *MAT*α *rad6*-1 *rad18*-2 *met1*-1 *arg4*-17 *his5*-2 *ade2*-1 *cyc1*-9 and CL1150-11b *MAT*a *rad1*-2 *rad2*-5.

The *S. pombe* strains were obtained from M. Yanagida, Kyoto University. They include HM123 (h⁻ *leu1 TOP1*⁺), 710 (h⁻ *leu1 top1*-710), and SP7 (h⁻ *leu1 top1*::*LEU2 top2*^{ts}-342). Strain 710 is derived from HM123 and has a mutation leading to temperature-sensitive topoisomerase I enzymatic activity (13). SP7 is a *top1* null mutant (11), which grows normally at 25°, the temperature used to test Cpt sensitivity for this strain.

Plasmid pWE3 GAL-TOP1 is derived from the yeast shuttle vector YCp50 (14). In addition to vector sequences, it has a 0.82-kb *Bam*H1-*Sal*I fragment with the GAL1-10 promoter (14) and a 3.3-kb *Bam*HI-*Hind*III fragment containing the entire yeast *TOP1*-coding sequence (9). The resulting plasmid gives galactose-dependent *TOP1* expression from the *GAL1* promoter.

Drugs. With the exception of four Cpt analogs, all compounds were provided by the Drug Synthesis and Design or Natural Products Branches of the National Cancer Institute. 9-Nitrocamptothecin was

ABBREVIATION: Cpt, camptothecin.

synthesized as described by Wani *et al.* (15). The 7-alkylated Cpt derivatives were prepared from the appropriate 10-substituted analogs (16).

Measurement of Cpt sensitivity. Yeast strains were generally grown in YPD medium (1% yeast extract, 2% Bactopeptone, 2% glucose). To measure drug sensitivity, about 10^7 cells were spread on a YPD agar plate. Wells, 5 mm in diameter, were introduced into the agar and 100 μl of an appropriate dilution of drug dissolved in water or dimethylsulfoxide/methanol (1:1) was placed in the well. Plates were incubated at 30° for 2 days and the diameter of the zone of inhibition surrounding the well was measured. IC_{12} is defined as the concentration of drug required to produce a zone of inhibition 12 mm in diameter. Experiments with strain RS190/pWE3 GAL-TOP1 were carried out as above, but in a supplemented minimal medium (17) lacking uracil, with either glucose or galactose as the carbon source.

Results

Yeast rad52 mutants are hypersensitive to Cpt. In the course of examining the effect of Cpt on the yeast *S. cerevisiae* we observed that, whereas most strains are minimally sensitive to Cpt, a DNA repair-deficient mutant, *rad52*, is hypersensitive to the drug. Fig. 1 shows the Cpt sensitivity of the *rad52* mutant compared with that of an isogenic DNA repair-proficient wild-type strain. Two other DNA repair-deficient mutants, belonging to the *rad6* and *rad3* epistasis groups (18), were also tested. The *rad6* mutant was found to have intermediate sensitivity (IC_{12} of 160 μg/ml) compared with the *rad52* (IC_{12} of 4 μg/ml) and wild-type (IC_{12} of 800 μg/ml) strains. The mutant belonging to the *rad3* epistasis group, actually a *rad1 rad2* double mutant, was indistinguishable from wild-type in its sensitivity to Cpt (data not shown).

Sensitivity to Cpt requires DNA topoisomerase I. From *in vitro* experiments, we knew that Cpt inhibited yeast DNA topoisomerase I in a manner similar to that found for the mammalian enzyme (data not shown). In order to determine whether topoisomerase I was responsible for the *in vivo* cytotoxic effects of Cpt on yeast, *top1* null mutants (9) totally lacking the enzyme were examined. Isogenic *TOP1*$^+$, *top1*, *rad52*, and *top1-rad52* mutants were constructed as described in Materials and Methods. As can be seen in Fig. 1, the *top1* mutants are totally resistant to Cpt. From these results we conclude that the presence of DNA topoisomerase I is necessary for the growth inhibition by Cpt.

Similar results were found for the fission yeast *S. pombe*. In this case, a DNA repair-proficient *TOP1*$^+$ strain was compared with a related strain carrying a *top1* null mutation (no well characterized DNA repair-deficient mutants of *S. pombe* were readily available). The *TOP1*$^+$ strain was found to be sensitive to Cpt (somewhat more so than the wild-type *S. cerevisiae* strain), and the *top1* null mutant again was found to be totally resistant to the drug (data not shown). An experiment with a strain carrying the *top1*-710 mutation was also informative. This allele leads to a temperature-sensitive enzyme, as judged by *in vitro* topoisomerase I assays, although growth of the strain is normal at all temperatures (13). Fig. 2 shows that the temperature sensitivity of the enzyme *in vitro* is mirrored by the *in vivo* Cpt sensitivity. The mutant is just as sensitive as wild-type at 22°, has intermediate sensitivity at 30°, and is almost totally resistant at 37°.

Topoisomerase I overproduction leads to increased Cpt sensitivity. An *S. cerevisiae* strain with a *top1* null mutation on the chromosome but carrying a wild-type *TOP1* gene on a plasmid was tested for Cpt sensitivity. On this plasmid, pWE3 GAL-TOP1, the *TOP1* gene is under the control of the tightly regulated GAL1-10 promoter (14). Thus, the strain has almost no DNA topoisomerase I activity when cells are grown on a noninducing carbon source, and, as is shown in Fig. 3A, it has about 5-fold more topoisomerase I activity than a wild-type strain when the cells are grown on the inducing sugar galactose. Fig. 3B shows that the Cpt sensitivity of this plasmid-bearing strain depends entirely on whether the *TOP1* gene is induced. After induction, the Cpt sensitivity is greater than that of a wild-type (*TOP1*$^+$ *RAD52*$^+$) strain but less than that of a *rad52* mutant (see Fig. 1). The uninduced culture is completely resistant to Cpt.

Interestingly, *rad52* mutants are sensitive to topoisomerase I overproduction, even in the absence of Cpt. Strain RS321 (*top1 rad52*) carrying the topoisomerase I-overproducing plasmid pWE3 GAL-TOP1 grows normally on glucose but cannot grow on galactose. The same strain without the plasmid grows on both carbon sources. When 10^7 cells of RS321/pWE3 GAL-TOP1 were plated on a galactose plate, a few colonies arose after about a week. Two of these mutants were studied further. They no longer overproduced topoisomerase I after galactose induction, showing lower enzymatic activity than TOP1$^+$ strains by *in vitro* enzymatic assays (data not shown), and thus presumably had spontaneous mutations in the *TOP1* gene or

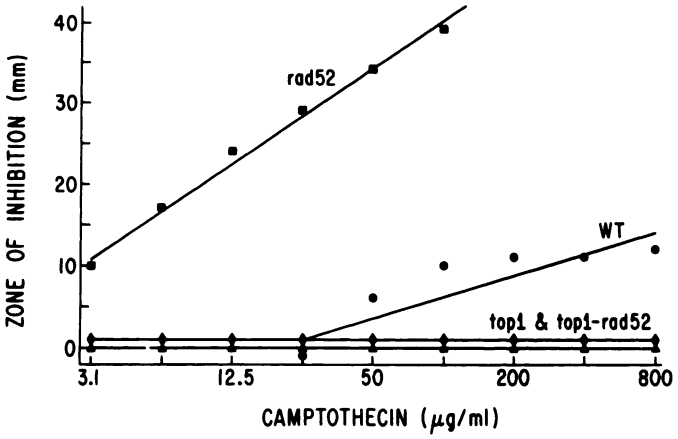

Fig. 1. Cpt sensitivity of different yeast strains. The zone of inhibition assay is described in Materials and Methods. The *S. cerevisiae* strains used are W303-1a (WT) (●); RS190 *top1* (◆); RS322 *rad52* (■); and RS321 *top1 rad52* (▲).

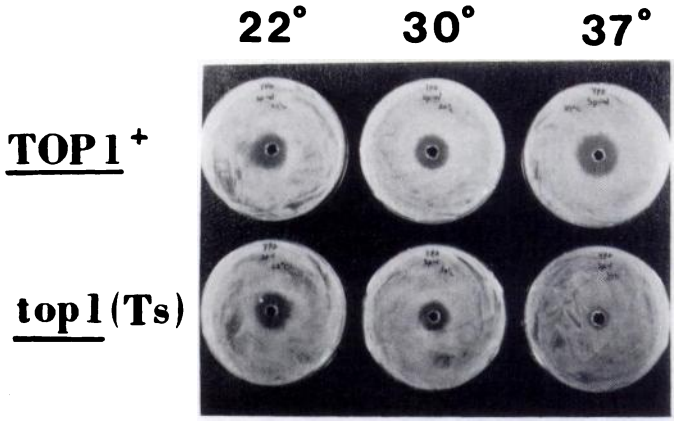

Fig. 2. Correlation of the level of functional *S. pombe* topoisomerase I and sensitivity to Cpt. Strains HM123 *TOP1*$^+$ and 710 *top1*(Ts) were assayed for Cpt sensitivity at three temperatures as indicated. The zones of inhibition can be seen as clear spots surrounding the wells where NaCpt (1 mg/ml) was applied.

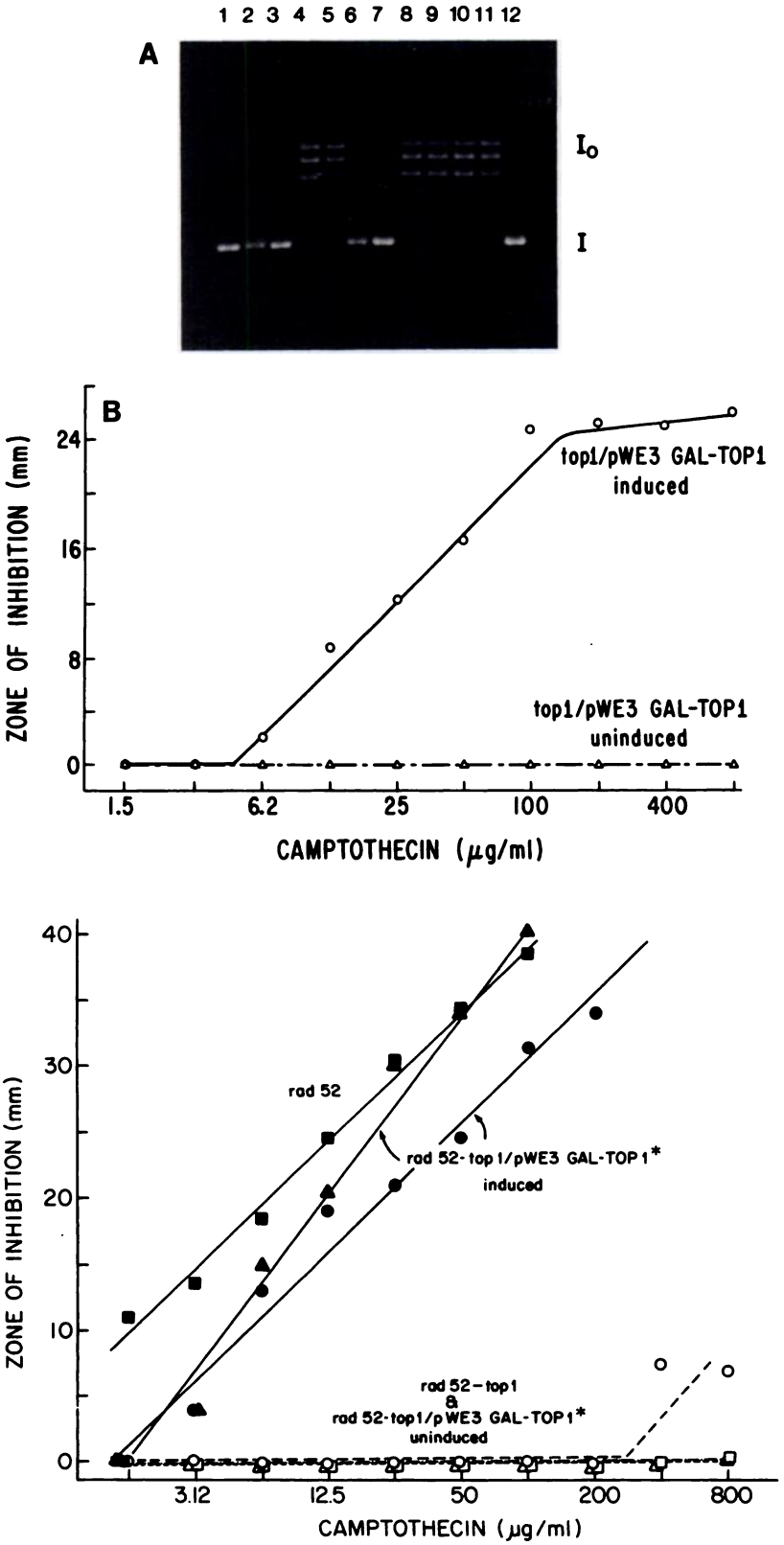

Fig. 3. Overproduction of topoisomerase I leads to increased Cpt sensitivity. A, DNA topoisomerase I activity, assayed as described (27). *Lane 1*, no extract added. *Lanes 2* and *3*, undiluted and 3-fold-diluted extracts of RS190/pWE3 GAL-TOP1 cells grown in the noninducible carbon source raffinose. *Lanes 4–7*, undiluted, 3-, 9-, and 27-fold-diluted extracts of W303-1a (*WT*) cells grown in galactose. *Lanes 8–12*; undiluted, 3-, 9-, 27-, and 81-fold-diluted extracts of RS190/pWE3 GAL-TOP1 cells grown in galactose. All three extracts had equal initial protein concentrations. *I* and I_o mark the positions of supercoiled and relaxed closed circular DNA, respectively. B, Cpt sensitivity. The strain used is RS190/pWE3 GAL-TOP1 grown on glucose (uninduced) (Δ) or galactose (induced) (○).

Fig. 4. Low level expression of topoisomerase I in a *rad52 top1* strain results in Cpt sensitivity nearly equivalent to that seen in a *rad52 TOP1*$^+$ strain. The strains used were RS322 *rad52* (■), RS321 *rad52 top1* (□), and the two spontaneous mutants of RS321/pWE3 GAL-TOP1 that had gained the ability to grow on galactose. These are both referred to as RS321/pWE3 GAL-TOP1*; their Cpt sensitivity was tested on glucose (uninduced) (Δ,○) or on galactose medium (induced) (▲,●).

its promoter. After galactose induction, these mutants are sensitive to Cpt (Fig. 4). The level of sensitivity is consistent with the decreased level of topoisomerase I in these mutants compared with a *TOP1*$^+$ strain.

Activity of Cpt analogs. A number of Cpt analogs were evaluated in yeast strains with DNA repair and/or topoisomerase I mutations (Table 1). Cytotoxicity or antitumor activity has been described for each of the analogs listed in Table 1 and we have demonstrated that each inhibits purified mammalian topoisomerase I[1]. None of the Cpt analogs was active in the DNA repair-proficient strain. In the *rad52* strain, cytotoxicity was seen for all the compounds except one. Substitutions that

[1] R. Hertzberg, M. Caranfa, and R. Johnson, unpublished results.

TABLE 1

Activity of Cpt analogs in repair-deficient and/or topoisomerase I-deficient yeast mutants

IC_{12} is the concentration required to produce a zone of inhibition of 12 mm diameter. Values are mean ± standard error of IC_{12} values determined in multiple independent concentration-response studies. Values without standard errors represent results of single concentration-response studies.

Drug	IC_{12}				
	Repair-proficient			Repair-deficient (*rad52*)	
	TOP1$^+$	*top1*	*TOP1*$^{++a}$	*Top1*$^+$	*top1*
			μg/ml		
Cpt	>800	>800	25 ± 3	4.5 ± 0.4	>800
9-CH_3O-Cpt	>800	>800	21	5.1 ± 2.1	>800
9-Nitro-Cpt	>800	>800	180 ± 38	39 ± 5	>800
10-CH_3O-Cpt	>800	>800	29 ± 3	5.0 ± 1.4	>800
10-CH_3O-7-ethyl-Cpt	>800	>800	>800	9.4 ± 3.6	>800
7-Methyl-Cpt	>800	>800	91 ± 6	16 ± 0.2	>800
10-HO-Cpt	>800	>800	>800	210 ± 60	>800
10-HO-7-ethyl-Cpt	>800	>800	>800	>800	>800

[a] *TOP1*$^{++}$ refers to strain RS190 bearing the topoisomerase I-overproducing plasmid pWE3 GAL-TOP1 under induced conditions.

increase polarity adversely affected potency. For example, 10-hydroxy or 7-alkyl substitutions (which increase basicity of the quinoline nitrogen in Cpt) resulted in less activity. A compound with both of these substituents had no detectable activity in the yeast strains. A number of Cpt analogs that were inactive as inhibitors of topoisomerase I were tested and none showed activity in the *rad52* strain (data not shown).

As with Cpt, deletion of the *TOP1* gene in a *rad52* background resulted in complete loss of sensitivity to the analogs. Overexpression of topoisomerase I resulted in hypersensitivity to a number of the Cpt analogs. The more polar analogs did not inhibit growth of the overproducing strain. These compounds probably do not enter yeast cells as easily as Cpt; the impermeability of the yeast cell membrane to many compounds, particularly polar ones, is well known.

Activity of topoisomerase II inhibitors and other compounds. In order to test the specificity of the effects observed with Cpt, we evaluated drugs that have been reported to inhibit DNA topoisomerase II on yeast strains with or without *top1* and/or *rad52* mutations. Table 2 shows that repair-proficient strains are resistant to these drugs, with the exception of ellipticine, and that the deletion of topoisomerase I increased sensitivity to ellipticine and anthracyclines slightly but had no effect on sensitivity to the other topoisomerase II inhibitors. The repair-deficient *rad52* strain, however, is hypersensitive to several of these agents. This is not surprising considering that, with the exception of merbarone and fostriecin (19, 20), these compounds are thought to act by trapping a topoisomerase II-DNA complex (21) in a manner analogous to that of Cpt for DNA topoisomerase I (4). Interestingly, the absence of topoisomerase I further increased the sensitivity of the *rad52* strain to several of these drugs (Table 2).

We tested a number of other cytotoxic drugs and antifungal agents in these yeast strains. The compounds that were toxic to repair-proficient and/or repair-deficient *rad52* strains are listed in Table 3. None of these agents is known to inhibit DNA topoisomerases by trapping an enzyme-DNA complex. The presence or absence of topoisomerase I did not affect the cytotoxicity of these drugs. The presence of the *rad52* mutation had a minimal effect on drug sensitivity, with two exceptions. Streptonigrin was much more cytotoxic for *rad52* mutants than for DNA repair-proficient strains whereas the converse was true for the glutamine antagonist acivicin.

TABLE 2

Activity of topoisomerase II inhibitors in repair-deficient and/or topoisomerase I-deficient yeast mutants

See legend to Table 1.

Drug	IC_{12}			
	Repair-proficient		Repair-deficient (*rad52*)	
	TOP1$^+$	*top1*	*TOP1*$^+$	*top1*
	μg/ml			
Ellipticine	690 ± 110	360 ± 140	39 ± 8	14 ± 4
Bisantrene	>800	>800	52 ± 7	18 ± 6
Amsacrine	>800	>800	120 ± 13	19 ± 3
Doxorubicin	>800	760	78 ± 33	30 ± 10
Daunorubicin	>800	760	140	100 ± 33
Mitoxantrone	>800	>800	>800	140 ± 7
Dactinomycin	>800	>800	480 ± 55	530 ± 39
Teniposide	>800	>800	>800	>800
Etoposide	>800	>800	>800	>800
Merbarone	>800	>800	>800	>800
Fostriecin	>800	>800	>800	>800

TABLE 3

Activity of nontopoisomerase inhibitors in repair-deficient and/or topoisomerase I-deficient yeast mutants

See legend to Table 1.

Drug	IC_{12}			
	Repair-proficient		Repair-deficient (*rad52*)	
	TOP1$^+$	*top1*	*TOP1*$^+$	*top1*
	μg/ml			
Rapamycin	0.041	0.054	0.028	0.039
Cinerubin A	4.4	6.5	1.6	1.4
CC-1065	5.9	5.4	3.4	3.5
Kanchanomycin	9.4	15	3.0	8.2
Nystatin	12	15	12	8.2
Amphotericin B	14	22	13	16
Hedamycin	28	29	7.4	6.8
Acivicin	46	41	380	174
Streptonigrin	110	120	3.0	5.4
Netropsin	150	140	100	110

Discussion

The results presented clearly demonstrate that DNA topoisomerase I is required for Cpt cytoxicity in yeast. Strains without topoisomerase I are totally resistant to Cpt and related drugs and a strain that overproduces the enzyme is hypersensitive. It is noteworthy that the same result was found for Cpt with two very different yeast species, *S. cerevisiae* and *S. pombe*, which diverged about 10^9 years ago (22). The *S. pombe* and

human *TOP1* genes have recently been sequenced (11, 23). The predicted amino acid sequences for the enzymes show a great deal of similarity to each other and to the *S. cerevisiae* sequence previously determined (9). Thus, it is likely that the site of interaction of Cpt with DNA topoisomerase I, and probably also the active site of the enzyme, has been conserved from yeast to humans.

There is strong evidence that DNA topoisomerase I is the target of Cpt in mammalian cells. Andoh and coworkers (7, 8) isolated a Cpt-resistant human lymphoblastic leukemia cell line and showed that topoisomerase I purified from this cell line is itself Cpt resistant. Their results led them to conclude that topoisomerase I is essential for the survival of mammalian cells. Based on our work, we would argue that their results are not sufficient to support this conclusion. Two different yeast species are sensitive to Cpt in a topoisomerase I-dependent manner, and yet topoisomerase I is not essential for viability in either species (9–11). It should be pointed out that efforts to isolate Cpt-resistant mutants of the *rad52* yeast strain have resulted in isolation of mutants deficient in topoisomerase I activity[2]. On the other hand, in the development of three different mammalian cell lines resistant to Cpt[2] (7) deletion of topoisomerase I has not been demonstrated. Thus, topoisomerase I may indeed be essential for viability in mammalian cells.

In our view, the cytotoxicity of Cpt is not due to inhibition of topoisomerase I per se but rather to the topoisomerase I-mediated damage to DNA. Cpt is thought to increase the lifetime of the topoisomerase I-DNA covalent intermediate (4) and apparently this intermediate is recognized as DNA damage by a DNA repair system. In *S. cerevisiae*, the *RAD52* DNA repair system (18) must be the predominant pathway used to repair the Cpt-stabilized enzyme-DNA covalent complex. Presumably *rad52* mutants are far more sensitive to Cpt than are repair-proficient strains because they are unable to complete the DNA repair process. It is likely that the lethal event in both yeast and mammalian cells may be the incomplete or improper repair of these DNA lesions. This also explains why Cpt causes DNA strand breaks in treated cells (6).

The fact that overproduction of topoisomerase I from plasmid pWE3 GAL-TOP1 kills a *rad52* mutant strain even in the absence of Cpt suggests that excess topoisomerase I bound to DNA can overwhelm the DNA repair systems remaining in this mutant. Repair-proficient cells can tolerate the same 5-fold overproduction of the enzyme, however. These results would suggest that Cpt stabilizes the same enzyme-DNA intermediate as is formed naturally in the absence of drug.

It is interesting that *rad52* strains also exhibit greater sensitivity to topoisomerase II inhibitors than do wild type strains (Table 2). This is probably because those inhibitors also function by stabilizing an enzyme-DNA intermediate that is recognized as a lesion by a DNA repair system. It has been proposed recently that the topoisomerase II inhibitors teniposide and amsacrine exert their cytotoxic effects in mammalian cells in an active process that occurs after stabilization of the enzyme-DNA complex (24). This process is likely to be part of a DNA repair pathway.

The *top1 rad52* strain is even more sensitive to topoisomerase II inhibitors than the *rad52* strain (Table 2). This observation is consistent with the finding that yeast topoisomerase I and topoisomerase II can partially substitute for each other (25, 26). When topoisomerase I is absent, cells are forced to rely on topoisomerase II and therefore become more sensitive to topoisomerase II inhibitors.

[2] Unpublished observations.

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