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Mutations in the *gyrB* Domain of Eukaryotic Topoisomerase II Can Lead to Partially Dominant Resistance to Etoposide and Amsacrine

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SUMMARY

Anti-topoisomerase II agents represent a major class of anticancer therapeutic agents. Resistance to this class of agents can be mediated by several possible mechanisms. One mechanism may involve mutations in the structural gene(s) for topoisomerases, altering the drug sensitivity of the enzymes. Several mutations have been described in mammalian cell lines that were selected for resistance to topoisomerase II-targeting drugs such as Adriamycin, etoposide, or amsacrine. The difficulty of performing genetic analysis in mammalian cell lines has complicated the determination of whether the observed mutations are responsible for drug resistance. We have reconstructed, in the yeast topoisomerase II gene, the arginine to glutamine mutation at position 450 of human topoisomerase IIα that was originally identified by Bugg *et al.* [*Proc. Natl. Acad. Sci. USA* **88**:7654–7658 (1991)]. Mutation of Lys_{439}, the equivalent amino acid in the yeast protein, to either glutamine or glutamic acid confers resistance to etoposide and amsacrine. Interestingly, in diploid yeast cells the heterozygous mutation can still confer partial drug resistance, compared with a diploid strain that is homozygous for wild-type topoisomerase II. Because mutations in the topoisomerase II gene that can confer dominant resistance to anti-topoisomerase II agents are relatively rare, mutations in the *gyrB* region may be important in the development of clinical drug resistance.

DNA topoisomerases are important targets for anticancer chemotherapeutic agents. Anti-topoisomerase agents are effective anticancer agents due to their ability to convert the enzyme into a cellular poison (1–3). The conversion of the enzyme into a cellular poison occurs by stabilization of an intermediate in the topoisomerase II reaction cycle that is termed the cleavage complex (4). The stabilized intermediate can then interfere with a variety of DNA metabolic events, including replication and transcription. Replication or transcription may convert the cleavage complex, a reversible lesion, into irreversible DNA damage (5).

A prediction of the poison hypothesis is that sensitivity to drugs targeting topoisomerase II should parallel enzyme activity, with low activity leading to drug resistance and high activity leading to drug hypersensitivity. Experiments in yeast have demonstrated that overexpression of topoisomerase II leads to drug hypersensitivity, whereas reduced enzymatic activity leads to specific resistance to anti-topoisomerase II drugs (3, 6).

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The mechanism of action of anti-topoisomerase drugs suggests that the presence of a drug-sensitive enzyme is sufficient for drug sensitivity. This implies that drug resistance due to a drug-resistant topoisomerase II should be recessive. This has been experimentally demonstrated with quinolone sensitivity in bacteria (7).

Several different cell lines have been selected for resistance to anti-topoisomerase II drugs (8, 9). In several cases, the cell lines carry mutations in the structural gene for topoisomerase II. For example, Bugg *et al.* (10) identified a mutation in the topoisomerase IIα gene that changes Arg_{450} to glutamine. Danks *et al.* (11) later found that amino acid 803 is changed from proline to serine, although it is not known whether the mutation is on the same allele of the *TOP2α* gene as the Arg_{450} mutation.

For most of the mutations identified in mammalian cell lines, it has been difficult to rigorously demonstrate the role that the identified mutation plays in drug resistance. This has been due to the difficulty of demonstrating drug resistance for mutations that act in a recessive fashion. In this report, we describe the construction of a mutation homologous to the Arg_{450}Gln mutation in yeast topoisomerase II. We demonstrate that the altered yeast enzyme confers resistance to etoposide and

ABBREVIATIONS: mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; PCR, polymerase chain reaction; YPDA, yeast extract/peptone/dextrose medium.

mAMSA, demonstrating that the change identified by Bugg *et al.* (10) is likely to be responsible for the drug-resistant enzyme. We also provide experimental evidence that the mutational change results in an enzyme that can confer partially dominant drug resistance.

Materials and Methods

Yeast growth and transformation. Yeast cells were typically grown in YPDA or, to select for plasmids carrying *URA3* as a marker, synthetic complete medium lacking uracil (12). Yeast transformation was carried out using the modified lithium acetate protocol of Schiestl and Gietz (13).

Yeast strains and plasmids. The yeast strain JN362a has been described previously (14). This strain carries the *ISE2* mutation, which allows sensitivity of yeast cells to anti-topoisomerase II agents such as etoposide. The etoposide and mAMSA sensitivity of cells carrying wild-type *TOP2* was determined with JN394, which is isogenic to JN362a but also carries the DNA repair mutation *rad52* to increase the sensitivity of cells to mAMSA and etoposide. The yeast strain JN514 is a mating type α strain that is closely related to JN362a and also carries the *ISE2* mutation. Diploid strains were created by mating JN514 and JN362a derivatives, using standard techniques (12). Typically, JN362a was transformed with plasmids carrying different *TOP2* alleles and was then converted to *rad52*$^-$ by one-step gene disruption. The construction of the isogenic *TOP2*$^+$ *rad52*$^-$ strain JN394 has been described previously (14). Similarly, JN514 was also converted to *rad52*$^-$ by one-step gene disruption before mating.

Construction of mutagenized alleles of *TOP2* was performed using the plasmid pMJ2. This plasmid carries most of the coding sequence of the yeast *TOP2* gene but lacks the promoter and the first 485 nucleotides of the coding sequence. The plasmid has a unique Asp_{718} site within *TOP2* for integration into the chromosomal yeast *TOP2* locus, using two-step gene replacement (15, 16), and also carries the yeast *URA3* gene for selection of transformants in yeast.

Oligonucleotide-directed mutagenesis. Mutations were constructed using the plasmid pMJ2 described above. Oligonucleotide-directed mutagenesis was performed using the technique of Kunkel *et al.* (17) with the Muta-gene kit (Bio-Rad), following the supplier's instructions. All of the mutations were introduced at amino acid 439 of yeast topoisomerase II, which corresponds to amino acid 450 of human topoisomerase IIα. The mutagenic oligonucleotides used were GCCGGTACA**C**AAGAAGGCTAT (changing Lys_{439} to glutamine), GCCGGTACAAA**C**GAAGGCTAT (changing Lys_{439} to asparagine), GCCGGTACAA**G**AGAAGGCTAT (changing Lys_{439} to arginine), GCCGGTACA**G**AAGAAGGCTAT (changing Lys_{439} to glutamate), and GCCGGTACAA**C**AGAAGGCTAT (changing Lys_{439} to threonine). The nucleotides shown in bold type differ from the wild-type sequence. All mutations introduced into pMJ2 were verified by DNA sequence analysis.

Determination of drug sensitivity. Drug sensitivity of yeast strains carrying different *TOP2* alleles was determined as described previously (18). All tests of drug sensitivity were performed at least three times with two independently constructed isolates.

Results

Construction and verification of mutations in yeast topoisomerase II corresponding to Arg_{450}Gln. The amino acid sequence around Arg_{450} in human topoisomerase IIα is Asn-Asp-Ala-Gly-Gly-Arg_{450}-Asn-Ser-Thr-Glu-Cys (19). The corresponding region of yeast topoisomerase II is Asn-Lys-Ala-Gly-Thr-Lys_{439}-Glu-Gly-Tyr-Lys-Cys (20). Four of the 10 amino acids in the region around Arg_{450} are identical in the two sequences, although the yeast sequence contains a conservative substitution of $lysine_{439}$ for arginine. Sequences flanking the 10 amino acids around Arg_{450} also show significant homology. For example, in yeast topoisomerase II the topoisomerase II signature sequence Glu-Gly-Asp-Ser-Ala is found at amino acids 450–454 (10, 21). The same sequence is found at amino acids 461–465 of human topoisomerase IIα. We introduced (by oligonucleotide-directed mutagenesis) several different substitutions into the yeast topoisomerase II gene at nucleotides corresponding to Lys_{439}, including mutations that change Lys_{439} to arginine, glutamine, asparagine, threonine, or glutamate.

To verify that the constructed mutations actually produce only the mutant topoisomerase II, RNA was isolated from yeast cells carrying the mutant topoisomerase II. The RNA was converted to DNA and amplified by PCR, and the PCR product was directly sequenced. In all cases, the sequence of the PCR product corresponded to the sequence of the mutagenic oligonucleotide.

Resistance to anti-topoisomerase II drugs conferred by mutating Lys_{439} of yeast topoisomerase II. We first examined the effect on drug sensitivity in yeast of mutating Lys_{439} to glutamine. The sensitivity to etoposide of the *rad52*$^-$ derivative of JN362a carrying the Lys_{439}Gln mutation is shown in Fig. 1. For wild-type topoisomerase II, 50 μg/ml etoposide is cytotoxic and 100 μg/ml levels of the drug result in more cell killing (strain JN394). In contrast, in cells carrying the Lys_{439}Gln mutation 50 μg/ml etoposide reduced the growth rate but was not cytotoxic. The higher etoposide concentration, 100 μg/ml, inhibited growth but also was not cytotoxic. Therefore, the mutation that is homologous to the mutation identified by Bugg *et al.* results in etoposide resistance in yeast.

The sensitivity to amsacrine of the strain carrying the Lys_{439}Gln mutation was also determined. The results of this determination are shown in Fig. 2. As we have reported previously, 5 μg/ml amsacrine results in cell killing in *rad52*$^-$ strains expressing wild-type topoisomerase II (3, 6). In comparison, drug concentrations of >20 μg/ml were needed to effect cytotoxicity in the Lys_{439}Gln mutant strain. Furthermore, at a concentration of 100 μg/ml amsacrine, survival of strains carrying wild-type topoisomerase II was <0.5%, compared with approximately 60% for strains carrying the Lys_{439}Gln mutation. These results clearly demonstrate that mutations at this position lead to a drug-resistant topoisomerase II.

We also examined the effect of other amino acid substitutions at Lys_{439}. We introduced the conservative substitution of arginine for lysine. We also changed Lys_{439} to threonine or asparagine and introduced the nonconservative change to glutamic acid. Neither the Lys_{439}Arg nor Lys_{439}Thr mutation had any significant effect on drug sensitivity. The Lys_{439}Asn mutation conferred modest resistance to both amsacrine and etoposide, whereas the Lys_{439}Glu mutation produced resistance to both etoposide and amsacrine at least as great as that produced by the Lys_{439}Gln mutation (data not shown). As a control experiment, we also carried out the two-step gene replacement procedure using pMJ2 and simply reintegrated wild-type topoisomerase II. Reintegration of wild-type *TOP2* did not change the sensitivity of cells to either mAMSA or etoposide (data not shown).

Partially dominant drug resistance conferred by the Lys_{439}Gln mutation in yeast topoisomerase II. Bugg *et al.* (10) suggested that the human Arg_{450}Gln mutation might be dominant, based on their detection of mRNA for both wild-type and mutant *TOP2*α. We tested the dominance of the

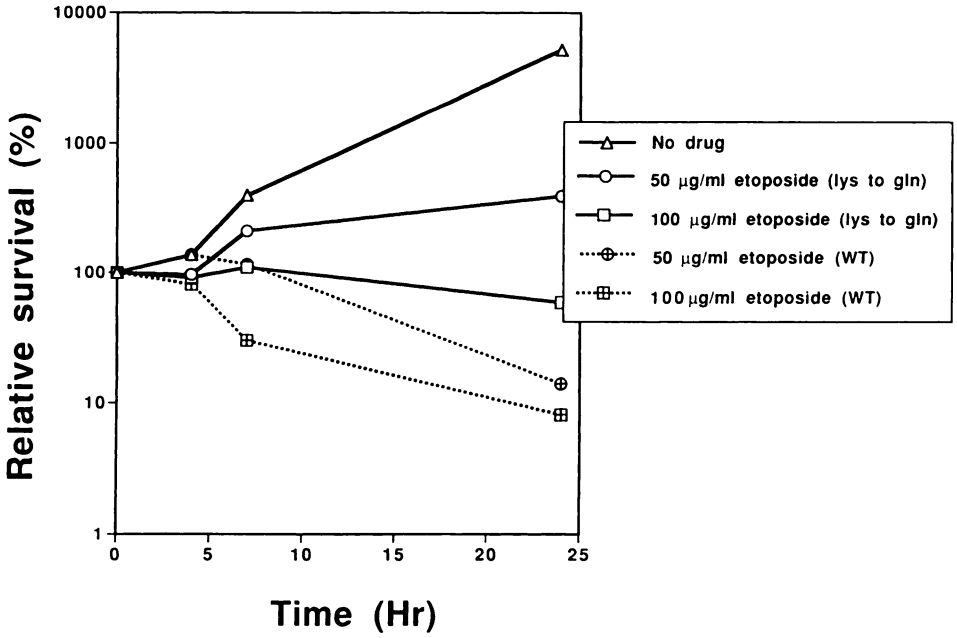

Fig. 1. The $Lys_{439}Gln$ mutation confers resistance to etoposide. Logarithmically growing yeast cells were suspended in YPDA at a titer of 2×10^6 cells/ml, and drugs were added at the indicated concentrations. The growth of JN394 and that of JN394 carrying the $Lys_{439}Gln$ mutation were essentially the same in YPDA in the absence of drug, so only the wild-type (*WT*) form is shown. At the indicated times, samples were removed and plated on solid YPDA plates to determine the viable counts; all data are shown relative to the viable titer at time 0. All drug sensitivity experiments were performed at least three times, with two independently constructed isolates; representative results are shown.

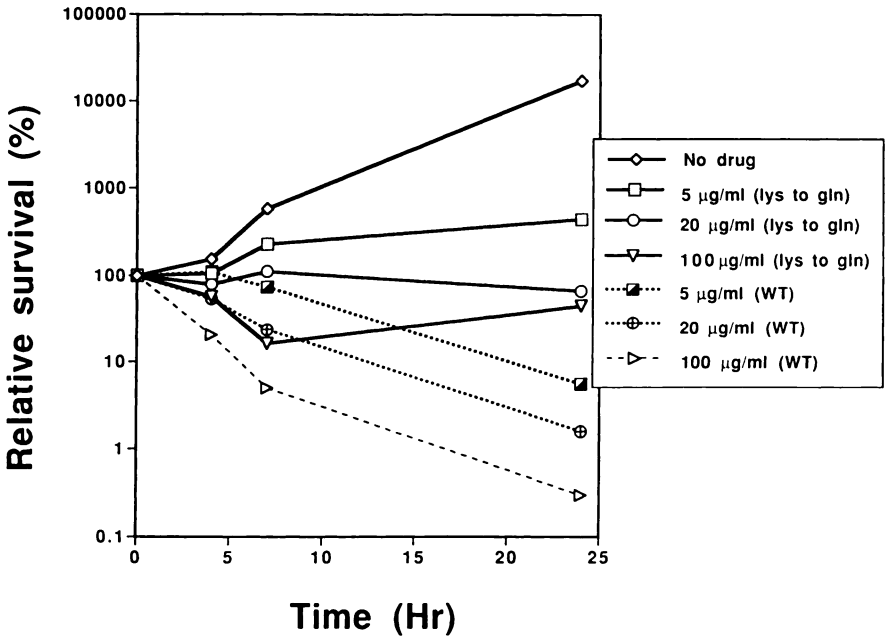

Fig. 2. The $Lys_{439}Gln$ mutation confers resistance to amsacrine. The sensitivity to amsacrine of JN394 and JN394 carrying the $Lys_{439}Gln$ mutation was determined using the conditions described for Fig. 1. Drug concentrations are indicated. The no-drug sample contained an equivalent volume of dimethylsulfoxide, the solvent used to dissolve amsacrine. *WT*, wild-type.

$Lys_{439}Gln$ and $Lys_{439}Glu$ mutations in yeast by mating JN394/$Lys_{439}Gln$ or JN394/$Lys_{439}Glu$ with JN514r52. The resultant diploid is homozygous for *rad52*$^-$ and *ISE2* and heterozygous for *TOP2*. We then tested the sensitivity of the resultant diploid to either amsacrine or etoposide and compared the sensitivity with that of an isogenic diploid that is homozygous for wild-type *TOP2*. The results with etoposide are shown in Fig. 3A. The diploid that is homozygous for wild-type *TOP2* showed some cytotoxicity at 20 μg/ml etoposide, with greater cell killing at 50 or 100 μg/ml etoposide. In contrast, 20 or 50 μg/ml etoposide reduced cell growth in the heterozygous diploid but was not cytotoxic. Increasing the drug concentration to 100 μg/ml etoposide did cause cell killing in the heterozygous diploid, although the cell killing was less than that observed in the strain that is homozygous for topoisomerase II.

Similarly, the $Lys_{439}Gln$ mutation also enhanced resistance to amsacrine. Concentrations of >20 μg/ml amsacrine were necessary to effect cytotoxicity in the heterozygous diploid, whereas concentrations of 5 μg/ml amsacrine or greater were sufficient to confer cytotoxicity in the strain homozygous for wild-type *TOP2* (Fig. 3B). The $Lys_{439}Glu$ mutation behaved similarly to the $Lys_{439}Gln$ mutation, inasmuch as it conferred a similar level of resistance to both mAMSA and etoposide when heterozygous (data not shown). These results are in contrast to results we previously reported with the *top2–5* allele (22). Yeast cells carrying the *top2–5* allele are very resistant to both etoposide and mAMSA, but when a wild-type *TOP2* gene is introduced drug sensitivity is indistinguishable from that of cells that are homozygous for wild-type topoisomerase II. Hence, the $Lys_{439}Gln$ and $Lys_{439}Glu$ mutations are members of a unique class of drug-resistant topoisomerase II mutations.

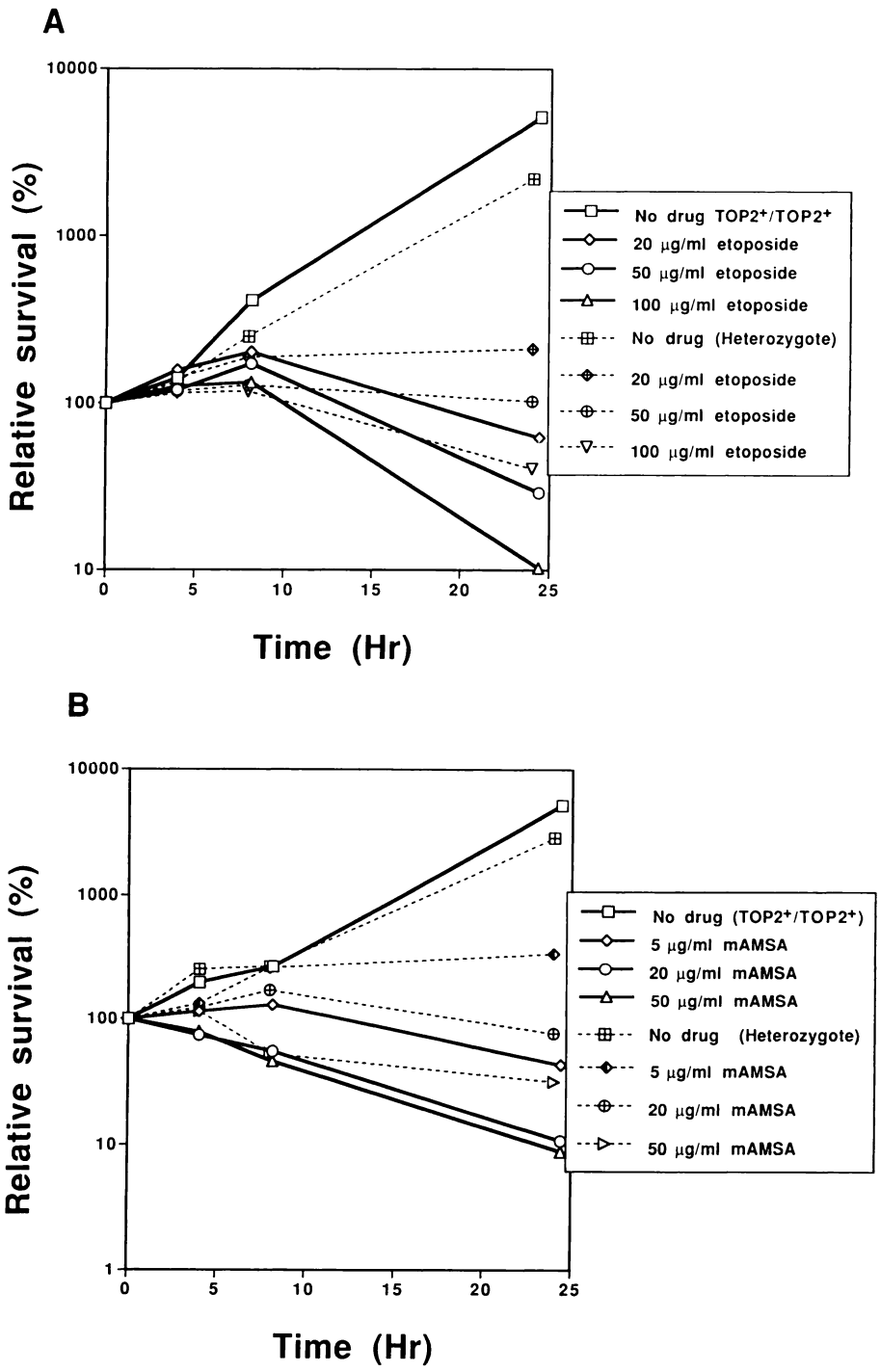

Fig. 3. The $Lys_{439}Gln$ mutation confers partially dominant resistance to etoposide and amsacrine. The *TOP2* allele of JN362a was converted to $Lys_{439}Gln$, and the strain was then converted to *rad52*$^-$ by one-step gene replacement. The strain carrying the mutation was then mated to a *rad52*$^-$ derivative of JN514. The resultant diploid, which is heterozygous for *TOP2* and homozygous for *rad52*$^-$, was then tested for sensitivity to etoposide and amsacrine using the same conditions as in Fig. 1. The control strain for this experiment was a diploid constructed using JN394 with wild-type topoisomerase II mated to a *rad52*$^-$ derivative of JN514. A, Sensitivity of the two diploids to etoposide. The drug concentrations are indicated. B, Sensitivity of the diploids to amsacrine.

Discussion

Several mutations that lead to resistance to anti-topoisomerase II agents have been identified in human cell lines. Many of the mutations that have been described fall within the region of amino acids 400–500 of topoisomerase IIα (23–25). Several other mutations have also been identified in other regions of topoisomerase II, frequently around the active site tyrosine (reviewed in Ref. 26). Several yeast mutations are also relatively close to the active site tyrosine or in the carboxyl-terminal third of the protein (22, 26). In the case of the yeast mutations, it has been straightforward to demonstrate that the observed mutational changes cause the observed drug resistance. This report represents the first direct demonstration that a mutation in topoisomerase II identified in a mammalian cell line can result in drug resistance.

It is not possible to directly compare the drug sensitivity of human and yeast cells, so our experiments do not exclude the possibility that other mechanisms of resistance are acting in the CEM/VM1 cells studied by Bugg *et al.* (10). Our experiments clearly show that the mutation at position 450 can confer resistance to anti-topoisomerase II agents; hence it is very likely that the mutation at amino acid 450 plays an important role in the drug resistance of CEM/VM1 cells.

The experiments presented here also confirm the hypothesis of Bugg *et al.* (10) suggesting that the mutation in topoisomerase IIα could confer semidominant drug resistance. This result may have important implications for the development of drug resistance in patients treated with anti-topoisomerase II agents. If mutations in the structural gene of topoisomerase II are important for the development of clinical drug resistance, it would be expected that mutations that can confer partially dominant drug resistance will be observed much more frequently than mutations that require multiple changes for the development of resistance. However, there is no published evidence on whether mutations in topoisomerase II occur in patient samples during treatment with anti-topoisomerase agents.

How might a mutation in topoisomerase II lead to partially dominant resistance to drugs that stabilize cleavage? One possibility may relate to the fact that topoisomerase II is a homodimer. If wild-type and mutant subunits are produced in the same quantities and have similar stabilities, then cells should contain three forms of the enzyme, i.e., wild-type homodimers, mutant homodimers, and wild-type/mutant heterodimers. The relative amounts of the three forms should be 1:1:2. We suggest that whether a particular mutation is recessive or semidominant depends on the drug sensitivity of the wild-type/mutant heterodimers. If the heterodimer is drug sensitive then 75% of the topoisomerase II holoenzymes in the cell are drug sensitive, whereas if the heterodimer is drug resistant then only 25% of the holoenzymes in the cell are drug sensitive. The stabilization of cleavage of the wild-type homodimeric enzyme would still cause some drug sensitivity; however, the overall reduction of drug-sensitive enzymatic activity would lead to enhanced drug resistance.

There is no specific information available at present regarding how a mutation in one subunit of topoisomerase II might lead to a drug-resistant holoenzyme. It has been previously demonstrated that, upon ATP binding, yeast topoisomerase II undergoes a conformational change (27). Perhaps the mutant subunit is able to enforce a conformational change in the enzyme that alters its ability to interact with drugs that target the enzyme.

One reason for studying topoisomerase II mutants that confer drug resistance is to define the binding sites on the protein that interact with anti-topoisomerase agents. Our results suggest that selection with anti-topoisomerase II drugs in mammalian cells may produce a limited spectrum of mutations, i.e., mutations that can confer at least partially dominant drug resistance. To fully define all mutations that can lead to drug resistance, it will be necessary to exploit systems such as yeast, where fully recessive drug-resistant topoisomerase II mutants can be recovered in an unbiased fashion.

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