Increased Drug Affinity as the Mechanistic Basis for Drug Hypersensitivity of a Mutant Type II Topoisomerase*

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Altered sensitivity of topoisomerase II to anticancer drugs profoundly affects the response of eukaryotic cells to these agents. Therefore, several approaches were employed to elucidate the mechanism of drug hypersensitivity of the mutant yeast type II topoisomerase, top2H1012Y. This mutant, which is ~5-fold hypersensitive to ellipticine, formed DNA cleavage complexes more rapidly than the wild-type yeast enzyme in the presence of the drug. Conversely, no change in the rate of DNA religation was observed. There was, however, a correlation between increased cleavage rates and enhanced drug binding affinity. The apparent dissociation constant for ellipticine in the mutant topoisomerase II-drug-DNA ternary complex was ~5-fold lower than in the wild-type ternary complex. Furthermore, the apparent $K_d$ value for the mutant binary (topoisomerase II-drug) complex was ~2-fold lower than the corresponding wild-type complex, indicating that drug hypersensitivity is intrinsic to the enzyme. These findings strongly suggest that the enhanced ellipticine binding affinity for topoisomerase II is the mechanistic basis for drug hypersensitivity of top2H1012Y.

Topoisomerase II is one of the most important targets currently available for the treatment of human cancers (1–5). Drugs targeted to this essential enzyme act by increasing levels of covalent topoisomerase II-cleaved DNA complexes that are normal but fleeting intermediates in the catalytic cycle of the enzyme (1–7). Treatment with these agents generates protein-associated breaks in the genome, which triggers a series of events that ultimately culminates in an apoptotic-like cell death (1, 3, 4, 8, 9).

There is a high degree of variability in the response of different cancers and/or patients to topoisomerase II-targeted drugs (1, 10–12). Although drug resistance or hypersensitivity greatly affects the success of cancer chemotherapy, mechanisms that alter drug sensitivity have yet to be fully defined. Several factors contribute to the sensitivity of cells toward agents targeted to the type II enzyme. First, altered rates of drug metabolism, cellular uptake, or efflux often result in resistance to a broad spectrum of agents (1, 8, 13–16). Second, mutations in enzymes that recognize or process topoisomerase II-induced lesions often lead to drug resistance, and diminished repair pathways render cells hypersensitive (1, 8, 14–21). Third, changes in cellular topoisomerase II content or activity dramatically affect the level of drug cytotoxicity (1, 2, 8, 14, 16, 20, 22–25). Finally, mutations within topoisomerase II that alter drug-induced DNA cleavage have produced a wide variety of phenotypes, ranging from high resistance (2, 3, 20, 26–36) to several-fold hypersensitivity (35, 36).

Although a number of mutant type II topoisomerases have been characterized in vitro (26–29, 31–36), the mechanistic basis for altered drug sensitivity of these enzymes has remained an enigma. It has been widely assumed that drug resistance of mutants that maintain high rates of catalytic activity is due primarily to decreased drug binding in the enzyme-drug-DNA ternary complex. While this has been demonstrated for one quinolone-resistant mutant DNA gyrase (a prokaryotic type II topoisomerase) (gyrA S83W, which has a Ser → Trp mutation at position 83 of its A subunit) (4, 37), drug binding studies have never been reported for any mutant eukaryotic enzyme. The strongest evidence for decreased drug binding in the eukaryotic ternary complex comes from dose-response relationships for quinolone-induced DNA cleavage mediated by a yeast mutant (top2H1012Y, which has a His → Trp mutation at position 1012) type II topoisomerase in which the potency of the drug was diminished (35).

The basis of drug hypersensitivity is even less well understood than resistance. To date, only two hypersensitive mutant type II topoisomerases have been described in eukaryotes, both of which are from yeast: top2H1012Y (hypersensitive to ellipticine) (35) and top2S741W (hypersensitive to etoposide) (36). Although the mechanistic basis of altered drug sensitivity has not been defined for either enzyme, hypersensitivity of the latter mutant correlated with a decreased rate of DNA religation in the presence of etoposide (36).

To elucidate a mechanism of drug hypersensitivity, the interactions of yeast top2H1012Y with ellipticine were characterized. The mutant type II enzyme established drug-induced enzyme-DNA cleavage complexes more rapidly than wild-type topoisomerase II, but religated DNA at the same rate. As determined by steady-state and frequency domain fluorescence spectroscopy, the apparent affinity of ellipticine for topoisomerase II in the mutant ternary complex was ~5-fold higher (comparable to the level of hypersensitivity; Ref. 35) than the wild-type complex. In addition, the affinity of top2H1012Y for ellipticine in the absence of DNA was ~2-fold higher than that of the wild-type enzyme. These results suggest a mechanistic basis for hypersensitivity of topoisomerase II-targeted drugs and indicate that interactions between ellipticine and
the enzyme are representative of drug action in the ternary complex.

**EXPERIMENTAL PROCEDURES**

**Materials and Yeast Strains—** A 40-mer oligonucleotide (corresponding to nucleotides 87–126 of pBR322; Ref. 38) containing a topoisomerase II cleavage site (39, 40) was synthesized and purified as outlined by FreiPhil-Ammon et al. (41). Oligonucleotides were diluted to the appropriate concentration in 5 mM Tris–HCl, pH 7.4, 0.5 mM EDTA. Negatively supercoiled pBR322 DNA was prepared as formerly described (42). Ellipticine was prepared as a 20 mM or 10 mM stock dissolved in dimethyl sulfoxide or ethanol, respectively, and stored at −20 °C. Ellipticine, ethidium bromide, and Tris–HCl were obtained from Sigma; UltraPure HEPES was from VWR; SDS was purchased from E. Merck Biochemicals; proteinase K was from U. S. Biochemical Corp.; yeast nitrogen base, yeast extract, and Bacto-agar were from Difco; Rose Bengal was from Aldrich; and dimethyl-POPOP was from Eastman. All other chemicals were analytical reagent grade.

The Saccharomyces cerevisiae yeast strains employed were J N934T2-4, which possesses the top2-4 temperature-sensitive mutant allele instead of the wild-type topoisomerase II gene, and has the genotype: ura3-52, leu2, trpl, his7, ade2-1, ISE2, rad52::LEU2 (24); and J E1.1, which has the genotype: leu2, trpl, ura3-52, prpl-1122, pep3-4, his3::PGAL10-GAL4 (43).

**Mutant Selection and Purification—** The H1012Y mutation was introduced into the yeast TOP2 gene by hydroxylamine-induced mutagenesis (44) and selected for resistance to quinolones as described by Elsea et al. (35). top2H1012Y displays resistance to CP-115,953 and etoposide, wild-type sensitivity to amsacrine, and ~5-fold hypersensitivity to ellipticine (35). Overexpression for purification of the wild-type and mutant type II enzymes was achieved using the plasmid YEpGAL1TOP2 (45). Mutant and wild-type enzymes were purified to apparent homogeneity (as determined by Coomassie or silver-stained polyacrylamide gels) using the protocol of Worland and Wang (45) as modified by Elsea et al. (35).

**Wild-type Topoisomerase II- and top2H1012Y-mediated DNA Cleavage—** Assays were performed by a modification of the protocol of Robinson and Osheroff (46). Cleavage reactions contained 100 nM wild-type topoisomerase II or top2H1012Y, 5 nM negatively supercoiled pBR322 DNA, and the appropriate drug concentration in a total of 20 μl of reaction buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, and 2.5% glycerol). Reaction mixtures were incubated at 28 °C for the designated times. Products were resolved by gel electrophoresis on ethidium bromide-containing 1% agarose gels (46) and DNA bands were visualized by transillumination with UV light (300 nm) and quantitated with an Alpha Innotech IS1000 imaging system. The density of the bands was proportional to the amount of DNA present. Control samples contained an equal amount of drug diluent. Drug-induced DNA cleavage was not seen in the absence of topoisomerase II.

**Wild-type Topoisomerase II- and top2H1012Y-mediated DNA Religation—** Assays were performed by a modification of the protocol of Robinson et al. (48). Reactions contained 5 nM negatively supercoiled pBR322 DNA, 100 nM yeast topoisomerase II, and 10 μM ellipticine in a total of 20 μl of reaction buffer. DNA cleavage/religation equilibria were established by incubation at 28 °C for 6 min. Topoisomerase II-mediated religation of cleaved DNA was induced by rapidly shifting samples from 28 °C to 65 °C. Religation was terminated by the addition of SDS at various time points followed by EDTA and proteinase K. Reaction products were analyzed by agarose gel electrophoresis and quantitated as described above.

**Steady-state and Frequency Domain Fluorescence Spectroscopy—** Steady-state and frequency domain fluorescence spectroscopy were performed as outlined previously (47). For all fluorescence experiments, samples were excited at 326 nm and emission light was monitored at 420 nm. The emission polarizer was fixed at the magic angle (54.7°), and a 420 nm interference band pass filter was employed to separate fluorescence from scattered light. Dimethyl-POPOP, with a lifetime of 1.45 ns, or rose Bengal, with a lifetime of 732 ps in EtOH (48), were used as references. Data were acquired at 25 °C. Samples contained 10 μM ellipticine, the designated concentrations of enzyme, and 200 nM 40-mer oligonucleotide (when DNA was present) in a final volume of 500 μl of 20 mM HEPES, pH 7.9, 100 mM NaCl, 5 mM MgCl2, and 0.1 mM EDTA and were incubated for 5 min prior to fluorescence measurements. All chemicals were ultrapure grade to minimize nonspecific fluorescence. The buffer background intensity was subtracted for binding calculations.

**RESULTS**

Rate of Topoisomerase II-DNA Cleavage Complex Formation—** The mutant type II topoisomerase, top2H1012Y, is hypersensitive to ellipticine as determined by the enhancement of DNA cleavage (Fig. 1, inset) (35). Yeast cells that harbor top2H1012Y (and lack wild-type enzyme activity) display a similar degree of hypersensitivity toward this drug (35), under-scoring the relationship between drug–enzyme interactions in vitro and cellular phenotype. Therefore, as a first step toward defining the mechanistic basis of enhanced ellipticine sensitivity of top2H1012Y, the rate of formation of drug-induced topoisomerase II-DNA cleavage complexes was determined for the wild-type and mutant enzymes.

As seen in Fig. 1 (inset), at equal concentrations of ellipticine (10 μM), top2H1012Y not only accumulated higher levels of DNA cleavage complexes but did so more rapidly than wild-type topoisomerase II. Even when cleavage levels for the two enzymes were normalized by decreasing the drug concentration to 4 μM in assays that employed the mutant enzyme, the rate of cleavage complex formation was severalfold faster for top2H1012Y than the wild-type enzyme (Fig. 1).

The enhanced rate of DNA cleavage complex formation observed with top2H1012Y may result from a number of possibilities. One possibility is that the mutant enzyme utilizes a broader spectrum of DNA cleavage sites than wild-type topoisomerase II. However, this does not appear to be the case. As determined by cleavage mapping experiments, the sites at which the two enzymes incised DNA were nearly identical (not shown). Two other possibilities to explain the enhanced rate of cleavage complex formation exist: 1) top2H1012Y may religate DNA more slowly, and/or 2) it may display a higher affinity for ellipticine than the wild-type enzyme. Experiments were performed to examine both of these alternatives.

**Topoisomerase II-mediated DNA Religation—** Ellipticine does not affect the rate of DNA religation mediated by wild-type topoisomerase II (47). Therefore, the drug apparently increases the level of DNA scission primarily by enhancing the forward rate of cleavage. It may be that the mechanism of ellipticine action on the mutant enzyme differs and that the hypersensitivity of top2H1012Y is due (at least in part) to an additional effect on DNA religation. As determined by DNA religation assays, this is not the case. The apparent first order rate of religation mediated by top2H1012Y was identical to that of the wild-type enzyme when ellipticine concentrations were equal (not shown) or corrected to normalize cleavage levels (Fig. 2).
Ellipticine Binding to top2H1012Y in the Ternary Enzyme—DNA Drug Complex—Recently, the binding affinities of ellipticine for DNA, yeast wild-type topoisomerase II, and the ternary complex were characterized by steady-state and frequency domain fluorescence spectroscopy (47). Therefore, these techniques were utilized to ascertain whether the drug hypersensitivity of top2H1012Y correlates with an increased binding affinity of ellipticine for topoisomerase II in the ternary complex. The apparent $K_D$ value was calculated from changes in fluorescence intensity of ellipticine in the presence of DNA with increasing concentrations of mutant topoisomerase II. As determined by double-reciprocal analysis (Fig. 3), the apparent dissociation constant of ellipticine for top2H1012Y was $-310 \text{ M}$ (Table I). This $K_D$ value is $-5$-fold lower than that previously reported (47) for the wild-type ternary complex ($1.5 \text{ M}$). The increase in binding affinity of ellipticine for topoisomerase II in the ternary complex is comparable to the level of drug hypersensitivity of top2H1012Y observed in vivo and in vitro (35). It should be emphasized that the lower dissociation constant of top2H1012Y in the ternary complex is not due to enhanced DNA binding by the mutant enzyme; indeed, the affinity of top2H1012Y for DNA is lower than that of the wild-type enzyme (35).

Ellipticine Binding to top2H1012Y—While protonated ellipticine binds to DNA, it is the deprotonated form of the drug that binds to topoisomerase II and is present in the ternary complex (47). On this basis, it has been suggested that ellipticine has contacts with topoisomerase II in the ternary complex and that the interactions between the enzyme and the drug in the absence of DNA (i.e. binary complex) may reflect those occurring within the topoisomerase II–drug–DNA complex. To investigate whether alterations that lead to hypersensitivity are intrinsic to the enzyme (in the absence of DNA), the binding of ellipticine to top2H1012Y was characterized (Fig. 4). The apparent $K_D$ value for the mutant enzyme ($-90 \text{ M}$) was $-2$-fold lower than that of wild-type topoisomerase II ($-160 \text{ M}$) (Table I) (47). The enhanced binding of ellipticine to top2H1012Y provides strong evidence that interactions within the binary complex mimic those observed for the ternary complex and contribute to the hypersensitivity of this mutant enzyme.

To examine interactions within the binary and ternary complex in greater detail, drug–enzyme binding was analyzed by frequency domain fluorescence spectroscopy (Table I). The fluorescence lifetime of free deprotonated ellipticine increased dramatically from $-60 \text{ ps}$ to $-24 \text{ ns}$ upon binding to the wild-type enzyme in the absence of DNA (47). A similar increase was observed following the formation of the wild-type ternary complex (47). In contrast, the lifetime of deprotonated ellipticine rose to $-15 \text{ ns}$ in the presence of top2H1012Y or upon formation of the mutant ternary complex. The fact that the lifetimes for ellipticine bound to the wild-type or mutant enzymes paralleled those for the respective ternary complexes supports the

**Table I**

| Sample                      | $r$    | $K_D$ (nM) |
|-----------------------------|--------|------------|
| WT topoisomerase II·ellipticine | 24.0 ± 1.4 | 160       |
| WT topoisomerase II·ellipticine·DNA | 21.3 ± 2.3 | 1500      |
| top2H1012Y·ellipticine       | 15.5 ± 0.1 | 90        |
| top2H1012Y·ellipticine·DNA   | 15.5 ± 0.2 | 310       |

*a* Samples contained $1 \mu M$ ellipticine in HEPES buffer as described under "Experimental Procedures." The topoisomerase II concentration was $100 \text{ nM}$, and when present, the DNA concentration was $200 \text{ nM}$. Samples were excited at $326 \text{ nm}$ and emission was monitored at $420 \text{ nm}$. Fluorescence lifetimes ($r$) were determined using frequency domain analysis. Data for the wild-type enzyme are from Froelich-Ammon et al. (47).

*b* Dissociation constants were determined by double-reciprocal analysis as outlined in "Experimental Procedures." Data for the wild-type enzyme are from Froelich-Ammon et al. (47).
Mechanistic Basis of Topoisomerase II Drug Hypersensitivity

Little is understood concerning the factors that govern the sensitivity of topoisomerase II to anticancer drugs. Changes in the cellular levels of topoisomerase II often correlate with either drug resistance or hypersensitivity (1, 2, 8, 14, 22–25); however, the mechanism(s) by which mutations within the enzyme contribute to drug sensitivity has not been defined. In an effort to delineate potential mechanisms underlying altered drug sensitivity, the hypersensitivity of top2H1012Y to ellipticine was characterized.

As determined by steady-state and frequency-based time domain fluorescence spectroscopy, the affinity of topoisomerase II-drug binding within the mutant ternary complex was higher than that of the wild-type complex. This increase in binding was comparable to the enhanced drug cytotoxicity of yeast harboring the top2H1012Y gene and the increased DNA cleavage mediated by the mutant enzyme in vitro (35). Thus, it appears that enhanced drug binding in the top2H1012Y ternary complex is the primary mechanistic basis of hypersensitivity to ellipticine.

Furthermore, the binding affinity of ellipticine for top2H1012Y in the absence of DNA was 2-fold higher than that of the wild-type binary complex. This indicates that drug hypersensitivity is intrinsic to topoisomerase II and provides compelling evidence for direct interactions between anticancer drugs and the enzyme. Moreover, these findings suggest that the enzyme-ellipticine interactions in the binary complex are indicative of those in the ternary complex. However, it is likely that DNA also modulates topoisomerase II-drug interactions as shown by the greater increase in binding affinity in the ternary complex.

A difference in fluorescence lifetimes of ellipticine in the binary and ternary complexes, and further argues for direct interactions between topoisomerase II and ellipticine.

binding and the sensitivity of cells to topoisomerase II-targeted drugs.

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