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Mutations at Arg486 and Glu571 in Human Topoisomerase IIα Confer Resistance to Amsacrine: Relevance for Antitumor Drug Resistance in Human Cells[1]

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ABSTRACT

Human topoisomerase II, a nuclear protein involved in chromosome segregation, is the target of amsacrine and other clinically important anticancer drugs. The enzyme is expressed as α and β isoforms whose mutation/down-regulation has been implicated in drug resistance. To understand the role of target mutations in cellular drug resistance, we have used yeast to select and characterize plasmid-borne human topoisomerase IIα mutants resistant to amsacrine. Single point changes of Glu571 to Lys (E571K) or Arg486 to Lys (R486K) in the conserved PLRGK motif, both of which reside in the GyrB homology domain of human topoisomerase IIα, were frequently selected and could be shown in vivo to confer >25-fold and >100-fold resistance, respectively, to amsacrine and ~3-fold cross-resistance to etoposide. Highly purified E571K and R486K human topoisomerase IIα proteins required 100-fold higher levels of amsacrine to induce DNA cleavage similar to that of wild-type protein, consistent with a resistance mechanism involving reduced cleavable complex formation. Our functional studies of the R486K mutation, previously identified in two amsacrine-resistant human cell lines and in human biopsy material, establish unequivocally that it confers resistance, and suggest mechanisms for its phenotypic expression in vivo. These results differ significantly from previous work using yeast topoisomerase II as a model system: introduction of the equivalent mutation to R486K (R476K) into the yeast enzyme did not give amsacrine resistance. We conclude that species-specific differences in topoisomerase II enzymes can affect the drug resistance phenotype of particular mutations and highlight the need to study the relevant human homolog.

Much of the current interest in DNA topoisomerases stems from their role as targets for anticancer therapeutics and their involvement in resistance to chemotherapy (Liu, 1989; Pommier, 1993; Wang, 1996). In particular, topoisomerase II is inhibited by a variety of clinically important antitumor agents, including etoposide, doxorubicin, and amsacrine. The enzyme is a dimer that acts as an ATP-driven clamp and catalyzes the crossing of one DNA duplex through another, thereby allowing chromosome condensation and segregation at mitosis (Berger et al., 1996; Wang, 1996). Most of the topoisomerase II inhibitors in clinical use form a ternary complex with enzyme and DNA (the 'cleavable complex') that converts the protein into a cellular poison (Pommier, 1993). We and others have shown that human topoisomerase II is expressed as two genetically distinct isoforms, α (p170) and β (p180) (Drake et al., 1987; Jenkins et al., 1992; Austin et al., 1993). Using recombinant human α and β proteins overexpressed and purified from yeast (Wasserman et al., 1993; Austin et al., 1995), we have established that they both undergo complex formation with cleavage enhancing drugs in vitro (Cornarotti et al., 1996; Marsh et al., 1996). More recently, it has been demonstrated that both α and β isozymes form cleavable complexes with etoposide in human leukemic cells (Willmore et al., 1998). Therefore, both isoforms should be considered potential drug targets in vivo.

Reduction in cleavable complex formation is a common mechanism of resistance to topoisomerase poisons (Liu, 1989). Studies using experimentally derived drug-resistant cell lines have identified various cellular changes associated with reduced cleavable complex levels. Examples include enhanced drug efflux (McKenna and Padua, 1997), reductions in topoisomerase IIα and β levels (Deffie et al., 1989; Harker et al., 1995; Dereuddre et al., 1997), alterations in the phosphorylation status of topoisomerase IIα (Aoyama et al., 1998), and qualitative changes involving topoisomerase IIα mutations (Vassetzky et al., 1995). Some deletion mutations seem to act dominantly, resulting in ectopic cytoplasmic expression of truncated topoisomerase IIα (through loss of nu-

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ABBREVIATIONS: DMSO, dimethyl sulfoxide; SC-URA, synthetic complete medium lacking uracil; bp, base pair(s); MLC, minimum lethal concentration.

clear localization signals), thus sparing the nucleus (Austin and Marsh, 1998). Although point mutations in topoisomerase IIα have been described in cell lines, with a few exceptions, their phenotype remains to be established.

Yeast provides a powerful system in which to characterize drug resistance mutations in topoisomerase II and several approaches have been described (Nitiss and Wang, 1988; Nitiss, 1994). The basic method exploits a drug-permeable, temperature-sensitive DNA topoisomerase mutant carrying either *top2–1* or *top2–4* mutations that permit growth at 25°C but not at 35°C. Complementation with a plasmid-borne *TOP2* gene rescues growth at 35°C and allows the selection and phenotypic analysis of putative drug resistance alleles. Mutations in the yeast topoisomerase II gene have been selected, conferring resistance to amsacrine or doxorubicin (Wasserman and Wang, 1994; Patel et al., 1997). Three classes of amsacrine resistance mutations were identified: multiple mutations in the conserved PLRGK motif, single point changes at residue A642, and carboxyl-terminal deletions that interfere with nuclear localization (Wasserman and Wang, 1994). A second model system has identified two amsacrine resistance mutations in the phage T4 enzyme: E457K in the GyrB′-like domain located 14 residues downstream of the PLRGK motif and equivalent to E495 in the yeast enzyme, and S79L in the DNA breakage-reunion subunit at the residue equivalent to S763 in human topoisomerase IIα (Freudenreich et al., 1998).

Human TOP2α and TOP2β plasmid constructs also complement in yeast (Wasserman et al., 1993; Hsiung et al., 1996; Jensen et al., 1996; Meczes et al., 1997) and site-directed mutagenesis has been used to introduce putative teniposide resistance mutations at particular codons in the *TOP2*α gene for analysis in yeast (Hsiung et al., 1996). Here, we describe the complementary approach of screening a randomly mutagenized human TOP2α plasmid library for the ability to confer amsacrine-resistant growth in yeast. In contrast to results in the phage T4 and yeast topoisomerase II systems (Wasserman and Wang, 1994; Freudenreich et al., 1998), we selected a different spectrum of mutations in the human topoisomerase IIα enzyme, namely E571K and R486K changes in the GyrB′ region. We compare their resistance phenotypes in vivo with the enzymatic properties of the corresponding mutant proteins purified after overexpression in yeast. Contrary to previous conclusions based on yeast topoisomerase II, our work provides the first functional evidence indicating that the R486K mutation mediates resistance to amsacrine and etoposide in amsacrine-resistant human leukemia cell lines and in biopsy material (Hinds et al., 1991; Lee et al., 1992; Kubo et al., 1998).

Materials and Methods

Chemicals and Drugs. Amsacrine [4′-(9-acridinylamino)methanesulfon-*m*-anisidide] was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Etoposide was obtained from Sigma Chemical Co. (Poole, UK). Doxorubicin was purchased from the Pharmacy Dept., St. George's Hospital, London, UK. Amsacrine and etoposide were dissolved in dimethyl sulfoxide (DMSO), doxorubicin was dissolved in water. Oligonucleotides were made in this department using an Applied Biosystems Synthesizer (Norwalk, CT). The Sequenase version 2.0 sequencing kit, [γ-^{32}P]ATP, and [α-^{35}S]dATP were obtained from Amersham International (Little Chalfont, Bucks, UK). Supercoiled pBR322 was purified by standard methods (Patel et al., 1997). All other chemicals were of analytical grade.

Yeast Strains and Plasmids. *Saccharomyces cerevisiae* strains JN394t2–4 (*MATa ISE2 ura3–52 top2–4 rad52::LEU2*) and JEL1 (*a leu2 trp1 ura3–52 prb1–1122 pep4–3*Δ *his3::PGAL1-GAL4*) were obtained from James Wang and Caroline Austin and have been described previously (Wasserman et al., 1993; Austin et al., 1995; Patel et al., 1997). Plasmid YEpWOB6 has been described previously (Wasserman et al., 1993). Yeast strains were grown in synthetic complete medium lacking uracil (SC-URA) (Nitiss et al., 1994) to select for plasmids bearing URA3 as a marker. Transformation of yeast was by the modified lithium acetate method (Patel et al., 1997).

Plasmid Mutagenesis and Selection of Amsacrine-Resistant Mutants. Hydroxylamine mutagenesis of plasmid YEpWOB6 and selection of mutants was carried out as described previously (Patel et al., 1997). Plasmid DNA samples mutagenized for 20 and 40 min were recovered and independently transformed into *Escherichia coli* XL-1 Blue. For each plasmid library, 40,000 colonies were pooled and plasmid extraction was performed using a QIAGEN maxi prep procedure. Each DNA pool was used independently to transform yeast JN394t2–4 to URA+. About 20,000 colonies were pooled in each case and suspended in SC-URA medium. After dilution to an appropriate level of absorbance, amsacrine was added to 25 μg/ml and the cells were incubated at 35°C for 96 h (fresh drug was added at 48 h) before spreading on SC-URA plates and incubating at 35°C for 5 days. Surviving colonies were replica plated onto SC-URA plates containing 25 μg/ml amsacrine and grown at 35°C to confirm the isolation of resistant clones. Plasmid DNA was purified from these clones by a standard procedure and analyzed by restriction digestion to eliminate those that had undergone deletions or rearrangements. Cytotoxicity assays were carried out using JN394t2–4 retransformed with these plasmids.

To establish that drug resistance was conferred by mutant TOP2 plasmids, a 2133-base pair (bp) *Bsp*1407I-*Kpn*I TOP2α fragment (codons 80–791) from each mutant allele was used to replace the corresponding fragment in wild-type YEpWOB6. DNA sequencing was used to confirm the presence of each mutation in the resulting chimeric plasmids, YEpWOB6-A1F and YEpWOB6-A2F. These plasmids were transformed into yeast strains JN394t2–4 or JEL1 for cytotoxicity and protein expression studies, respectively.

Cytotoxicity Assays. Drug sensitivities of JN394t2–4 transformants were determined as described previously (Patel et al., 1997).

DNA Sequencing. The DNA sequence of *TOP2*α genes in mutant and wild-type YEpWOB6 plasmids was determined by the chain termination method (Sanger et al., 1977) using the Sequenase version 2.0 kit and double-stranded plasmid DNA as template. For each gene, overlapping coding sequence spanning codons 1 to 1081 was obtained using a panel of oligonucleotide primers spaced at 200-bp intervals. The presence of single point changes seen in the two mutant alleles was confirmed by sequencing of the complementary DNA strand. The human TOP2α cDNA sequence has recently been corrected (Tsai-Pflugfelder et al., 1988; Hsiung et al., 1996). However, we have retained the original nucleotide and protein numbering system (which places the catalytic tyrosine as residue 804) to facilitate comparison with earlier studies.

Protein Overexpression and Purification. Wild-type and mutant topoisomerase IIα proteins were overexpressed in yeast by galactose induction of protease-deficient yeast strain JEL1 transformed with YEpWOB6, YEpWOB6-A1F, or YEpWOB6-A2F. This procedure and the protocol for enzyme purification by Polymin P fractionation, ammonium sulfate precipitation, and phosphocellulose column chromatography have been described previously (Wasserman et al., 1993). Active fractions were frozen as aliquots in liquid nitrogen and stored at −70°C.

Topoisomerase II and ATPase Assays. Enzymatic relaxation of supercoiled pBR322 DNA was assayed as described previously (Patel et al., 1997). Decatenation of kinetoplast DNA from *Crithidia*

fasciculata (TopoGen, Columbus, OH) was carried out by the method of Keller et al. (1997).

ATP hydrolysis was measured using a coupled enzyme assay that links ATP hydrolysis by topoisomerase II to NADH oxidation (Lindsley and Wang, 1993). Each reaction containing topoisomerase II (50 nM enzyme dimers), 30 μM bp of pBR322 DNA, 2 mM phosphoenolpyruvate, 0.1 mM NADH, 3.5 U pyruvate kinase, and 5 U lactate dehydrogenase in 490 μl of topoisomerase II relaxation buffer was preincubated at 37°C for 3 min. The reaction was started by the addition of ATP (10 μl) to a final concentration of 1 mM. Absorbance was measured at a constant temperature of 37°C using a Cecil CE4400 UV VIS double-beam scanning spectrophotometer. The reaction was monitored for 5 min after an initial equilibration period. The rate of ATP hydrolysis was calculated from the average change in A_{340}/min, measured over 2 min. In assays for inhibition of ATPase activity, DMSO or amsacrine (0–100 μg/ml) was added to each reaction mix and preincubated for 3 min. The final DMSO concentration was always 1%. Reactions were monitored using appropriate blanks.

DNA Cleavage. Reaction mixtures (final volume 20 μl) contained 50 mM Tris · HCl, pH 7.5, 125 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM dithiothreitol, 30 μg/ml BSA, 1 mM ATP, a 4333-bp *Eco*RI-*Hin*dIII fragment of pBR322 uniquely 5′-^{32}P labeled at its *Eco*RI end (5000 cpm Cerenkov), 0.5 μg of wild-type or mutant human topoisomerase IIα, and various concentrations of either amsacrine or etoposide. The concentration of DMSO was 2.5% in all cases. Samples were incubated at 37°C for 30 min and DNA cleavage was induced by addition of 1 μl of 10% SDS. Proteinase K (1 μl of 1.5 mg/ml) was added and samples were incubated at 56°C for 30 min before electrophoresis in a 1% agarose gel run in Tris/borate/EDTA buffer. Gels were blotted on to DE81 paper, exposed to a phosphoscreen and the radioactivity quantitated on a Molecular Dynamics Storm 840 PhosphorImager. Gels were subsequently examined by autoradiography.

Results

Isolation and Functional Analysis of Amsacrine-Resistant Human Topoisomerase IIα Mutants in Yeast. To identify drug-resistant topoisomerase IIα mutants, we exploited *S. cerevisiae* strain JN394t2–4, which carries an ISE2 permeability mutation facilitating drug uptake and a rad52 defect in double-strand DNA break repair sensitizing the yeast to the action of topoisomerase II poisons (Wasserman et al., 1993; Patel et al., 1997). The strain also has a temperature-sensitive *top2–4* mutation in its chromosomal *TOP2* gene that allows growth at 25°C but not at 35°C. Crucially, a variety of plasmid-borne fungal and human *TOP2* genes are able to complement the defect, allowing growth at 35°C (Wasserman et al., 1993; Wasserman and Wang, 1994; Keller et al., 1997; Meczes et al., 1997). In principle, by selecting for drug resistance at 35°C, it should be possible to identify mutant TOP2 plasmids conferring resistance. For our work, we used the TOP2 plasmid YEpWOB6, which expresses an enzyme in which residues 29 to 1531 of human topoisomerase IIα are fused to the first five residues of yeast topoisomerase II under the control of a galactose-inducible GAL1 promoter (Fig. 1)(Wasserman et al., 1993). High level expression of human topoisomerase IIα is detrimental to yeast growth (Wasserman et al., 1993). However, by using glucose as the carbon source, the GAL1 promoter is repressed and YEpWOB6 will then complement in JN394t2–4. Galactose activation of the GAL1 promoter provides a useful system for protein overexpression, purification, and in vitro characterization of drug-resistant mutants.

Plasmid YEpWOB6 was randomly mutagenized in vitro by treatment with hydroxylamine and subsequently amplified in *E. coli*. The resulting plasmid DNA library was used to transform yeast JN394t2–4 to URA+ before plating at 25°C on SC medium lacking uracil (SC-URA). Approximately 20,000 URA+ transformants were pooled and grown for 96 h at 35°C in SC-URA medium containing 25 μg/ml amsacrine, a drug concentration that is 25-fold greater than that needed to inhibit growth of YEpWOB6 transformed JN394t2–4. Viable cells were recovered by plating on SC-URA and incubation at 35°C. From two independent selections using plasmid mutagenized for different time periods, a total of 600 yeast clones was obtained. Subsequent replica plating of 46 clones showed that a majority of these had the ability to grow (albeit slowly) at 35°C on 25 to 50 μg/ml amsacrine. Another six clones grew on plates containing 10 μg/ml amsacrine. Two resistant yeast transformants, clones 1 and 2, derived from the two independent selections, were examined in some detail.

Plasmids A1 and A2 recovered from clones 1 and 2 were retransformed into JN394t2–4, and the amsacrine response of these transformants was compared in liquid culture at 35°C with that of the strain carrying the wild-type YEpWOB6 (Fig. 2). In the absence of drug, all three plasmids supported similar levels of yeast growth at 35°C, resulting in a >10-fold increase in viable counts after 24 h. All three transformants showed a drug-dependent inhibition of yeast growth. For cells transformed with wild-type YEpWOB6, inclusion of 0.2 or 0.5 μg/ml amsacrine led to growth inhibition, whereas >2 μg/ml produced cell killing (Fig. 2A). For this transformant, the amsacrine minimum lethal concentration (MLC; the concentration required to kill rather than inhibit yeast growth) was ~1 μg/ml. Much higher amsacrine concentrations were required to inhibit growth of the A1 and A2 yeast transformants (Fig. 2, B and C) whose MLC values were >25 and >100 μg/ml, respectively.

Amsacrine-Resistant Human top2 Mutants Encode E571K or R486K Mutations. DNA sequence analysis of the

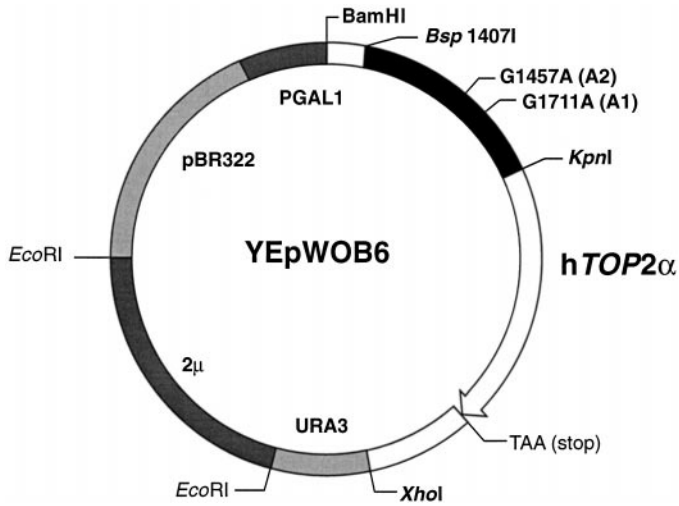

Fig. 1. Structure and restriction map of plasmid YEpWOB6, a yeast-*E. coli* shuttle plasmid that allows inducible expression of a human *TOP2α* gene from a GAL1 promoter (PGAL1). Sites for *Bam*HI, *Bsp*1407I, *Eco*RI, *Kpn*I, and *Xho*I are indicated (names in bold). Filled section denotes a *Bsp*1407I-*Kpn*I region used in fragment exchange to express TOP2α cDNAs containing amsacrine resistance mutations at nucleotide positions 1457 and 1711 in the TOP2α coding region (mutant YEpWOB6 plasmids A2 and A1, respectively).

TOP2 genes in these plasmids (and comparison with that of YEpWOB6) revealed each had a single nucleotide change resulting in a single amino acid change in the human topoisomerase II protein. The *TOP2* gene of plasmid A1 exhibited a G1711-to-A mutation, producing an E571K alteration in topoisomerase IIα. A G1457-to-A nucleotide mutation was found in the *TOP2* allele of plasmid A2 that encoded an R486K mutation at the protein level. These two mutations were selected frequently by our screening procedure. The E571K mutation was present in two other clones and the R486K alteration was found in 10 other plasmid isolates. By fragment exchange, a 2100-bp *Bsp*1407I-*Kpn*I TOP2 fragment bearing one mutation or the other was introduced into the wild-type plasmid YEpWOB6 (Fig. 1). The resulting plasmids YEpWOB6-A1F and YEpWOB6-A2F were isolated and by DNA sequence analysis were confirmed to carry the appropriate mutations. Transformation of JN394t2–4 with the chimeric plasmids and subsequent cytotoxicity assays revealed that the E571K and R486K mutations, respectively conferred >25- and >100-fold resistance to amsacrine (Table 1). These results were reproduced in a second experiment and were the same as those obtained with the A1 and A2 plasmid transformants (Fig. 2, B and C). Thus, the fragment exchange data showed unequivocally that each mutation is responsible for amsacrine resistance and established that human topoisomerase IIα is an intracellular target for the drug.

We also examined whether the mutations reduced susceptibility of yeast to other cleavable complex-forming agents that were structurally distinct from amsacrine. Figure 3 shows a representative experiment comparing the response of yeast transformants to etoposide, an epipodophyllotoxin. Yeast growth was generally less susceptible to inhibition by etoposide than by amsacrine; therefore, cytotoxicity was measured only after a 24-h drug exposure. The etoposide MLC for yeast cells expressing wild-type topoisomerase IIα was 50 μg/ml (Fig. 3A). Both the E571K and R486K proteins expressed by the respective A1F and A2F plasmids resulted in a small increase in resistance to etoposide compared with wild-type (Fig. 3, B and C) giving MLC values of 100 and 150 μg/ml, respectively. Given the relatively small differences in MLC values, the same experiment was repeated three times for yeast cells expressing wild-type or R486K protein. In each instance, the R486K protein conferred a higher MLC than the wild-type protein. The results summarized in Table 1 indicate that the A2F transformant expressing the R486K protein exhibited a 3-fold increase in resistance that was statistically significant ($P < .01$). Thus, the R486K mutant topoisomerase IIα conferred low-level resistance to etoposide.

Table 1 also presents the results obtained from duplicate experiments carried out for ellipticine. The ellipticine MLC for yeast transformed with the wild-type TOP2α plasmid was 25 μg/ml and was essentially unchanged for transformants bearing plasmid A1F or A2F. Doxorubicin was somewhat more potent, with an MLC of 10 μg/ml against yeast transformed with wild-type plasmid. However, this value was unchanged for yeast carrying plasmid A2F expressing the R486K mutant. It seems that the R486K alteration had little effect on susceptibility to either ellipticine or doxorubicin.

Enzymatic Properties of the Purified E571K- and R486K-Enzymes. To examine the enzymatic effects of mutations identified in the yeast system, it was important to characterize the mutant topoisomerase II proteins. The *TOP2* gene in YEpWOB6 and its A1F and A2F mutants lies downstream of a GAL1 promoter allowing expression in yeast of the respective topoisomerase proteins on induction with galactose. Accordingly, each of the three plasmids was transformed into JEL1, a protease-deficient yeast strain. All three proteins were expressed and purified to >90% homogeneity by a procedure involving cell lysis, fractionation with Polymin P, followed by ammonium sulfate precipitation, and finally phosphocellulose column chromatography (Fig. 4)(Wasserman et al., 1993). The specific activities of the wild-type, E571K, and R486K proteins measured in a DNA relaxation assay using supercoiled pBR322 as substrate were 1.4×10^5, 9×10^4, and 1×10^5 U/mg, respectively. Similarly, the three proteins were comparably efficient in catalyzing the decatenation of kinetoplast DNA (not shown). It seems that the drug resistance mutations do not greatly affect intrinsic topoisomerase IIα activity.

We also measured the ATPase activities of the mutant enzymes and compared them with those of wild-type enzymes using an enzyme assay that couples ATP hydrolysis to the oxidation of NADH. In each assay, 50 nM enzyme dimers

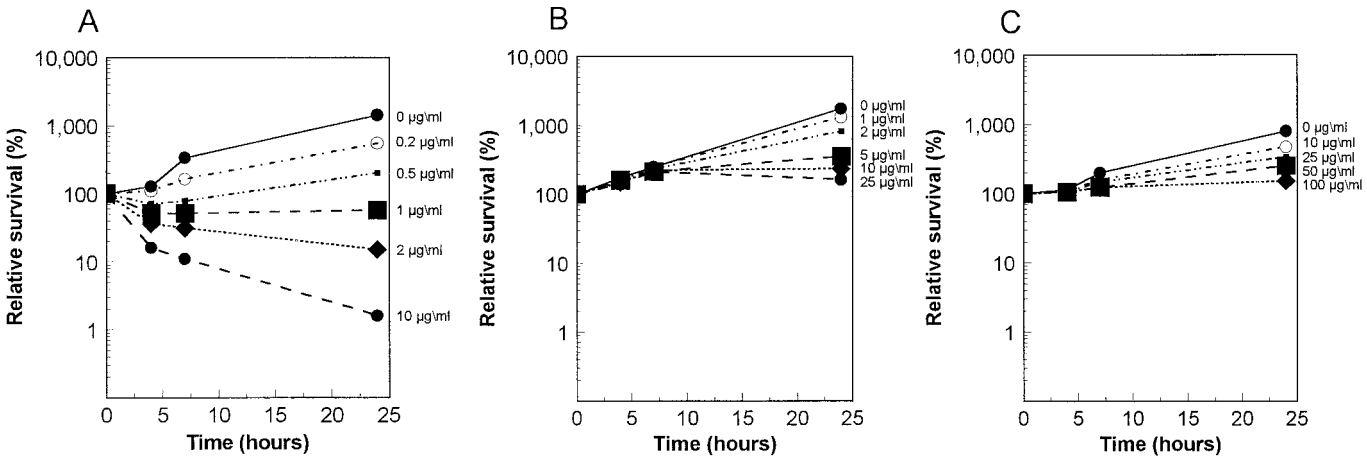

Fig. 2. Effects of amsacrine on the survival at 35°C of yeast strain JN394top2–4 transformed with wild-type TOP2α plasmid YEpWOB6 (A) or its mutant derivatives, plasmids A1 (B) and A2 (C). Yeast transformants were grown in the absence or presence of amsacrine at the concentrations indicated, and viable counts, determined at 4, 7, and 24 h, are expressed as a percentage of viable counts measured at time zero.

and 1 mM ATP were included in the absence or presence of 30 μM bp of pBR322 DNA (Lindsley and Wang, 1993). The DNA-dependent ATPase rates of wild-type, E571K, and R486K proteins were very similar at 1, 1.6, and 1.3 ATP molecules/s/dimer, respectively. Although we did not examine enzyme activities at subsaturating ATP concentrations, the three enzymes were similarly efficient as judged by their turnover numbers. Amsacrine inhibited the ATPase activities of the wild-type and mutant enzymes. For the wild-type enzyme, inclusion of the drug led to a dose-dependent reduction in DNA-dependent ATPase activity with an IC_{50} (the dose producing 50% inhibition) of 5 μg/ml (in duplicate experiments). The amsacrine IC_{50} values for E571K and R468K proteins were 5-fold and 2- to 4-fold higher, as measured in duplicate experiments (data not shown).

Mutant Proteins Are Defective in Amsacrine-Promoted DNA Cleavage. Resistance to topoisomerase II poisons has been ascribed to reduced levels of cleavable complex formation. Therefore, we tested the ability of the wild-type and mutant proteins to promote drug-mediated DNA cleavage using ^{32}P-end-labeled pBR322 DNA as substrate (Fig. 5). Equal weights (0.5 μg) of wild-type and mutant topoisomerase IIα were incubated with the labeled DNA substrate and 1 mM ATP in the absence or presence of either amsacrine or etoposide. DNA breakage was induced by addition of SDS. After proteinase K digestion, the DNA was analyzed by agarose gel electrophoresis. The extent of DNA cleavage was quantified on a PhosphorImager and visualized by autoradiography (Fig. 5). The amsacrine cleavage results are shown in Fig. 5A. Consistent with previous work (Cornarotti et al., 1996), the wild-type enzyme was able to promote some DNA cleavage even in the absence of amsacrine (Fig. 5A, lane 2). The level of drug-independent DNA breakage was much lower for the E571K and R486K proteins (Fig. 5A, mt 571 and mt 486, lanes 8 and 14, respectively). The effects of 0.5, 1, 5, 10, and 50 μg/ml of amsacrine are indicated for wild-type enzyme (Fig. 5A, lanes 3–7), and for mutant proteins (Fig. 5A, lanes 9–13 and 15–19). For the wild-type enzyme, cleavage enhancement was apparent even at the lowest drug concentration of 0.5 μg/ml (Fig. 5A, lane 3), where, based on PhosphorImager analysis, 80% of the input DNA was cleaved. Conversion of linear DNA to smaller fragments was almost complete at 5 μg/ml (Fig. 5A, lane 5). By contrast, very much higher levels of amsacrine were needed to yield detectable DNA breakage with the mutants. Even with amsacrine at 50 μg/ml, the E571K and R486K proteins cleaved only 50 and 60% of the input DNA, respectively (Fig. 5A, lanes 13 and 19). Thus, the two mutant proteins behaved comparably in requiring at least 100-fold higher drug levels to achieve comparable DNA cleavage to that induced by wild-type enzyme. Interestingly, the R486K protein was, if anything, somewhat more proficient in amsacrine-induced cleavage than its E571K counterpart (Fig. 5A, compare lanes 17–19 with 11–13).

Figure 5B shows a comparable experiment using etoposide as the cleavage enhancing drug. As seen in Fig. 5A, the wild-type enzyme exhibited a drug-independent cleavage activity converting some of the linear substrate DNA (Fig. 5B, lane 1) into smaller fragments (Fig. 5B, lane 2): this activity was much less marked for the mutant proteins (Fig. 5B, lanes 8 and 14). Etoposide promoted dose-dependent DNA cleavage by the wild-type protein with detectable depletion of linear

TABLE 1
Cross-resistance profile at 35°C of JN394t2-4 transformed with wild-type and mutant *TOP2α* alleles

Plasmid	Mutation in Topoisomerase IIα	MLC			
		Amsacrine	Etoposide[a]	Ellipticine[b]	Doxorubicin
			μg/mL		
YEpWOB6	Wild-type	1	50 ± 25	25, 15	10
YEpWOB6-A1F	E571K	>25	100	25, 20	ND
YEpWOB6-A2F	R486K	>100	166 ± 28*	25, 20	10

[a] MLC values represent mean of three independent experiments ± S.D.
[b] Values are the results of two independent experiments.
* $P < .01$.

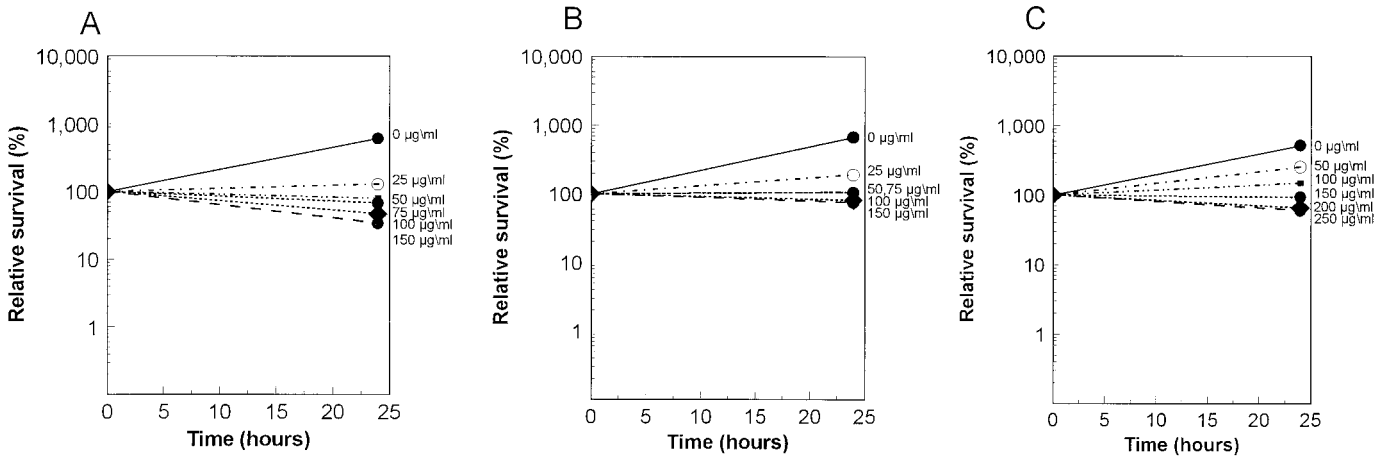

Fig. 3. Cytotoxicity of etoposide at 35°C against JN394top2–4 transformants carrying TOP2α plasmid YEpWOB6 (A) or mutant plasmids A1F (B) and A2F (C). Yeast cells were grown in the absence or presence of various concentrations of etoposide, and viable counts were determined at 24 h as described in the legend for Fig. 2.

DNA at 0.5 μg/ml etoposide (Fig. 5B, lane 3) and extensive breakage at 5 to 500 μg/ml (Fig. 5B, lanes 4–7). By contrast, for the mutant proteins, there was little breakage of linear DNA in the presence of etoposide at 0.5 μg/ml (Fig. 5B, lanes 8 and 9, lanes 14 and 15). Drug-mediated cleavage of linear DNA to smaller fragments was apparent only at 5 to 50 μg/ml etoposide for the E571K protein (Fig. 5B, lanes 10 and 11) and at 5 μg/ml for the R486K protein (Fig. 5B, lane 16). Higher drug levels resulted in complete DNA fragmentation (Fig. 5B, lanes 12, 13, 17–19). Thus, based on the depletion of linear DNA, cleavage was severalfold less efficient for the mutant proteins (Fig. 5B, lanes 8–10, 14–16) than for wild-type (Fig. 5B, lanes 2–4). It is clear that the mutations in topoisomerase IIα have much less effect on DNA cleavage promoted by etoposide than those promoted by amsacrine, consistent with results determined in vivo (Table 1).

Discussion

A complete understanding of the mechanisms of cellular resistance to amsacrine and other topoisomerase poisons will require the phenotypic analysis of target mutations detected in vivo. This objective is difficult to achieve using human cells because of the normal presence of two topoisomerase II isoforms and the recessive nature of resistant *TOP2* alleles. As a complementary approach to this issue, we have used a yeast system to screen a library of randomly mutagenized human TOP2α plasmids for the ability to confer resistance to amsacrine. We show that point mutations of E571K or R486K in the GyrB′ homology region of human topoisomerase IIα are frequently selected and confer, respectively >25- and >100-fold increases in resistance to amsacrine in vivo and a small (~3-fold) increase in resistance to etoposide. The highly purified E571K and R486K topoisomerase IIα proteins expressed in yeast exhibited wild-type specific activities in DNA relaxation and DNA-dependent ATPase assays. However, the mutant enzymes were each some 100-fold less efficient at promoting DNA breakage by amsacrine than the wild-type protein, indicating that the mutations cause resistance by interfering with cleavable complex formation (Fig. 5). This is the first genetic and biochemical analysis of amsacrine resistance mutations in the human drug target and has implications for elucidating the molecular basis of drug resistance in tumor cells.

The E571K topoisomerase IIα mutation is novel and has not been identified thus far in human cells. However, the R486K change has been detected in two human leukemia cell lines selected for resistance to amsacrine (Hinds et al., 1991; Lee et al., 1992) and in a bronchial biopsy sample from a patient with small cell lung carcinoma treated with etoposide (Kubo et al., 1998). One of the cell lines, KBM-3/AMSA, was some 200-fold more resistant to amsacrine compared with parental cells and exhibited 6-fold cross-resistance to etoposide (Lee et al., 1992). The HL-60/AMSA line was 100-fold more resistant to amsacrine than parental cells and 2- to 3-fold more resistant to etoposide (Zwelling et al., 1989). Recently, it has been found that HL-60/AMSA cells, in addition to expressing a mutant topoisomerase IIα, do not produce topoisomerase IIβ (Herzog et al., 1998). This observation is consistent with previous experiments showing that mammalian cells lacking β are viable whereas loss of α is lethal (Dereuddre et al., 1997; reviewed in Austin and Marsh,

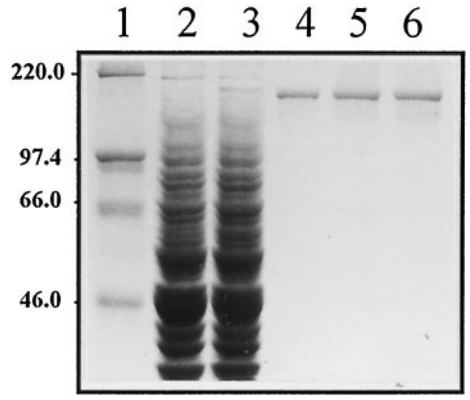

Fig. 4. Purification of wild-type, E571K, and R486K human topoisomerase IIα proteins. Proteins were overexpressed in protease-deficient *S. cerevisiae* strain JEL1 from plasmid YEpWOB6 bearing the appropriate allele, purified and displayed on a 6.5% polyacrylamide-SDS gel. Lysate from uninduced yeast transformed with YEpWOB6 (lane 2) and induced with galactose (lane 3). Peak fractions from phosphocellulose column chromatography of wild-type, E571K, and R486K- protein preparations are depicted in lanes 4 to 6. Protein markers (sizes indicated in kDa) were run in lane 1.

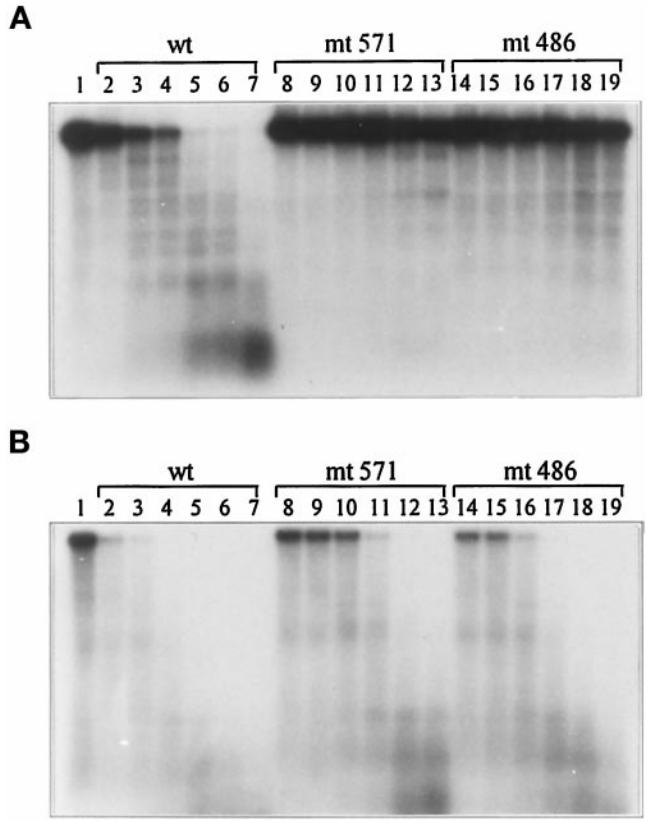

Fig. 5. Drug-enhanced DNA cleavage mediated by wild-type and amsacrine-resistant human topoisomerase IIα. A *Hin*dIII-*Eco*RI DNA fragment from plasmid pBR322 was end labeled at its *Eco*RI site and incubated with wild-type (wt), E571K (mt571), or R486K(mt486) topoisomerase IIα proteins (in each case 0.5 μg) in the absence or presence of amsacrine (A) or etoposide (B) at 37°C for 30 min. All of the reactions contained 2.5% DMSO. After addition of SDS and proteinase K digestion, DNA samples were examined by electrophoresis in 1% agarose before autoradiography. A, lanes 2 to 19 contained enzymes. Lanes 2 to 7, 8 to 13, and 14 to 19 contained amsacrine at 0, 0.5, 1, 5, 10, and 50 μg/ml, respectively. Lane 1, no enzyme addition. B, as in A except etoposide replaced amsacrine in lanes 2 to 7, 8 to 13, and 14 to 19 at 0, 0.5, 5, 50, 200, and 500 μg/ml, respectively.

1998). Herzog et al. hypothesize that amsacrine specifically targets the β isoform and therefore its absence from HL-60/AMSA cells may confer resistance. However, the paper is equivocal about the role of the mutant α isoform, largely because introduction of the human R486K mutation at the equivalent position of yeast topoisomerase II (R476K) does not produce resistance in yeast (Wasserman and Wang, 1994), and therefore the mutation has been considered unimportant for HL-60/AMSA resistance.

Contrary to the inference from yeast topoisomerase II data, we have shown unequivocally that the R486K mutation in human topoisomerase IIα confers high-level amsacrine resistance and low-level etoposide resistance. Indeed, our studies in vivo and using purified mutant enzyme (Table 1, Fig. 5) suggest that the R486K mutation alone would be sufficient to account for most of the amsacrine- and etoposide-resistance phenotype of the KBM-3/AMSA and HL60/AMSA cells. Although resistant *TOP2α* alleles are usually recessive, the abnormal karyotype of HL-60 cells suggests a mechanism for expression of the mutant R486K topoisomerase IIα gene involving loss, inactivation, or down-regulation of other *TOP2* genes. It is thought that HL-60 cells have a single chromosome 17 bearing the *TOP2α* gene, which, in the observed absence of topoisomerase IIβ expression in the HL-60/AMSA cell line, would have a dominant phenotype. Thus, in explaining the drug resistance of HL-60 cells, it may not be necessary to propose that the β isoform is the primary target for amsacrine (Herzog et al., 1998). In fact, it is known that the purified isoforms are targeted similarly by amsacrine and by etoposide in vitro and in yeast and both are etoposide targets in human leukemic CCRF-CEM cells (Austin et al., 1995; Cornarotti et al., 1996; Marsh et al., 1996; Meczes et al., 1997; Willmore et al., 1998). Therefore, loss of β expression in HL-60 cells may simply allow full (rather than partial) expression of the mutant α phenotype. It is remarkable that by using an independent selection method and a yeast system to express and test human topoisomerase II, we have been able to establish the importance of the R486K mutation in cellular resistance to amsacrine. Clearly, the functional approach reported here should be broadly applicable in examining the role of mutated topoisomerase isoforms in human tumor cell lines made resistant to anticancer drugs.

Unlike the HL-60/AMSA cells, the genetic background of the small-cell lung cancer cells in biopsy material carrying the R486K topoisomerase IIα mutation has not been reported (Kubo et al., 1998). However, given that the R486K mutation does confer low-level resistance to etoposide (Fig. 3), down-regulation of other topoisomerase genes could allow its dominant expression in small-cell lung cancer, thus accounting for its selection during etoposide therapy. Alternatively, it is conceivable that the R486K α allele might be partially dominant even in the presence of wild-type *TOP2* alleles. There is precedent for this idea in that studies in yeast have shown that K439E and K439Q mutations in the GyrB homology region of yeast topoisomerase II confer partially dominant resistance to both amsacrine and etoposide (Nitiss et al., 1994). Attempts were made to examine this aspect by growing JN394t2–4 transformants at 25°C where both the yeast and human enzymes are expressed. In this heterologous situation, the sensitivity of the yeast cells was determined by the most sensitive allele consistent with a recessive phenotype for the R486K allele (S.P. and L.M.F., data not shown). The genetic requirements that allow topoisomerase II mutations to determine cellular drug resistance merit further investigation. Furthermore, it will be interesting to examine clinical samples for the presence of the E571K mutation.

It is interesting that the R486K and E571K changes in the GyrB′ homology region of human topoisomerase IIα uniquely conferred high level resistance to amsacrine but had little or no effect on sensitivity to other cleavable complex forming agents belonging to different structural classes. Thus, resistance to etoposide was only ~3-fold and there was no cross-resistance to ellipticine and doxorubicin (Table 1). The results suggest there are sufficient differences in the structures of cleavable complexes formed by amsacrine vis-a-vis other topoisomerase IIα poisons such that the R486K and E571K changes do not interfere with complex formation by structurally unrelated drugs. At present, there is little information on the structure of the ternary complex involving amsacrine except from photolabeling studies indicating that the drug binds DNA at the sites of DNA breakage mediated by the catalytic tyrosine residues (Freudenreich and Kreuzer, 1994). However, recent work on yeast topoisomerase II indicates that the GyrB′ region of one subunit interacts in *trans* with the GyrA homology domain of the other subunit containing the catalytic tyrosine, to form the DNA binding site and drug-binding pocket (Liu and Wang, 1999). Were this to occur in human topoisomerase IIα, it would provide an explanation as to how the R486K and E571K mutations in the GyrB′ region interfere with ternary complex formation and thereby confer resistance. Further work will be necessary to delineate the structural basis by which the mutant enzyme activities are modified.

Finally, it is important to recognize that the amsacrine resistance mutants we selected in human topoisomerase IIα are distinct from those seen in model systems. Unlike the phage T4 enzyme (Freudenreich et al., 1998), we did not identify mutations in the DNA breakage-reunion domain. Similarly, although we used the same random mutagenesis procedure as previous studies on yeast topoisomerase II (Wasserman and Wang, 1994), we recovered different drug-resistant mutants. Although these differences could reflect differences in selection procedures or analysis of relatively small numbers of mutants, we believe it likely that particular enzymes interact uniquely with the drug. In support of this idea, it is striking that the R486K mutation in human topoisomerase IIα confers resistance to amsacrine, whereas the equivalent change in yeast topoisomerase II is without effect. Moreover, other recent studies indicate that the yeast enzyme and human topoisomerase IIα have different drug-mediated DNA cleavage specificities (Strumberg et al., 1999). Thus, there seem to be subtle differences in the drug-binding pockets of yeast and human topoisomerases II that can influence drug interactions and the ability of particular mutations to generate resistance. Our results emphasize the importance of studying the relevant human target.

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