Probing the Interaction of the Cytotoxic Bisdioxopiperazine ICRF-193 with the Closed Enzyme Clamp of Human Topoisomerase IIα

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ABSTRACT

Topoisomerase II is an ATP-operated protein clamp that captures a DNA helix and transports it through another DNA duplex, allowing chromosome segregation at mitosis. A number of cytotoxic bisdioxopiperazines such as ICRF-193 target topoisomerase II by binding and trapping the closed enzyme clamp. To investigate this unusual mode of action, we have used yeast to select plasmid-borne human topoisomerase IIα alleles resistant to ICRF-193. Mutations in topoisomerase IIα of Leu-169 to Phe (L169F) (in the N-terminal ATPase domain) and Ala-648 to Pro (A648P) (in the core domain) were identified as conferring >50-fold and 5-fold resistance to ICRF-193 in vivo, respectively. The L169F mutation, located next to the Walker A box ATP-binding sequence, resulted in a mutant enzyme displaying ICRF-193-resistant topoisomerase and ATPase activities and whose closed clamp was refractory to ICRF-193-mediated trapping as an annulus on closed circular DNA. These data imply that the mutation interferes directly with ICRF-193 binding to the N-terminal ATPase gate. In contrast, the A648P enzyme displayed topoisomerase activities exhibiting wild-type sensitivity to ICRF-193. We suggest that the inefficient trapping of the A648P closed clamp results either from the observed increased ATP requirement, or more likely, from lowered salt stability, perhaps involving destabilization of ICRF-193 interactions with the B-‘B’ interface in the core domain. These results provide evidence for at least two different phenotypic classes of ICRF-193 resistance mutations and suggest that bisdioxopiperazine action involves the interplay of both the ATPase and core domains of topoisomerase IIα.

Topoisomerase II, a nuclear enzyme expressed in mammalian cells as α- and β-isoforms, is essential for eukaryotic chromosome condensation and segregation (Wang, 1996; Austin and Marsh, 1998). The protein is a dimer that passes one DNA duplex through a transient enzyme-bridged double-strand break in a second DNA segment. A variety of structurally diverse cytotoxic drugs exert their lethal effects by interfering with different aspects of the enzyme cycle (Sehested and Jensen, 1996). For example, topoisomerase II poisons, such as the clinically important anticancer agents doxorubicin, etoposide, and amsacrine, stabilize a covalent enzyme-DNA complex termed the “cleavable complex”, which cellular processes convert into a lethal double-strand DNA break (Chen and Liu, 1994). In contrast, bisdioxopiperazine inhibitors such as ICRF-154, ICRF-159, ICRF-187, and ICRF-193 do not induce DNA breakage but lock the enzyme as a closed clamp on DNA (Andoh, 1998; Andoh and Ishida, 1998). Although originally classed as catalytic inhibitors along with aclacinomycin, merbarone, and suramin, recent work suggests that bisdioxopiperazine inhibition of topoisomerase II kills cells by a novel poison mechanism (van Hille and Hill, 1998; van Hille et al., 1999; Jensen et al., 2000). These results are of interest in cancer chemotherapy. First, ICRF-154 is therapeutically useful in protecting against doxorubicin cardiotoxicity (Sehested et al., 1993). The drug is thought to act as an antagonist of the free radical-mediated effects of doxorubicin rather than by blocking cleavable complex formation. Second, MST-16, a masked ICRF-154 derivative with increased bioavailability, is currently used to treat malignant lymphoma and adult T-cell leukemia (Andoh, 1998). Despite the relevance to cancer therapeutics, little is known about the molecular basis of bisdioxopiperazine interactions with topoisomerase II.

Much of our current understanding of bisdioxopiperazine action has come from studies on yeast topoisomerase II. The enzyme functions as a protein clamp whose closure and open-
ing are operated by the respective binding and hydrolysis of ATP (Wang, 1998). The complex first binds a DNA duplex (the G or gate segment) and in the presence of ATP, the jaws of the protein clamp close, capturing a second DNA helix called the T or translocated segment. A transient double-strand break is introduced into the G segment through which the T segment is passed. The G gate is resealed and the T segment passes out of the enzyme complex by the opening of a protein gate formed by an interface between the two protein subunits of the dimer. The protein gate recloses and the enzyme clamp opens ready for another reaction cycle (Berger et al., 1996; Wang, 1998). Bisdioxopiperazine inhibition involves drug-induced trapping of the closed clamp form (Roca et al., 1994; Ishida et al., 1995). Recent kinetic work indicates that the drug traps a closed clamp intermediate bound to one ADP (Morris et al., 2000).

In mammalian cells, bisdioxopiperazines target topoisomerase II and exert their cytotoxicity during transit through the G2-M phase of the cell cycle, resulting in incomplete chromosome segregation and production of polyploid cells (Morris et al., 1994; Ishida et al., 1995). Recent kinetic work indicates that drug-induced trapping of the closed clamp form (Roca et al., 1996; Wang, 1998). Bisdioxopiperazine inhibition in vivo involves drug-induced trapping of the closed clamp (Roca et al., 1994; Ishida et al., 1995). Recent kinetic work indicates that the drug traps a closed clamp intermediate bound to one ADP (Morris et al., 2000).

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Given the conflicting data, we have followed a complementary approach to investigate the mode of action of bisdioxopiperazines against the biologically relevant target, involving the use of a yeast system to select and overexpress human topoisomerase IIα mutants resistant to ICRF-193, the most potent bisdioxopiperazine (Fig. 1) (Hasinoff et al., 1995). Two novel topoisomerase IIα mutations, L169F (in the ATPase region) and A644P (in the core domain), were identified and the mutant enzymes were characterized in vivo and in vitro. The data indicate that in addition to the ATPase region, the core domain may participate in ICRF-193 action.

**Materials and Methods**

**Drugs and Chemicals.** ICRF-193 was dissolved in dimethyl sulfoxide (DMSO) and made up fresh for each experiment, except for enzyme assays in which aliquots stored at −20°C were used. Etoposide was obtained from Sigma (Poole, Dorset, UK) and was dissolved in DMSO. The Sequenase version 2.0 sequencing kit, [α-35S]-dATP (3000 Ci/mmol), and [γ-32P]ATP (3000 Ci/mmol) were from Amersham International (Little Chalfont, Bucks, UK). Oligonucleotides were synthesized at St. George’s Hospital Medical School, London, UK.

**Yeast Strains and Plasmds.** Saccharomyces cerevisiae strains JN9342-4 (MATa Ise2 ura3-32 top2-4 rad52::LEU2) and JEL1 (a leu2 trp1 ura3-52 prb1-112 pep4-3 his3::PGAL10-GAL4) were kindly provided by Professor J. C. Wang (Harvard University, Cambridge, MA) and have been described previously (Patel et al., 1997). Both strains were transformed by using the lithium acetate method and grown in synthetic complete medium lacking uracil (SC-URA) to select for plasmids carryingURA3 as a marker (Patel et al., 1997). Plasmid YepWOB6 and preparation of supercoiled plasmid pBR322 have been described (Wasserman et al., 1993; Patel et al., 1997).

**Selection of ICRF-193-Resistant top2 Mutants.** A standard method was followed with minor modifications (Patel et al., 1997). Briefly, plasmid YepWOB6 was mutagenized in vitro by hydroxylamine treatment for 20 or 40 min. Each mutagenized DNA sample, after amplification in Escherichia coli XL-1, was used to transform yeast JN9342-4. In each case, 20,000 yeast transformants were pooled and suspended in SC-URA at an A600 of 2.0 in the presence of either 10 or 25 μg/ml ICRF-193. Cultures were incubated at 35°C for 96 h with addition of fresh drug after 48 h. The cells were finally plated on SC-URA agar and incubated at 35°C. Resulting colonies were replicated onto SC-URA plates containing ICRF-193 at the selection concentration. Plasmids were isolated from positive clones and transformed back into drug-sensitive JN9342-4. The drug sensitivity of the retransformants was determined by using a drug cytotoxicity assay already described (Patel et al., 1997).

To confirm that the drug resistance conferred by plasmids YepWOB6-E1 and -E2 was due to the identified mutations, a 2.13-kb Bsp1407I-KpnI restriction fragment from each resistant allele was used to replace the corresponding fragment in wild-type YepWOB6, yielding plasmids YepWOB6-E1F and -E2F. The presence of each mutation in the chimeric constructs was confirmed by DNA sequencing. These constructs were used to transform JN9342-4 for drug cytotoxicity studies and strain JEL1 for protein purification.

**DNA Sequencing.** The DNA sequence of the entire coding region of the TOP2 gene in plasmids YepWOB6, YepWOB6-E1, and YepWOB6-E2 was determined by the chain termination method (Sanger et al., 1977) with a panel of overlapping oligonucleotide primers spaced at 200-base pair intervals. The single point mutation identified in each mutant TOP2 gene was confirmed by sequencing the complementary strand.

**Topoisomerase II Protein Purification.** YepWOB6 and the two constructs YepWOB6-E1F and YepWOB6-E2F carrying the drug-resistance mutations were transformed into JEL1 for overexpression and purification of the respective proteins. Wild-type and mutant proteins were purified according to the method of Wasserman et al. (1993) in which the final step is phosphocellulose column chromatography with a linear KCl gradient (0.2–1.0 M). Topoisomerase II eluted at approximately 0.5 M KCl. Active fractions were flash frozen and stored at −70°C.

**Enzyme Assays.** Topoisomerase II activity was assayed by the ATP-dependent relaxation of supercoiled pBR322 as already described (Patel et al., 1997). Decatenation activity was determined in a similar fashion to the relaxation assay but at pH 7.5 by using 150 mM KCl, 50 μg/ml BSA, and 250 ng of kDNA as the substrate. The effect of ICRF-193 or etoposide on the relaxation or decatenation activity of wild-type or mutant enzymes was determined with the following modification: wild-type or mutant enzyme, ATP, and vari-

![Fig. 1. Structure of ICRF-193.](image-url)
ous concentrations of the drug were preincubated in the assay mixture for 10 min at room temperature before the addition of supercoiled pBR322 or kDNA. The reaction was then incubated for 30 min at 37°C and processed as for the standard relaxation or decatenation assay.

ATP hydrolysis was measured with a coupled enzyme assay that links ATP hydrolysis by topoisomerase II to NADH oxidation (Lindsay and Wang, 1993). Each reaction containing topo II (50 nM enzyme dimers), 30 μM base pairs of pBR322 DNA, 2 mM phosphoenolpyruvate, 0.1 mM NADH, 5 U of lactate dehydrogenase, and 3.5 U pyruvate kinase in 490 μl of topo II relaxation buffer was preincubated at 37°C for 3 min. The reaction was started by the addition of ATP to a final concentration of 1 mM. Absorbance was measured at a constant temperature of 37°C with 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 A260/min measured over 2 min. For assays of ATPase inhibition, protein, ATP, and DMSO (1% final) and ICRF-193 (0–100 μg/ml) were preincubated in reaction mix for 10 min at 37°C before the addition of pBR322 to start the reaction. DNA cleavage assays were carried out as described previously (Keller et al., 1997; Patel et al., 1997).

**Filter-Binding Assay.** A GF/C glass fiber filter method was used to measure trapping of topoisomerase II proteins on DNA (Roca et al., 1994). Each reaction mixture contained wild-type or mutant topoisomerase IIα (285 ng) and supercoiled pBR322 DNA (0.6 μg) in 20 μl (final volume) of 50 mM Tris-HCl, 150 mM KCl, 8 mM MgCl2, 1 mM 2-mercaptoethanol, and 100 μg/ml BSA. The solution was incubated for 10 min at 37°C in the presence of 2.5% DMSO or 1 mM ICRF-193 before addition of 1 mM ATP and further incubation for 30 min at 37°C. The reaction was stopped by the addition of NaCl to a final concentration of 1 M. After 15 min at room temperature, the solution was passed through a glass fiber filter (prepared by previous overnight soaking in a buffer containing 50 mM Tris-HCl, 8 mM MgCl2, 1 mM EDTA, 7 mM 2-mercaptoethanol, 100 μg/ml BSA, and 100 μg/ml salmon sperm DNA followed by copious washing with filter-soaking buffer without salmon sperm DNA). The filter was washed three times with 50 μl of reaction buffer plus 1 M NaCl. A solution containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS, and 500 μg/ml proteinase K was applied to the filter and after a 30-min incubation at 37°C, the filter was eluted. Samples of the high-salt wash and SDS elution were analyzed by electrophoresis in a 0.8% agarose gel. The positive control for clamp formation involved preincubating wild-type or mutant topoisomerase II with pBR322 and yeast 2μ DNA. The reaction was then incubated for 30 min at 37°C and processed as for the standard relaxation or decatenation assay.

**Results**

Novel A648P and L169F Mutations in Human Topoisomerase IIα Confer Resistance to ICRF-193 In Vivo: Mutant Selection and Genetic Analysis in Yeast. We used a yeast system to isolate plasmid-encoded human top2α mutants resistant to ICRF-193. Briefly, URA-3 plasmid YEpyWOB6 expressing human topoisomerase IIα (residues 28–1531 fused to the first five residues of yeast topoisomerase II) (Fig. 2) was chemically mutagenized and used to rescue growth at 35°C of drug-permeable ura-yeast strain JN3942-4 that carries a temperature-sensitive mutation in its single chromosomal TOP2 gene (Nitiss and Wang, 1988; Wasserman et al., 1993; Wasserman and Wang, 1994; Patel et al., 1997). Two libraries were grown at 35°C for 96 h in the presence of 10 or 25 μg/ml ICRF-193 (a drug concentration 5–10-fold higher than that required to inhibit growth of JN3942-4 transformed with wild-type YEpyWOB6). Plating on SC-URA and incubation at 35°C for 5 days yielded 10,000 and 3,000 colonies from selections with 10 and 25 μg/ml ICRF-193, respectively. Twelve clones from each selection were then restreaked on to SC-URA plates containing ICRF-193 at the original selecting concentration. Plasmids were isolated from positive clones and retransformed into sensitive JN3942-4 to confirm that the resistance was plasmid encoded.

Survival curves were determined for JN3942-4 transformed with YEpyWOB6 and for two retransformants carrying plasmids YEpyWOB6-E1 and YEpyWOB6-E2 (Fig. 3). All three plasmids supported yeast growth at 35°C producing a 3- to 10-fold increase in viable counts at 24 h. Low concentrations of ICRF-193 (0.5–1.0 μg/ml) resulted in growth inhibition of cells transformed with YEpyWOB6 and cell killing was observed with 2 to 10 μg/ml drug (Fig. 3A). The minimum lethal concentration or MLC (the drug concentration required to kill the yeast rather than inhibit growth) was 2 μg/ml. Plasmids E1 and E2 also supported growth in the absence of drug but viable counts were reproducibly some 3-fold lower at 24 h compared with the wild-type transformant (Fig. 3, B and C). The E1 transformant was somewhat resistant to ICRF-193 with an MLC of 10 μg/ml (Fig. 3B). In contrast, plasmid E2 conferred a high level of resistance: ICRF-193 at 10, 50, and 75 μg/ml (data not shown) and at 100 μg/ml (Fig. 3C) had no effect on cell growth. Essentially identical results to those in Fig. 3 were obtained when the experiment was repeated. Thus, plasmids E1 and E2 conferred 5-fold and >50-fold increases in resistance to ICRF-193.

The TOP2α gene of plasmids E1 and E2 was sequenced and compared with that of YEpyWOB6. The E1 and E2 sequences were identical with wild-type except for a single nucleotide
change in each case. A G-to-C mutation was identified at nucleotide position 1942 in the TOP2α allele of plasmid E1, resulting in an Ala-to-Pro mutation at codon 648 (A648P). Nucleotide numbering is according to the corrected TOP2α cDNA sequence (Tsai-Pflugfelder et al., 1988; Hsiung et al., 1996). This residue is located in the catalytic breakage-reunion domain of human TOP2α and is not well conserved in the topoisomerases II of other species. Plasmid E2 carried a G-to-T mutation at position 507, generating a Leu-to-Phe substitution at residue 169 (L169F) (Fig. 9). Four other clones carried the TOP2α mutation, giving the L169F protein change: four additional clones specified the A648P alteration (data not shown).

To verify that the plasmid-mediated resistance was due to the identified point mutations, a fragment exchange was carried out in which a 2.13-kb Bsp1407I-KpnI fragment from mutant plasmids E1 and E2 was used to replace the corresponding fragment in TOP2α of the wild-type plasmid (Fig. 2). The presence of each mutation in the resulting chimeric constructs, YEpWOB6-E1F and YEpWOB6-E2F, was confirmed by DNA sequencing. The plasmids were transformed into JN394t2-4 and the ICRF-193 sensitivities were measured at 35°C. E1F and E2F transformants displayed MLC values of 10 and >100 μg/ml, respectively. These results demonstrate that the A648P and L169F mutations in topoisomerase IIα are responsible for resistance to ICRF-193 in vivo. In other experiments, the E1F and E2F transformants retained sensitivity to etoposide (data not shown).

**Purified Mutant Proteins Have Wild-Type Catalytic Turnover Numbers but Altered Topoisomerase Activities at Limiting ATP.** Wild-type and mutant human topoisomerase IIα proteins were overexpressed in yeast and purified to >90% homogeneity (Wasserman et al., 1993) (Fig. 4). When assayed at saturating ATP (1 mM), the mutant enzymes exhibited similarly efficient topoisomerase activities to each other and to wild type with specific activities for DNA relaxation and kinetoplast DNA decatenation of 7 to 8 × 10⁵ U/mg and 1 to 2 × 10⁵ U/mg, respectively. Similarly, the DNA-dependent ATPase activities measured at 1 mM for wild-type, A648P, and L169F proteins were 1.0, 0.9, and 0.5 ATP molecules hydrolyzed/s/dimer, respectively. Thus, the turnover numbers of the mutant proteins were comparable to those of the wild-type enzyme.

Given that mutations in the ATP-binding region could reduce ATP affinity (O’Dea et al., 1996), we examined the effects of decreasing ATP concentration on the DNA relaxation and kDNA decatenation activities of mutant and wild-type enzymes (Fig. 5). At limiting ATP concentrations, the mutant enzymes were less active than wild-type protein. Thus, ATP at 0.025 and 0.01 mM supported complete and almost complete relaxation of pBR322 DNA by the wild-type protein (Fig. 5A, lanes 5 and 6). DNA relaxation by the mutant proteins was partial at 0.025 mM ATP (lanes 11 and 17) and inhibited at 0.01 mM (lanes 12 and 18). The same result was obtained in three independent experiments. In the decatenation assay (Fig. 5B), complete decatenation by the wild-type protein occurred at 0.025 mM ATP (lane 4), whereas 0.1 mM ATP was required for the mutant proteins (lanes 8 and 14). Thus, both the L169F and A648P mutants have an increased requirement for ATP.

**A648P Protein Exhibits Wild-Type Sensitivity to ICRF-193 at Saturating ATP but the L169F Enzyme Is Highly Drug Resistant.** To allow comparison with in vivo results in yeast (Fig. 3), we examined ICRF-193 inhibition of DNA relaxation and decatenation by mutant enzymes in the presence of 1 mM ATP. Figure 6A presents the effects of ICRF-193 on enzymatic DNA relaxation. In the absence of drug, wild-type and mutant enzymes converted supercoiled pBR322 DNA to the relaxed form (Fig. 6A, lanes 2, 8, and 14). The A648P protein exhibited a wild-type sensitivity to drug (cf. lanes 2–7 with 8–13) with partial inhibition of relaxation at 0.1 and 1 μg/ml (lanes 9 and 10) and complete inhibition at >10 μg/ml (lanes 11–13). Strikingly, relaxation by the L169F enzyme was refractory to inhibition by ICRF-193 even at 250 μg/ml (lanes 14–19) consistent with its effects in vivo (Fig. 3C). When DNA relaxation was examined similarly in the presence of etoposide (0–100 μg/ml) as the inhibiting drug, the A648P and L169F proteins showed a wild-type pattern of inhibition (data not shown). Thus, neither mutation affected the etoposide response.

When the topoisomerase proteins were incubated with ICRF-193 and 1 mM ATP plus kinetoplast DNA (Fig. 6B), no

**Fig. 3.** Cytotoxicity of ICRF-193 at 35°C against yeast strain JN394top2-4 carrying plasmid YEpWOB6 expressing a wild-type hTOP2α gene (A) or mutant plasmids E1 (B) and E2 (C). Yeast cells were grown in the absence or presence of ICRF-193 at the indicated concentrations, and viable counts determined at 4, 7, and 24 h are expressed as a percentage of viable counts determined at time 0. Because the E2 transformant was totally resistant to ICRF-193 at all concentrations tested, only data obtained in the absence or presence of the highest drug concentration of 100 μg/ml are shown in C.
difference was seen in the sensitivity of wild-type and A648P decatenation activities (cf. lanes 2–7 with 8–13), with 10, 100, and 250 μg/ml drug producing complete inhibition (lanes 5–7 and 11–13). The L169F enzyme required 100 μg/ml drug to inhibit decatenation (lane 18), i.e., 10-fold greater than that for wild type. Both the relaxation and decatenation experiments were repeated numerous times with identical results. In experiments with etoposide, both mutant proteins displayed the same decatenation inhibition profile as the wild-type enzyme (data not shown).

**Mutant Enzymes Are Competent in Mediating Etoposide-Promoted DNA Cleavage.** We compared the ability of the wild-type and mutant topoisomerase II proteins to undergo cleavable complex formation in the presence of ICRF-193 or etoposide. Proteins were incubated with supercoiled pBR322 and 1 mM ATP in the absence or presence of drug. After addition of SDS (to disrupt cleavable complexes and induce DNA breakage) and proteinase K treatment, DNA was analyzed by agarose gel electrophoresis. None of the proteins induced significant DNA breakage in the presence of ICRF-193 at drug levels up to 1 mM (data not shown). However, with etoposide, the two mutant proteins were as efficient as wild type in producing linear DNA (Fig. 7 cf. lanes 3–5, 8–10, and 13–15), consistent with the etoposide sensitivities of the proteins seen in vivo. It appears that the mutant proteins retain the ability to mediate DNA breakage by a known topoisomerase II poison.

**ATPase Activity of the L169F Enzyme Is Resistant to Inhibition by ICRF-193.** To examine ATPase inhibition, wild-type and L169F enzymes were preincubated with vari-
uous concentrations of ICRF-193 and 1 mM ATP for 10 min at 37°C before initiating the reaction with pBR322 DNA (there was no measurable ATPase activity in the absence of DNA; data not shown). For the wild-type protein, there was a dose-dependent inhibition of ATPase activity, yielding an IC₅₀ value (the drug concentration required to inhibit activity by 50%) of 0.85 μg/ml. In contrast, the ATPase activity of the L169F protein was resistant to inhibition by ICRF-193 even at 100 μg/ml (data not shown). Thus, the L169F mutation abrogates the inhibitory effect of ICRF-193 on ATPase activity.

L169F and A648P Mutations Inhibit ICRF-193-Induced Trapping of the Closed Enzyme Clamp. We examined the ability of ICRF-193 to trap topoisomerase II proteins in the closed clamp form by using incubation with the nonhydrolysable ATP analog AMP-PNP as a positive control for clamp formation (Roca et al., 1994). In each case, to follow the reaction, we exploited the finding that trapping of the closed clamp form as an annulus on circular DNA results in a DNA-protein complex resistant to dissociation with high salt. This complex binds to a glass filter (through its protein component) in the presence of 1 M NaCl and the DNA can only be released from the clamp (and filter) by denaturation of the complex with SDS (Roca et al., 1994).

Superciled pBR322 DNA was incubated with equal amounts of wild-type or mutant proteins (DNA:protein molar ratio of 1:4) in the presence of 2 mM AMP-PNP, or 1 mM ATP and 1 mM ICRF-193. Reactions were stopped by addition of NaCl to 1 M and applied to a glass filter, which was washed several times with 1 M NaCl in reaction buffer before elution with SDS. DNA in the eluates was examined by agarose gel electrophoresis (Fig. 8, NaCl wash and SDS eluates are in odd and even lanes, respectively). With AMP-PNP (lanes 1–6), some DNA eluted in the 1 M NaCl wash (lanes 1, 3, and 5) predominantly as the nicked form. However, for all three proteins, input DNA was trapped on the filter and was eluted by SDS (lanes 2, 4, and 6). In control experiments, the presence of both AMP-PNP and topoisomerase II was shown to be necessary for filter binding (data not shown). In addition to nicked DNA circles and a small amount of linear DNA, the filter-bound DNA consisted mainly of partially relaxed closed DNA circles. Partial relaxation results from stoichiometric DNA strand crossing promoted by AMP-PNP before enzyme capture as a closed clamp on DNA. Similar results have been seen for trapping of the protein clamp of yeast topoisomerase II on DNA (Roca et al., 1994). Quantitation by laser densitometry indicated that for wild-type, A648P, and L169F en-

zymes, the proportion of total DNA trapped on the filter was 54, 37, and 48%, respectively. Thus, from Fig. 8, it appears that both mutant proteins form the AMP-PNP-induced closed clamp, although the A648P mutant appeared to be somewhat less proficient than wild type.

In the presence of 1 mM ATP, ICRF-193 was able to trap the wild-type protein on circular DNA in the closed clamp form but this reaction was markedly less effective with the mutant enzymes (Fig. 8, lanes 13–18). Thus, for wild-type enzyme, 63% of the input DNA was bound to the filter, largely as nicked or relaxed circles plus a small amount of linear DNA (cf. lanes 13 and 14), suggesting that ICRF-193-mediated clamp trapping is inefficient compared with ATP-dependent DNA relaxation. Omission of either ATP, ICRF-193, or enzyme components abrogated filter binding (data not shown). For the A648P enzyme, only 25% of the DNA were retained on the filter (lane 16): the majority appeared in the NaCl eluate (lane 15). For the L169F enzyme, essentially all the DNA was present in the NaCl wash (lane 17) with <10% bound to the filter (lane 18). This experiment was repeated three times and the results clearly showed that the A648P enzyme is partly resistant, and the L169F enzyme is highly resistant to trapping in the closed clamp conformation by ICRF-193. Interestingly, little or no linear DNA was observed with the mutant proteins, suggesting its production by wild-type enzyme is dependent on ICRF-193 (Fig. 8).

Previous studies have shown for yeast topoisomerase II that incubation with ATP and ICRF-193 results in conversion to the closed clamp conformation such that the enzyme is then unavailable to form an annulus on DNA (Roca et al., 1994). Experiment ICRF-193 (a) in Fig. 8 was included to test this possibility for human topoisomerase IIα and its mutants. Enzymes, ATP, and ICRF-193 were preincubated before a second incubation with pBR322 DNA. It can be seen that essentially the same results were obtained (lanes 7–12) as in experiment ICRF-193 (b) described above in which DNA was present at the outset. In contrast to yeast topoisomerase II, it appears that in the absence of DNA, closed clamp formation by human topoisomerase IIα is relatively inefficient.

![Fig. 7](image-url)  
**Fig. 7.** Etoposide-promoted DNA breakage by topoisomerase II proteins. Superciled pBR322 (0.4 μg) was incubated with topoisomerase II proteins (25 ng) in the presence of 1 mM ATP and various concentrations of etoposide. DNA breakage was induced by addition of SDS, and after digestion with proteinase K, DNA samples were analyzed by gel electrophoresis. Lanes 1 to 5, 6 to 10, and 11 to 15 contained etoposide at 0, 5, 25, 50, and 100 μg/ml, respectively. Lane A, supercoiled pBR322 DNA with no enzyme or drug addition. N, L, and S indicate nicked, linear, and supercoiled pBR322 DNA, respectively.

![Fig. 8](image-url)  
**Fig. 8.** Trapping of the closed clamp form of topoisomerase II proteins by AMP-PNP and by ICRF-193. Wild-type or mutant topoisomerase IIα proteins (285 ng) were incubated with supercoiled pBR322 DNA (0.6 μg) and 2 mM AMP-PNP (lanes 1–6). Topoisomerase II proteins, pBR322, and 1 mM ICRF-193 were preincubated in experiment ICRF-193 (a) (lanes 13–18) before addition of 1 mM ATP. In experiment ICRF-193 (b), topoisomerase II proteins were preincubated with 1 mM ATP and 1 mM ICRF-193 before addition of pBR322 DNA and further incubation (lanes 7–12). Reactions were stopped by the addition of NaCl to 1 M and applied to glass filters. Filters were washed with 1 M NaCl before treatment with 1% SDS and proteinase K followed by elution with 1% SDS. Contents of the 1 M NaCl washes (odd lanes) and SDS elutions (even lanes) were examined by electrophoresis in 0.8% agarose. Lane 19, supercoiled pBR322 DNA. N, R, and S denote nicked, relaxed, and supercoiled pBR322 DNA, respectively.
Discussion

Bisdioxopiperazine inhibitors of topoisomerase II have a unique mode of action that involves trapping of a closed clamp enzyme intermediate in the topoisomerase II reaction cycle accessed through the binding and hydrolysis of ATP. However, the nature of the bisdioxopiperazine-topoisomerase II complex and the cellular consequences of its formation are poorly understood. To study the interactions of bisdioxopiperazines with topoisomerase II, we have used a yeast genetic system to select, from a randomly mutagenized plasmid library, human TOP2α alleles mediating resistance to ICRF-193. We found that novel point mutations of L169F in the ATPase domain, and of A648P in the core domain of human topoisomerase IIs, were commonly selected and conferred >50-fold and ~5-fold increases in ICRF-193 resistance in yeast, respectively. The highly purified mutant enzymes had increased ATP requirements at subsaturating ATP levels, but in the presence of saturating (1 mM) ATP, exhibited broadly wild-type-specific activities in DNA relaxation, decatenation and ATPase assays, and in etoposide-mediated DNA cleavage. Unlike the A648P enzyme, which displayed a wild-type sensitivity to ICRF-193 at 1 mM ATP, the catalytic activities of the L169F protein were highly refractory to drug inhibition. Despite these phenotypic differences, the mutant enzymes were both resistant to trapping by ICRF-193 in the closed clamp conformation as an annulus on closed circular DNA. This is the first detailed enzymatic analysis of bisdioxopiperazine resistance mutants of human topoisomerase IIs and suggests both ATPase and core domains influence drug action.

The L169F topoisomerase IIα enzyme conferred high-level resistance to ICRF-193 in yeast (Fig. 3C). Moreover, its topoisomerase and ATPase activities were particularly refractory to inhibition by ICRF-193 (Fig. 6) and the enzyme showed no evidence of trapping in the closed clamp form by ICRF-193 (Fig. 8). These data suggest that the L169F mutation may interfere directly with binding of ICRF-193 to a site in the ATPase domain. Residue Leu-169 in human topoisomerase IIα is highly conserved in type II topoisomerases and lies next to a glycine-rich Walker A box found in the ATP-binding sites of many proteins (Walker et al., 1982) (Fig. 9). The equivalent residue to Leu-169 in GyrB is Val-122, which although not directly involved in ATP binding, lies immediately adjacent to the ATP site. It is plausible that Leu-169 interacts directly with the ICRF-193, or at least allows drug binding, resulting in inhibition of ATP hydrolysis and capture of the closed enzyme clamp form in a manner akin to AMP-PNP-mediated clamp trapping. By interfering with ICRF-193 binding, the L169F mutation would lead to the observed release of enzyme inhibition and clamp capture by ICRF-193 (Figs. 6 and 8). Although the mutation had some effect on the binding affinity for ATP (Fig. 5), there was a less than a 2-fold effect on ATP turnover number (under Results).

Our studies of the L169F mutation obtained by random mutagenesis and selection in yeast complement and extend the analysis of topoisomerase IIα mutations identified in three bisdioxopiperazine-resistant cell lines. Thus, I48T and Y49F topoisomerase IIα mutations have been found in CHO cell lines made resistant to ICRF-187 and ICRF-159, respectively (Sehested et al., 1998; Yalowich et al., 1998). The Y49F topoisomerase IIα activity present in a 0.35 M NaCl nuclear extract from ICRF-159-resistant CHO cells behaved like the human L169F enzyme: it proved impossible to inhibit decatenation activity with ICRF-187 (Sehested et al., 1998). Furthermore, functional expression in yeast of the human Y50F protein (equivalent to Y49F in the CHO enzyme) confirmed a high-level bisdioxopiperazine resistance phenotype (Sehested et al., 1998). More recently, an R162Q topoisomerase IIα mutation identified in an ICRF-187-resistant human small cell lung cancer line was shown to confer severalfold resistance when the allele was expressed in yeast (Wessel et al., 1999).

Each of these three mutations reported in cell lines is likely to be at or near the ATP-binding site of topoisomerase II. Thus, in the closed clamp structure of E. coli GyrB, the equivalent residues to Ile-48 and Tyr-49 in CHO topoisomerase IIα are Met-25 and Tyr-26, which lie in a protein loop adjacent to the ATP site and spatially close to Val-122. Moreover, the R162Q mutation lies directly in the Walker A box consensus region (Fig. 9) (Wessel et al., 1999). Thus, the clustering of L169F, I48T, Y50F, and R162Q mutations near the ATP site of human topoisomerase IIα could indicate a bisdioxopiperazine-binding site, consistent with studies showing that ICRF-193 inhibits ATP hydrolysis by an N-terminal fragment of yeast topoisomerase II (Olland and Wang, 1999). We note that bisdioxopiperazines are bifunctional (Fig. 1) and therefore one molecule may be sufficient to bind and bridge sites across the closed ATPase clamp. Structural studies will be needed to test this idea.

Unlike previously reported bisdioxopiperazine-resistance mutations, the A648P alteration resides in the core domain rather than the ATPase region and produced a significantly different phenotype to that of the L169F enzyme. First, the A648P protein conferred only a 5-fold increased resistance to ICRF-193 when expressed in yeast (Fig. 3) and exhibited only partial trapping in the closed clamp form (Fig. 8). Second, its DNA relaxation and decatenation activities were as sensitive
to ICRF-193 as the wild-type enzyme when assayed in the presence of 1 mM ATP (Fig. 6). Thus, it seems that mutations causing bisdioxopiperazine resistance belong to at least two different phenotypic classes and likely act by two distinct mechanisms. The phenotype of the L169F mutant is consistent with a direct effect on drug action in the ATPase domain: the properties of the A648P protein are more complex and admit several possible explanations.

First, the A648P mutation could alter clamp trapping by acting allosterically on ATP binding. In fact, we have shown that the A648P enzyme does have an increased requirement for ATP at subsaturating concentrations and is less effectively trapped as a clamp by AMP-PNP (Figs. 5 and 8). A decreased affinity for ATP would alter the enzyme equilibrium to favor the pre-ATP-bound open clamp state, thereby making it more difficult to trap the closed clamp form, resulting in resistance to bisdioxopiperazines. A similar mechanism has been suggested for the R162Q enzyme that shares the same phenotype as A648P (Wessels et al., 1999). However, to explain the severalfold resistance conferred by these mutations in yeast, the intranuclear levels of ATP would need to be subsaturating in strain JN394t2-4 either through compartmentation or as a secondary effect of bisdioxopiperazine action. [It is known that intracellular ATP levels are maintained at 1 to 2 and 3 mM, respectively, in aerobically growing yeast and mammalian cells (Buxeda et al., 1993; van den Boogaart, 1995). Moreover, an effect mediated through diminished ATP affinity is inconsistent with the results of the ICRF-193 trapping experiments in Fig. 8. In these experiments, the A648P protein was incubated with ICRF-193 in the presence of 1 mM ATP. From the enzyme turnover number, we calculate that <10% ATP hydrolysis would have occurred, thus maintaining ATP at saturating levels. On the basis of relaxation and decatenation assays (Fig. 6), we would expect the enzyme to exhibit wild-type sensitivity to ICRF-193 and thus be trapped as an annulus on circular DNA. In fact, little or no trapping of the A648P protein on DNA was observed (Fig. 8).

To reconcile these observations, we suggest that the closed clamp of the A648P enzyme is in fact captured as an annulus on DNA by ICRF-193 and 1 mM ATP but, unlike wild-type enzyme, is disrupted by the addition of 1 M NaCl (used to eliminate nonspecific DNA interactions) before filter binding (Fig. 8). Salt sensitivity of the A648P protein clamp could arise through allosteric effects of the mutation on the N-terminal ATPase gate. Alternatively, the mutation could affect the ICRF-193-trapped clamp by destabilizing the B’-B’ interface. Precedent for this idea comes from previous studies showing that a truncated Dro sophila topoisomerase II protein lacking the ATPase region can be trapped on DNA by ICRF-159 and that this protein annulus has lower salt stability than full-length protein (Chang et al., 1998). Given the instability of the A648P closed clamp and the observed resistance to ICRF-193 conferred by the A648P protein in yeast, it is tempting to suggest that formation of the closed clamp rather than inhibition of catalytic activity is responsible for ICRF-193-induced killing of yeast cells expressing topoisomerase IIα, as recently suggested (Jensen et al., 2000). Further studies will be needed to clarify precisely how the A648P mutation affects closed clamp stability and confers cellular resistance.

In summary, by using a versatile yeast genetic system in combination with biochemical analysis of mutant proteins, we have shown that alterations in both the ATPase and core domains of human topoisomerase IIα can modulate bisdioxopiperazine action and that resistance alleles belong to at least two phenotypically distinct classes. Access to such mutants will be important in understanding the mechanism of the topoisomerase II clamp and in developing its potential as a novel antitumor drug target.

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