N-terminal and core-domain random mutations in human topoisomerase II α conferring bisdioxopiperazine resistance

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Abstract Random mutagenesis of human topoisomerase II α cDNA followed by functional expression in yeast cells lacking endogenous topoisomerase II activity in the presence of ICRF-187, identified five functional mutations conferring cellular bisdioxopiperazine resistance. The mutations L169F, G551S, P592L, D645N, and T996L confer > 37, 37, 18, 14, and 19 fold resistance towards ICRF-187 in a 24 h clonogenic assay, respectively. Purified recombinant L169F protein is highly resistant towards catalytic inhibition by ICRF-187 in vitro while G551S, D645N, and T996L proteins are not. This demonstrates that cellular bisdioxopiperazine resistance can result from at least two classes of mutations in topoisomerase II; one class renders the protein non-responsive to bisdioxopiperazine compounds, while an other class does not appear to affect the catalytic sensitivity towards these drugs. In addition, our results indicate that different protein domains are involved in mediating the effect of bisdioxopiperazine compounds.

Key words: Human topoisomerase II α; Bisdioxopiperazine resistance; Mutation

1. Introduction

Type II topoisomerase is a family of highly conserved nuclear enzymes capable of transferring one DNA double helix through a transient double strand break in another DNA double helix [1]. The importance of this unique mechanistic action which is absolutely required in chromosome segregation following cell division is illustrated by the fact that all living cells possess topoisomerase II enzymes. Higher vertebrates including humans express two isoforms of topoisomerase II; a 170 kDa α isoform mainly found in proliferating cells where its expression is highest in the G_2/M phase of the cell cycle, and a 180 kDa β isoform found in both proliferating and non-proliferating cells whose expression is independent of the cell cycle status [2].

Besides being an essential enzyme human topoisomerase II is also an important target in cancer therapy. One major class of anti-cancer drugs acts by stabilizing a transient configuration of topoisomerase II, where the enzyme is covalently attached to DNA [3]. These are referred to as cleavable complex stabilizing drugs and are represented by such clinically important compounds as the anthracyclines (doxorubicin, daunorubicine), the epipopdohyllotoxins (VP-16, VM-26), and the aminoacridines (*m*-AMSA). Another major class of drugs are catalytic inhibitors of topoisomerase II exemplified by merbarone and aclarubicin which do not induce DNA breaks [4].

Bisdioxopiperazines are topoisomerase II-directed drugs which lock topoisomerase II as a closed clamp on DNA by stabilizing an otherwise transient N-terminal dimer interaction formed upon ATP binding [5]. These drugs are also known to inhibit the ATPase activity of topoisomerase II enzymes [6]. Originally classified as catalytic inhibitors believed to exert their cytotoxic effect through inhibition of decatenation activity [7,8], recent data indicate that the bisdioxopiperazine compounds kill cells through the formation of non-covalent closed clamp complexes in vivo interfering with basic DNA metabolic processes resulting in more rapid and pronounced cell death than seen by inhibition of enzymatic activity [9,10]. Although much information concerning mechanistic and thermodynamic properties of topoisomerase II trapped on DNA in the presence of bisdioxopiperazine analogues has emerged during recent years [6,11–13], the connection between the action of these on topoisomerase II and cytotoxicity has been less investigated.

In order to study the mechanism of bisdioxopiperazine-mediated cell killing, we have used a random mutagenesis protocol to generate cellular bisdioxopiperazine resistance mediated through mutations in human topoisomerase II α.

2. Materials and methods

2.1. Constructs

The expression vector for human topoisomerase II α in yeast pMJ1 [14] comprises the entire coding region of human topoisomerase II α under control of a constitutive yeast topoisomerase I promoter, an URA3 selectable marker, a yeast origin of replication and a yeast centromere sequence for the introduction and maintenance in yeast cells. The plasmid pYX113hTOPII [15] comprises the same functions as pMJ1, but in this construct the expression of human topoisomerase II α is controlled by an galactose inducible GAL-1 promoter.

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Abbreviations: SC−URA, synthetic medium lacking uracil; YPDA, complete medium containing yeast extract, peptone, dextrose and adenine; *m*-AMSA, amsacrine; TRIS, tris[hydroxymethyl]aminomethane; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; T-DNA, transfer-DNA; G-DNA, gate-DNA; wt, wild type; kDNA, kinetoplast DNA

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2.2. Yeast strains

The hyper-permeable temperature sensitive and RAD52 deficient yeast strain JN394t2-4 (*MAT**a**, ura52, leu2, trp1, his7, ade1-2, ISE2, rad52::LEU2, top2-4*) [14] was used for the isolation of bisdioxopiperazine resistance conferring mutations, in clonogenic assays and for the assessment of topoisomerase II decatenation activity in crude extracts. The protease deficient topoisomerase I negative yeast strain JelΔTop1 (*trp1, leu2, ura-52, pbr-1122, pep4-3*, Δ*his3::PGAL10-GAL4, TOP1::LEU2*) [15] was used for the over expression of wild type (wt) and mutant human topoisomerase II α to be subsequently purified.

2.3. Drugs

ICRF-187 (Cardioxane; Chiron) was dissolved in sterile H_2O at 50 mg/ml just prior to use. ICRF-193 a kind gift from Dr. Donald Witiak, University of Wisconsin (WI, USA) was dissolved in DMSO at 10 mg/ml. VP-16 (Etoposide; Bristol-Myers) was purchased ready for infusion and was not diluted further. *m*-AMSA (Parke-Davis) was diluted in DMSO to 10 mg/ml and merbarone (kindly provided by The Drug Synthesis Chemistry Branch, Development Therapeutics Program, Division of Cancer Treatment and Diagnostics, National Cancer Institute, Bethesda, MD, USA), was dissolved in DMSO at 10 mg/ml.

2.4. In vitro mutagenesis of plasmid DNA

20 μg supercoiled pMJ1 plasmid DNA was added to 1 ml reaction mixture consisting of 1 M hydroxylamine (Sigma), 100 mM NaCl and 100 mM sodium pyrophosphate, pH = 7.0. The reaction mixture was incubated at 75°C for 1 h. After desalting, 200 ng mutagenized plasmid DNA was transformed into competent TOP10 *Escherichia coli* cells (Invitrogen) which resulted in approximately 20 000 transformed bacterial colonies which were pooled and grown for 9 h with selection in 300 ml LB medium. Plasmid DNA was isolated using a tip 500 Quiafilter plasmid purification kit (Qiagen) and used to transform JN394t2-4 cells.

2.5. Isolation of ICRF-187 resistant clones

Yeast cells were transformed using a modified lithium acetate protocol with single-stranded DNA as carrier. Cells were plated to generate approximately 500 colonies per 100 mm petri dish. A total of approximately 20 000 yeast colonies were obtained on selective synthetic medium lacking uracil (SC−URA) plates at the permissive temperature of 25°C. 7 days after transformation the SC−URA plates were replica-plated onto complete medium containing yeast extract, peptone, dextrose and adenine (YPDA) plates containing 75 μM ICRF-187 which were incubated at the non-permissive temperature 35°C for 72 h to score resistant clones. For each resistant clone a 100 ml culture grown overnight at 34°C in YPDA medium was used for the isolation of plasmid DNA.

2.6. Rescue of plasmid DNA from ICRF-187 resistant cells

Plasmid DNA was isolated from 100 ml cultures grown to saturation as described in [16].

2.7. Sequencing of human topoisomerase II α cDNA

Human topoisomerase II α cDNA in recovered pMJ1 plasmids was sequenced using a set of primers described previously [17]. Sequencing was performed using a dRhodamine Terminator Cycle Sequencing kit (Perkin Elmer) as described by the manufacturer. Cycle sequencing was performed using a Perkin Elmer GeneAmp 2400 thermocycler. Samples were analyzed using an ABI PRISM 377 sequencer.

2.8. Reconstruction of ICRF-187 conferring mutations

Identified mutations were reconstructed in pMJ1 and pYX113hTOPII by oligo nucleotide-directed mutagenesis using a Quick change site-directed mutagenesis kit (Stratagene) as previously described [17] using the mutagenic primers depicted in Table 1. The presence of reconstructed mutations was verified by sequencing as described above.

2.9. Determination of drug sensitivity in vivo

Overnight cultures of cells in log phase were diluted to 2×10^6 cells/ml in pre-warmed YPDA medium, and 3 ml cultures were exposed to different concentrations of VP-16, *m*-AMSA, ICRF-187, and ICRF-193 for 24 h, 200 rpm, 34°C. 1 ml samples removed after 0 and 24 h were washed twice in distilled sterile H_2O, diluted 0–10^4 times in distilled sterile H_2O. Next, 200 μl of diluted cells were plated to SC−URA plates which were incubated for 6 days at 25°C prior to counting. Plates containing 200–600 colonies were used for counting. Finally, relative cell growth after 24 h compared to 0 h was calculated for all conditions used. All experiments were performed in duplicate.

2.10. Isolation of crude extract

Cells were grown overnight in 100 ml YPDA medium at 34°C, 200 rpm to a density of $1–2\times10^7$ cells/ml and were harvested by centrifugation for 2 min at 4000 rpm, 4°C. Isolation of crude extract was done as described in [14].

2.11. Purification of human topoisomerase II α

The purification of wt and mutant human topoisomerase II α from overexpressing yeast cells was carried out as described in [18].

2.12. Preparation of ^{3}H-labeled kinetoplast DNA (kDNA)

Tritium-labeled kDNA was isolated from *Crithidia fasiculata* as described in [19].

2.13. Determination of topoisomerase II decatenation activity

Decatenation of *C. fasiculata* kDNA was used to determine the amount of decatenation activity in crude extract and to assess the drug sensitivity and ATP requirement of purified mutant human topoisomerase II α. In experiments with purified enzyme 1 U of decatenation activity (defined as the amount required to decatenate 200 ng k-DNA at 37°C in 15 min) was incubated with 200 ng kDNA and increasing concentration of drug or ATP in a 20 μl reaction volume at 37°C for 15 min in topoisomerase II buffer (10 mM tris[hydroxymethyl]aminomethane (TRIS)–HCl pH = 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 15 μg/ml bovine serum albumin (BSA) and 1 mM ATP) as described in [17–19]. For the determination of decatenation activity in crude extracts, increasing amounts of extract were incubated with 100 ng kDNA for 60 min at 37°C using the same buffer and reaction volume.

3. Results

3.1. Identification of mutations in human topoisomerase II α

It has previously been shown that topoisomerase II is the target for bisdioxopiperazine compounds in yeast [20]. Further, in two studies we have shown that mutations in topoisomerase II α identified in mammalian cell lines resistant to bisdioxopiperazines conferred resistance to JN394t2-4 cells

Table 1
Primers used in oligonucleotide-directed mutagenesis

L169F-SN:	GGC TAT GGA GCC AAA TT**T** TGT AAC ATA TC AGT ACC
L169F-ASN:	GGT ACT GAA TAT GTT ACA **A**AA TTT GGC TCC ATA GCC
G551S-SN:	GGT TCC CAC ATC AAA **A**GC TTG CTG ATT AAT TTT ATC C
G551S-ASN:	GGA TAA AAT TAA TCA GCA AGC **T**TT TGA TGT GGG AAC C
P592L-SN:	GGC ATT TTA CAG CCT TC**T** TGA ATT TGA AGA GTG G
P592L-ASN:	CCA CTC TTC AAA TTC A**A**G AAG GCT GTA AAA TGC C
D645N-SN:	CTG GTC CTG AA**A** ATG ATG CTG CTA TCA GC
D645N-ASN:	GCT GAT AGC AGC ATC AT**T** TTC AGG ACC AG
T996L-SN:	CTC CAA ACT AGT CTC A**T**A TGC AAC TCT ATG GTG C
T996L-ASN:	GCA CCA TAG AGT TGC AT**A** TGA GAC TAG TTT GGA G
P1248L-SN:	CTG AAG GAA GCC **T**TC AAG AAG ATG GTG TGG
P1248L-ASN:	CCA CAC CAT CTT CTT GA**A** GGC TTC CTT CAG

The primers are presented in the 5′ to 3′ direction.
Nucleotides responsible for introduction of mutations in the human topoisomerase IIα coding sequence are underlined.

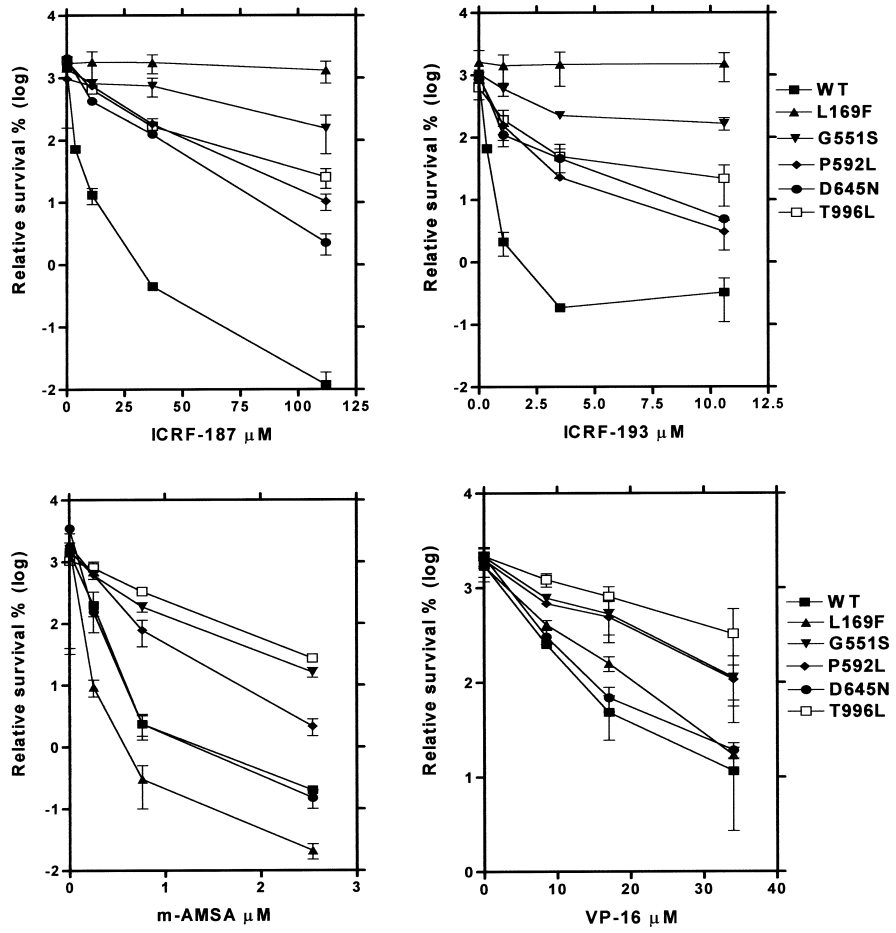

Fig. 1. Determination of drug sensitivity in clonogenic assay. JN394t2-4 cells comprising a temperature sensitive chromosomal topoisomerase II allele were transformed with pMJ1 – a plasmid capable of episomal expression of human topoisomerase II α in yeast. The pMJ1 plasmid comprised no mutation (wt) or single base pair changes resulting in the following amino acid substitutions in the human topoisomerase II α gene product (L169F, G551S, P592L, D645N and T996). Transformed cells were grown overnight to log phase and diluted to 2×10^6 cell/ml in prewarmed YPDA medium. The cells were incubated in the presence of different concentrations of ICRF-187 (left upper panel), ICRF-193 (right upper panel), *m*-AMSA (left lower panel) and VP-16 (right lower panel) for 24 h at the non-permissive temperature and were plated to produce 200–600 viable colonies per 10 cm petri dish. Error bars represent S.E.M. of two experiments performed with individually reconstructed mutations.

when expressed in these cells [17,18]. Transformation of JN394t2-4 cells with mutagenized human topoisomerase II α cDNA resulted in 13 ICRF-187 resistant colonies which were able to grow at the non-permissive temperature in the presence of 75 μM ICRF-187. Eight resistant clones were used for isolation of plasmid DNA. Six single base pair changes each responsible for the change of one amino acid in the polypeptide chain were identified namely: G507T (L169F), G1651A (G551S), C1778T (P592L), G1933A (D635N), C2987T (T996L) and C3743T (P1248L). All identified mutations were reconstructed by oligonucleotide-directed mutagenesis, and were found to be functional except the C3743T mutation (P1248L) which was not studied further.

3.2. *In vivo drug sensitivity*

24 h clonogenic assays were performed with JN394t2-4 cells transformed with pMJ1-wt plasmid or with pMJ1 plasmids comprising the five functional mutations L169F, G551S, P592L, D645N and T996L. Clonogenic assay was performed at 34°C where human topoisomerase II α is the only active topoisomerase II in these cells. The sensitivity of JN394t2-4 cells expressing the five mutants and wt human topoisomerase II α towards ICRF-187, ICRF-193 and the two cleavable complex stabilizing drugs *m*-AMSA and VP-16 are shown in Fig. 1A–D, respectively. The minimal lethal concentration (MLC) which results in a relative survival of 100% was estimated from the killing curves and used to determine the relative resistance of cells expressing mutant proteins (Table 2).

3.3. *Cellular bisdioxopiperazine resistance is not caused by alterations in topoisomerase II decatenation activity*

The observed ICRF-187 resistance in vivo could be caused by altered decatenation activity in cells expressing the mutant proteins compared to cells expressing the wt protein. To exclude this possibility, decatenation activity in cells grown under conditions equal to those used in determination of cellular drug sensitivity was determined using crude extracts. Decatenation was performed at 37°C where endogenous yeast topoisomerase is inactive. Of the five mutations identified only the D645N mutation caused a significant reduction in the decatenation activity of crude extract (Fig. 2). It can therefore be concluded that cellular bisdioxopiperazine resistance is generally not caused by alterations in the decatenation activity of human topoisomerase IIα in resistant cells.

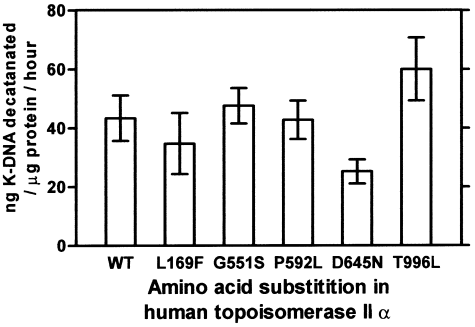

Fig. 2. Determination of catalytic topoisomerase II decatenation activity in crude extract isolated from JN394t2-4 cells expressing wt human topoisomerase II α or mutant L169F, G551S, P592L, D645N and T996L from the pMJ1 vector. Cells were grown under the same conditions used in clonogenic assay. Increasing amounts of crude extract were incubated with 100 ng *C. fasiculata* kDNA in 20 μl topoisomerase II reaction buffer at 37°C for 60 min. Next, catenated substrate DNA left unprocessed was separated from decatenated mini circles by gel electrophoresis and visualized by ethidium bromide staining. Finally, the specific decatenation activity of the crude extracts was determined as ng kDNA fully decatenated per μg protein per hour. Error bars represent S.E.M. of five experiments using individually prepared crude extracts.

3.4. In vitro drug sensitivity of purified mutant enzymes

In order to test the inhibitory effect of various anti-cancer drugs on decatenation activity wt, L169F, G551S, D645N, and T996L proteins were purified from overexpressing yeast cells. Purified L169F protein did not display any ICRF-187-mediated inhibition of decatenation. In contrast, none of the G551S, D645N and T996L proteins displayed any substantial resistance to ICRF-187-mediated inhibition of decatenation (Fig. 3A and Table 2). Decatenation was also carried out in the presence of merbarone, another catalytic inhibitor of topoisomerase II. With merbarone the L169F protein displayed wt sensitivity while the three core-domain mutations analyzed D645N, G551S, and T996L all conferred some resistance (Fig. 3B and Table 2). It is also evident that the G551S and T996L mutations which both display cross-resistance to VP-16 and *m*-AMSA in clonogenic assay are resistant to inhibition of decatenation by VP-16 (Fig. 3D and Table 2). These mutations also confer reduced sensitivity to *m*-AMSA in the decatenation assay although this is not significant (Fig. 3C).

3.5. Increased ATP requirements of mutant enzymes in decatenation

The ATP requirement of the mutant enzymes was also assessed by performing decatenation at low ATP concentrations. Using this assay it is evident from Fig. 3E that the L169F mutation increases the ATP requirement of the protein as it is inactive at 250 μM ATP where the wt enzyme is fully active. Surprisingly all mutations found in the core-domain of the protein (Fig. 4) corresponding to the central part of yeast topoisomerase II [1] also increase the ATP requirement of the protein although to a much lesser extent.

4. Discussion

Random mutagenesis in vitro followed by functional expression in the presence of ICRF-187 identified five novel functional mutations in the human topoisomerase II α structural gene conferring bisdioxopiperazine resistance (Fig. 4). Four of these map to the catalytic core-domain involved in DNA cleavage and religation while only one mutation L169F maps to the N-terminal ATP clamp region where three previously identified mutations were found (Fig. 4) [17,18,21].

The high level of resistance towards both ICRF-187 and ICRF-193 in vivo (Fig. 1A,B) and ICRF-187 in vitro (Fig. 3A) indicates that the L169F mutation renders the protein non-responsive to the action of bisdioxopiperazine compounds. Further, the lack of cross resistance towards the catalytic inhibitor merbarone as well as the cleavable complex stabilizing compounds VP-16 and *m*-AMSA indicates that the effect of the L169F mutation is specific to bisdioxopiperazine compounds. This agrees well with the location of the L169F mutation at the N-terminal clamp region of the protein that forms a closed clamp upon ATP binding, which is stabilized by the bisdioxopiperazine compounds [4–6]. Also, the increased requirement for ATP in decatenation fits with the location of the L169F mutation next to the Walker A ATP binding motif 161-GXGXXG-166 (Fig. 4).

Homology alignment of human topoisomerase IIα with 46 other type 2 topoisomerases of both eukaryotic and prokaryotic origin [22] shows that the L169F mutation maps to a region generally displaying a high degree of conservation. In 45 of 46 cases, the residue homologous to human topoisomerase II α L169 is either leucine or valine, which are both structurally and chemically very similar. This indicates that this residue is highly conserved among type 2 topoisomerases.

The lack of response towards bisdioxopiperazine compounds in vivo and in vitro seen with the L169F protein suggests that the L169 residue of human topoisomerase II α may be involved in drug binding. A previously described mutation changing Y50 to phenylalanine also results in complete lack of response to bisdioxopiperazine compounds [17]. Interestingly, the two homologous residues in the structure of

Table 2
Drug sensitivity in vivo and in vitro

Mutation	MLC in 24 h clonogenic assay (μM)/relative resistance (fold)				IC_{50} values in decatenation assay (μM)			
	ICRF-187	ICRF-193	*m*-AMSA	VP-16	ICRF-187	merbarone	*m*-AMSA	VP-16
None	3.0	0.3	0.35	13.0	15	45	45	90
L169F	112 (>37)	>10.6 (>35)	0.15 (0.4)	20.5 (1.6)	>1500	35	42	130
G551S	112 (37)	>10.6 (>35)	1.30 (3.7)	35.0 (2,7)	15	>200	65	>200
P592L	54 (18)	2.0 (7)	0.80 (2.3)	35.0 (2.7)	nd	nd	nd	nd
D645N	41 (14)	1.5 (5)	0.35 (1.0)	16.0 (1.2)	15	100	40	70
T996L	58 (19)	2.5 (8)	1.60 (4.6)	>35.0 (>2.7)	25	>200	50	>200

The MLC is defined as the drug concentration resulting in no cell growth and no cell killing i.e. 100% relative survival.
Relative resistance is defined as MLC-mutant/MLC-wt.
nd: not determined.

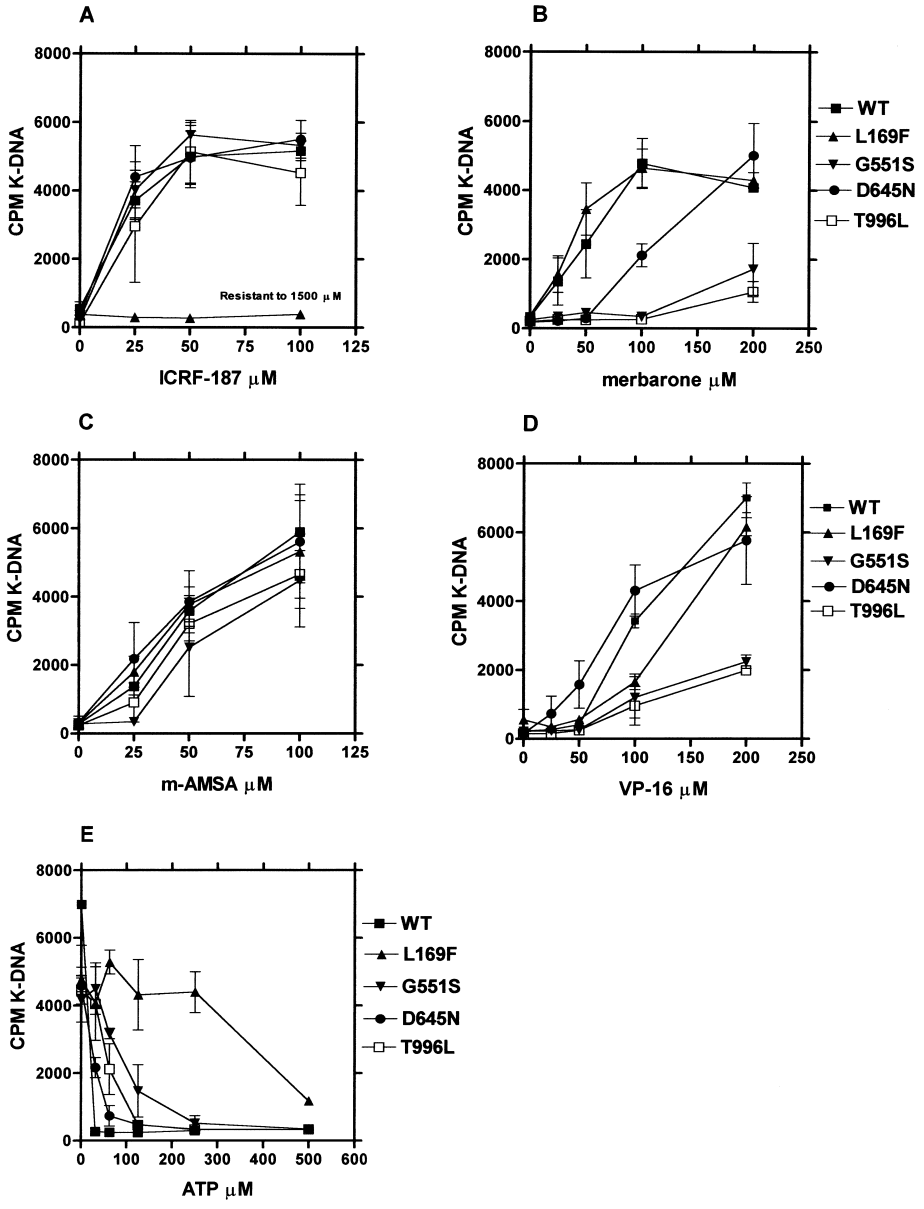

Fig. 3. Assessment of drug sensitivity and ATP dependence with purified mutant proteins. 1 U of purified wt, L169F, G551S, D645N, or T996L protein was incubated with 200 ng of ^{3}H-labeled *C. fasiculata* kDNA in 20 μl topoisomerase II reaction buffer for 15 min at 37°C at increasing concentrations of ICRF-187 (A), merbarone (B), *m*-AMSA (C), VP-16 (D) or at varying ATP concentrations (E). Experiments assessing the drug sensitivity were performed at 1 mM ATP. Unprocessed substrate kDNA was separated from decatenated mini circles as described in Fig. 2 and were cut out of the agarose gel and subjected to liquid scintillation counting. Finally, the amount of radioactivity expressed as CPM was plotted against drug or ATP concentration to reveal dose response curves. Error bars represents S.E.M. of three independent experiments.

E. coli gyrase B [23] Y26 and V122 are in close proximity suggesting that they may both contribute to a drug binding site. However, it is important to note that *E. coli* gyrase does not display sensitivity to bisdioxopiperazine compounds [11].

The fact that the human topoisomerase IIα L169 residue is so highly conserved among type 2 topoisomerases suggests that this residue contributes to protein function, which is confirmed in this study by the increased ATP requirement of the L169F protein in decatenation (Fig. 3E). The functional importance of this residue makes it possible that the L169F mutation destabilizes the closed clamp which might per se be sufficient to cause bisdioxopiperazine resistance. Due to the complete lack of drug response seen with the L169F mutation, we favor the drug binding hypothesis. In any case our results strongly suggest that the N-terminal ATP-operated clamp is a significant target for bisdioxopiperazine compounds. This is supported by data from Olland and Wang who found that ATP hydrolysis catalyzed by a N-terminal fragment of yeast topoisomerase II constituting this protein region (amino acid 1–409) is inhibited by ICRF-193 [13].

The core-domain mutations G551S, P592L, D645N and T996L display a quite different phenotype. These mutations confer less cellular bisdioxopiperazine resistance than the Y50F [17] or L169F (Fig. 1A,B) mutations. The level of cellular bisdioxopiperazine resistance conferred by these core-domain mutations and a previously described R162Q muta-

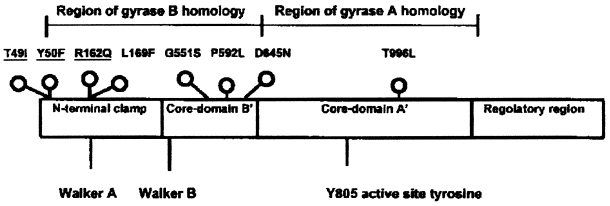

Fig. 4. Schematic linear presentation of human topoisomerase II α showing the N-terminal ATP-operated clamp which is believed to capture the transfer-DNA (T-DNA) segment upon ATP binding, the catalytic core-domain, which is involved in the cleavage and religation of the gate-DNA (G-DNA) segment and the hyper variable region. The Walker A and Walker B ATP binding motives and the Y805 active site tyrosine are also shown. The L169F, G551S, P592L, D645N, and T996L mutations are all identified in the present study while the underlined T49I, Y50F, and R162Q mutations were previously identified in mammalian cell lines selected for bisdioxopiperazine resistance [18,19,22].

tion [18] are similar. In addition, no substantial resistance towards ICRF-187-mediated inhibition of decatenation is observed in vitro with purified core-domain mutant proteins (Fig. 3A), which was also the case with purified R162Q protein [18]. Since the cellular bisdioxopiperazine resistance conferred by these mutations is not caused by altered decatenation activity in the presence of bisdioxopiperazines, the activity of these mutant proteins must be changed in other ways.

Among the four core-domain mutations analyzed, G551S has the highest in vivo bisdioxopiperazine resistance. Alignment of human topoisomerase IIα with 44 other type 2 topoisomerases of both eukaryotic and prokaryotic origin [22] shows a high level of conservation of the residues surrounding human topoisomerase IIα G551 in general. The residue homologous to human topoisomerase IIα G551 is conserved in 17 of 17 other eukaryotic type 2 topoisomerases while 0 of 29 prokaryotic topoisomerases display glycine at the homologous position [22]. It is thus interesting that eukaryotic type 2 topoisomerases which are sensitive to bisdioxopiperazine compounds all have glycine at this position while this is never the case with prokaryotic type 2 topoisomerases which are insensitive [11]. It has been demonstrated that a core-domain fragment of *Drosophila* topoisomerase II lacking the N-terminal 406 amino acids as well as the C-terminal 240 amino acids can form a closed clamp on DNA in the presence of ICRF-159 suggesting the existence of a B′B′ clamp in type II topoisomerases [12]. Another biochemical study also suggests that residues in the B′ region (residue 409–681) of yeast topoisomerase II are involved in mediating monomer–monomer interactions [13]. In the Berger and Wang structure [1] of yeast topoisomerase II, residues L551, L557, and Y602 which are homologous to L565, L570, and Y612 in human topoisomerase IIα play an important role mediating B′B′ interactions between the monomers [1], and alignment analysis shows that only conservative substitutions are allowed at these positions [22]. Further, in the structure of yeast topoisomerase II the residue homologous to human topoisomerase IIα G551, which is G537 is in close proximity to both L551 and L557 indicating that this residue may be important for the stability of the B′B′ clamp. The high level of homology in this protein region suggests that this may also be the case in human topoisomerase IIα. If so, the G551S mutation could affect the stability of the B′B′ interaction.

We have previously presented data, which strongly suggests a model, where bisdioxopiperazine compounds elicit their cytotoxic effect primarily through the stabilization of human topoisomerase IIα closed clamp complexes on DNA in vivo [10]. One consequence of this model is that cellular bisdioxopiperazine sensitivity is expected to correlate positively to the level of catalytic activity in cells expressing human topoisomerase IIα which has been shown experimentally [9]. Therefore, any mutation causing reduced catalytic activity will confer cellular bisdioxopiperazine resistance per se. Another important consequence of this model is that mutations which destabilizes the B′B′ clamp could also result in cellular bisdioxopiperazine resistance without affecting the catalytic sensitivity of topoisomerase II in decatenation assay. It is then possible that the G551S mutation identified in the present study causes cellular resistance via the latter mechanism.

The P592L mutation also maps to the B′ part of human topoisomerase IIα but this mutation falls within a region of less conserved homology than the L169F and G551S mutations and the P592 residue is not conserved among 18 eukaryotic type 2 topoisomerases aligned in [22]. In the Berger and Wang structure the homologous yeast residue P582 proximates Y602 which is also involved in mediating B′B′ dimer interactions. It is then possible that the P592L mutation affects the stability of the B′B′ clamp causing cellular bisdioxopiperazine resistance as described above, although the P592L protein has not been characterized in vitro.

The D645N mutation also falls within a region of limited sequence homology to other type 2 topoisomerases, however the D645 residue is well conserved among 18 eukaryotic type 2 topoisomerases aligned in [22]. The homologous residue in yeast topoisomerase II D635 is not resolved in the Berger and Wang structure and is probably part of the linker region which connects the B′ and A′ part of the protein [1]. Clearly, the function of this linker region must be important for protein function which is also suggested by the fact that the D645N protein displays reduced decatenation activity (Fig. 2). It is possible that this reduction in enzymatic activity is sufficient to cause bisdioxopiperazine resistance. Alternatively, the D645N mutation may affect the stability of the closed clamp leading to resistance as described above.

The T996L mutation maps to a region of human topoisomerase II α which has only limited sequence homology to other eukaryotic type 2 topoisomerases [22]. Also, this mutation maps to the gyr A homology part of the core-domain and is localized very distant to parts in the three dimensional protein structure involved in forming the N-terminal clamp(s) [1]. Instead this mutation maps to the vicinity of the C-terminal dimerization region of the protein [1,24]. According to the two gate mechanism of strand passage [25,26], the G-segment exits through the N-terminal gate which makes it difficult to explain how the T996L mutation can affect bisdioxopiperazine action. It is also noteworthy that the T996L mutation results in a drug resistance profile very similar to the G551S mutation even though these mutations map to very different parts of the protein (Figs. 1 and 3).

In summary we conclude that cellular bisdioxopiperazine resistance can result from at least two classes of mutations; (i) mutations that render the protein non-responsive to these drugs (Y50F and L169F) and (ii) mutations which do not affect the catalytic sensitivity towards these drugs in decatenation (R162Q, G551S, D645N and T996L). In addition, our

results indicate that different protein domains are involved in mediating the effect of the bisdioxopiperazine compounds.

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