0026-895X/03/6305-1159–1168$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:1159–1168, 2003

Vol. 63, No. 5
2027/1060489
Printed in U.S.A.

Probing the Role of Linker Substituents in Bisdioxopiperazine Analogs for Activity against Wild-Type and Mutant Human Topoisomerase IIα

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Received July 24, 2002; accepted February 6, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The bisdioxopiperazines are catalytic inhibitors of eukaryotic type II DNA topoisomerases capable of trapping these enzymes as a salt-stable closed-clamp complex on circular DNA. The various bisdioxopiperazine analogs differ from each other because of structural differences in the linker connecting the two dioxopiperazine rings. Although the composition of this linker region has been found to be important for potency, the structural basis for this is largely unknown. To elucidate the role of the linker region in drug action, we have analyzed the effect of different linker substituents in otherwise identical analogs by studying their interaction with wild-type and mutant human topoisomerase IIα. Two mutations, L169I and R162Q, displayed differential sensitivity toward closely related analogs, suggesting that the linker region in these compounds plays a highly specific role in protein drug interaction. The finding that the L169I mutation, which probably represents a subtle structural change, was sufficient to confer resistance further emphases the importance of this region of the protein for bisdioxopiperazine inhibition of topoisomerase II. Comparing the sensitivity profiles of different bisdioxopiperazines against wild-type and mutant proteins with that of mitindomide, we observed a spectrum of sensitivity closely resembling that of ICRF-154, a bisdioxopiperazine with no linker substituents. We discuss the implications of these observations for the understanding of the mechanism of bisdioxopiperazine action on topoisomerase II.

Topoisomerase II constitutes a family of nuclear enzymes essential to all living cells (Wang, 1996). These enzymes are capable of transferring one DNA double helix through a transient break in another DNA double helix (Roca and Wang, 1992, 1994). Type II topoisomerases play important roles in DNA metabolic processes, in which they are involved in DNA replication, transcription, chromosome condensation and de-condensation, DNA recombination, and untangling of replicated chromosomes (Nitiss, 1994; Wang, 1996). Topoisomerase II is also the cellular target for a number of widely used anticancer agents currently in clinical use, such as the anthracyclines (daunorubicin and doxorubicin), the epipodophyllotoxins (etoposide and teniposide), and the aminoacridines [4′-(9-acridinylamino)-3′-methoxymethanesulfonanilide]. These agents stimulate the topoisomerase II-cleavable complex, which is a transient configuration of topoisomerase II on DNA in which topoisomerase II is covalently attached to DNA. This causes the accumulation of cytotoxic nonreversible DNA double-strand breaks generated by the processing of such complexes by DNA metabolic processes (Liu, 1989; Chen and Liu, 1994).

Bisdioxopiperazines are anticancer agents capable of stabilizing topoisomerase II as a salt-stable closed-clamp on circular DNA, thereby preventing enzymatic turnover (Roca et al., 1994; Andoh and Ishida, 1998; Morris et al., 2000). Therefore, these compounds have been classified as catalytic topoisomerase II inhibitors. However, recent data suggest that the closed-clamp configuration of topoisomerase II on DNA may act as a new kind of noncovalent poison. Thus, it has been found that the expression of bisdioxopiperazine-sensitive human topoisomerase IIα in yeast cells also expressing resistant yeast topoisomerase II confers dominant sensitivity to ICRF-187 and ICRF-193, suggesting that these

This work was supported by a European Community Marie Curie Fellowship (HPMF-CT-2000-00501) (to A.R.-C.); the Toyota Foundation, Danish Cancer Society, H:S Research Council (to T.K.S.); The Danielsen Foundation, The National Cancer Institute grant CA52814 (to J.L.N.) and core grant CA21765 (to J.L.N.), and The American Lebanese Syrian Associated Charities (to J.L.N.).

ABBREVIATIONS: ICRF-187, (+)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane; ICRF-154, 4,4′-(1,2-ethanediyl)-bis(2,6-piperazinedione); ICRF-193, meso-4,4′-(2,3-butanediyl)-bis(2,6-piperazinedione); ICRF-202, 2,6-piperazinedione, 4,4′-(1-ethyl-2-methyl-1,2-ethanediyl)bis-, (*R**,*S**)-(.+ −.)-; AMP-PNP, adenylylimidodiphosphate; BSA, bovine serum albumin; kDNA, kinetoplast DNA; SPR, surface plasmon resonance.

compounds act as topoisomerase II poisons (van Hille and Hill, 1998; Jensen et al., 2000a). It has further been demonstrated that ICRF-193 is capable of stimulating sequence-specific DNA cleavage using purified human topoisomerase IIβ in vitro, suggesting that it is a true topoisomerase II poison (Huang et al., 2001). However, in this study, the cleavage activity of ICRF-193 was dependent totally on the agent used to trap the covalent complex, indicating that ICRF-193–stabilized complexes are different in nature from those stabilized by the classic topoisomerase II poisons. A clear difference in the mode of action between the bisdioxopiperazine compounds and the classic poisons also has been demonstrated in vivo, because the bisdioxopiperazines efficiently antagonize DNA damage and cytotoxicity induced by classic topoisomerase II poisons (Jensen and Sehested, 1997). Furthermore, the bisdioxopiperazine analog ICRF-187 can antagonize the cytotoxicity of etoposide in the mouse (Holm et al., 1996, 1998), and ICRF-187 is capable of protecting against necrosis induced by subcutaneous doxorubicin and daunorubicin injection in mice (Langer et al., 2000). These findings point to catalytic inhibition as the principal mode of action of the bisdioxopiperazine compounds in vivo.

The molecular interaction between the bisdioxopiperazine compounds and topoisomerase II has been the course of some debate. Two independent studies have demonstrated inhibition of the ATPase activity of N-terminal topoisomerase II fragments, suggesting that the N-terminal ATP-operated clamp is the main target of these compounds (Olland and Wang, 1999; Hu et al., 2002). On the other hand, a core fragment of *Drosophila melanogaster* topoisomerase II lacking the N-terminal clamp region was clearly capable of forming a salt-stable closed-clamp complex on DNA (Chang et al., 1998), indicating that the core region is also involved. Mutational analysis also points to the involvement of both the N-terminal and core region in the bisdioxopiperazine interaction because resistance-conferring mutations are found in both regions of the enzyme (Sehested et al., 1998; Wessel et al., 1999, 2002; Jensen et al., 2000b; Patel et al., 2000).

In the present study, to probe the interactions between the bisdioxopiperazine compounds and human topoisomerase IIα, we generated a panel of mutations within and next to the Walker A ATP binding site in human topoisomerase IIα and analyzed their effect on DNA strand passage, closed-clamp formation, and cytotoxicity in the presence of different bisdioxopiperazines (Fig. 1). An L169I mutation likely to represent only a very small structural change was sufficient to cause bisdioxopiperazine resistance in vitro and in vivo, demonstrating that bisdioxopiperazines have very specific structural demands for activity. Also, the L169I and R162Q mutations were nonresponsive toward inhibition by ICRF-154, although retaining significant sensitivity toward other bisdioxopiperazines, suggesting that the linker region plays a specific role in protein drug interaction. Furthermore, the L169I mutation is the first bisdioxopiperazine resistance-conferring mutation described that does not impair DNA strand passage at subsaturating ATP levels, suggesting that its resistance is not caused by indirect mechanisms. In summary, our results indicate that the linker region connecting the dioxopiperazine rings in different bisdioxopiperazines seems to play a highly specific role in protein drug interaction.

Materials and Methods

Yeast Strains. The hyperpermeable temperature-sensitive and RAD52-deficient yeast strain JN394t2–4 (*MATa, ura5–2, leu2, trp1, his7, ade1–2, ISE2, rad52::LEU2, top2–4*) was used in all clonogenic assays. The protease-deficient topoisomerase I-negative yeast strain JelΔTop1 (*Mat a, trp1, leu2, ura-52, pbr-1122, pep4–3,. his3::PGAL10-GAL4, TOP1::LEU2*) was used for the overexpression of wild-type and mutant human topoisomerase IIα to be purified.

Constructs. The pMJ1 vector for the expression of human topoisomerase IIα in yeast under control of the constitutive yeast topoisomerase I promoter was used for functional expression of wild-type and mutant human topoisomerase IIα in yeast cells (clonogenic assay) and has been described by Hsiung et al (1996). The vector YepWOB6, which was used for the overexpression of wild-type and mutant human topoisomerase IIα under control of a galactose-inducible GAL1 promoter, has been described in Wasserman et al. (1993). The first 28 amino acids of human topoisomerase IIα expressed from this construct are substituted for the first five residues of yeast topoisomerase II.

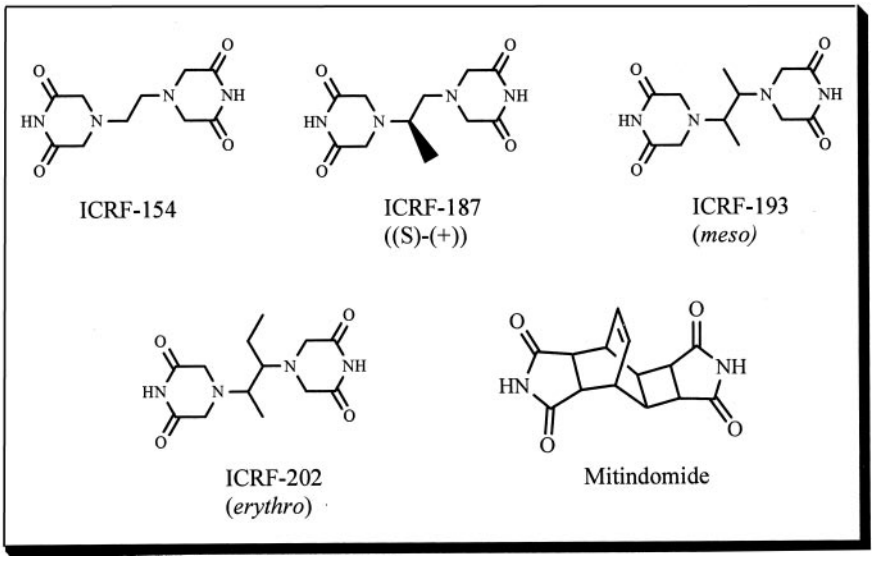

Fig. 1. The structural formula of the compounds used.

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out using a quick-change kit (Stratagene, La Jolla, CA) as described by Sehested et al. (1998). Primers used to construct the R162Q and L169F mutations are described by Wessel et al. (1999) and Jensen et al. (2000b). Other primers used in site-directed mutagenesis are depicted in Table 1. When mutations were introduced into the YepWOB6 vector, the numbering of these mutations refers to the intact human topoisomerase IIα sequence and does not reflect the distance to the ATG (start) codon in this vector (Wasserman et al., 1993).

Yeast Transformation. Yeast cells were transformed using a lithium acetate protocol with single-stranded salmon sperm DNA as the carrier, in accordance with standard procedures.

Drugs. ICRF-187 (Cardioxane, Chiron Group, the Netherlands) was dissolved in sterile water and kept at −80°C. ICRF-154 and ICRF-193 (BIOMOL Research Laboratories, Plymouth Meeting, PA) were dissolved in dimethyl sulfoxide and kept at −80°C. ICRF-202 and mitindomide were generous gifts from The Drug Synthesis Chemistry Branch, Development Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD) and were dissolved in dimethyl sulfoxide and kept at −80°C.

Preparation of ^{3}H-Labeled kDNA. Tritium-labeled kDNA was isolated from *Crithidia fasciculata* as described previously (Sahai and Kaplan, 1986).

Purification of Human Topoisomerase IIα. The purification of wild-type and mutant human topoisomerase IIα from overexpressing yeast cells was carried out as described previously (Worland and Wang, 1989) with modifications described previously (Sehested et al., 1998).

Clonogenic Assay. Clonogenic assay was performed as described previously (Sehested et al., 1998). Briefly, an overnight culture of cells in log phase was diluted to 2×10^6 cells/ml in prewarmed medium containing yeast extract, dextrose, and peptone, and 3-ml cultures were exposed to different concentrations of drug at 34°C. Samples removed after 0 and 24 h were diluted 0 to 10^4 times in distilled sterile water. Next, 200 μl was transferred to plates containing synthetic medium lacking uracil, which were incubated for 7 days at 25°C before counting. Finally, relative cell survival after 24 h compared with results at 0 h was calculated for all conditions used. All experiments were performed at least twice with identical results.

Decatenation Assay. Topoisomerase II catalytic activity was measured by kDNA decatenation assay as described previously (Jensen et al., 2002) with minor modifications. Briefly, 200 ng of ^{3}H-labeled kDNA isolated from *C. fasciculata* was incubated with increasing concentrations of drug in 20 μl of reaction buffer containing 10 mM Tris-HCl, pH 7.7, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 15 μg/ml BSA, and 1 mM ATP using 3 U of purified wild-type or mutant topoisomerase IIα for 20 min at 37°C (where 1 U of activity is defined as the amount of enzyme required for complete decatenation in the absence of drug). After the addition of 5× stop buffer (5% Sarkosyl, 0.0025% bromphenol blue, and 50% glycerol), unprocessed kDNA network and decatenated DNA circles were separated by filtering, and the amount of unprocessed kDNA in each reaction was determined by scintillation counting. The resulting values were finally normalized, which is when 100% inhibition corresponds to the radioactivity retained on the filter when no enzyme is added.

TABLE 1
Primers used for site-directed mutagenesis

Primers	Primer Sequence
L169I-SN	5′-GGC TAT GGA GCC AAA ATC TGT AAC ATA TTC-3′
L169I-ASN	5′-GAA TAT GTT ACA GAT TTT GGC TCC ATA GCG-3′
L169M-SN	5′-GGC TAT GGA GCC AAA ATG TGT AAC ATA TTC-3′
L169M-ASN	5′-GAA TAT GTT ACA CAT TTT GGC TCC ATA GCG-3′
L169A-SN	5′-GGC TAT GGA GCC AAA GCG TGT AAC ATA TTC-3′
L169A-ASN	5′-GAA TAT GTT ACA CGC TTT GGC TCC ATA GCG-3′
L169W-SN	5′-GGC TAT GGA GCC AAA TGG TGT AAC ATA TTC-3′
L169W-ASN	5′-GAA TAT GTT ACA CCA TTT GGC TCC ATA GCG-3′
L169V-SN	5′-GGC TAT GGA GCC AAA GTG TGT AAC ATA TTC-3′
L169V-ASN	5′-GAA TAT GTT ACA CAC TTT GGC TCC ATA GCG-3′
R162E-SN	5′-GTG ACA GGT GGT GAA AAT GGC TAT GGA GCC-3′
R162E-ASN	5′-GGC TCC ATA GCC ATT TTC ACC ACC TGT CAC-3′
R162K-SN	5′-GTG ACA GGT GGT AAG AAT GGC TAT GGA GCC-3′
R162K-ASN	5′-GGC TCC ATA GCC ATT CTT ACC ACC TGT CAC-3′

SN, sense; ASN, antisense.

Surface Plasmon Resonance Assay. To analyze quantitatively the stability of the closed-clamp complex intermediate form of wild-type and mutant human topoisomerase IIα on circular DNA formed in the presence of different bisdioxopiperazine analogs, we used a Biacore 3000 (Biacore Inc., Uppsala, Sweden)-based surface plasmon resonance (SPR) assay (Renodon-Cornière et al., 2002). Briefly, a 5-kilobase pair supercoiled circular DNA molecule carrying eight successive peptide nucleic acid-linked biotin labels at one known position (pGeneGrip biotin blank vector; Gene Therapy Systems Inc., San Diego, CA) was bound to streptavidin-coated sensor chips (Sensor Chip SA; Biacore). Next, 10 nM wild-type or mutant human topoisomerase IIα in 10 mM Tris-HCl, pH 7.7, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 30 μg/ml BSA, and 1 mM ATP was allowed to bind DNA on the sensor chip at a flow rate of 30 μl/min at 20°C. Association was then followed for 2 min. Next, dissociation was followed for 1 or 2 min by injecting running buffer (10 mM Tris-HCl, pH 7.6, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 30 μg/ml BSA) at the same flow rate. The amount of salt-stable complex was then determined as the percentage of protein bound to DNA at the end of the association phase that was resistant to high salt (1 M KCl)-washing conditions during 1 min. Another flow cell containing no DNA was used to correct for refractive index changes and nonspecific binding of topoisomerase IIα to the sensor chip.

Results

Site-Directed Mutagenesis. We have identified previously two mutations in human topoisomerase II, R162Q (Wessel et al., 1999) and L169F (Jensen et al., 2000b; Patel et al., 2000), that confer resistance to ICRF-193 and ICRF-187 when functionally expressed in JN394t2–4 yeast cells. To investigate the role of these residues in the bisdioxopiperazine protein interaction, we examined the effect of other amino-acid substitutions at these positions. The R162Q mutation represents a loss of charge. To test the importance of charge at residue 162 for bisdioxopiperazine sensitivity, we constructed R162K and R162E mutations representing no loss of charge and loss of two positive charges, respectively. To investigate in more detail the role of residue 169 in bisdioxopiperazine action, additional site-directed mutagenesis was performed by constructing L169A, L169V, L169I, L169M, and L169W mutations. These substitutions represent, together with the L169F, a listed increase in bulk when going from alanine up to tryptophan.

Complementation Analysis. The mutations indicated above were introduced into pMJ1 and transformed to drug-permeable JN394t2–4 cells having a temperature-sensitive endogenous topoisomerase (as described under *Materials and Methods*), and the ability of the mutant human topoisomerase IIα alleles to support growth at a nonpermissive temperature (34°C) was assessed. Cells carrying pMJ1 with either the R162E or L169W mutations failed to grow at the nonpermissive temperature, suggesting that these mutations substantially affect topoisomerase II catalytic activity. Cells expressing human topoisomerase IIα carrying the mutations R162Q, R162K, L169V, L169I, L169F, or L169M all grew at 34°C, with growth rates similar to those of cells expressing

the wild-type human enzyme. Cells expressing the L169A protein grew at a somewhat slower rate than did wild-type cells (data not shown). These results suggest that the R162Q, R162K, L169V, L169F, L169I, and L169M single amino-acid substitutions have no major impact on topoisomerase II function when expressed in yeast cells.

Clonogenic Sensitivity of Yeast Cells Expressing Wild-Type or Mutant Human Topoisomerase IIα toward Different Bisdioxopiperazine Analogs. To test the effect of these single amino-acid substitutions on bisdioxopiperazine sensitivity in vivo, we exposed the transformed cells to different bisdioxopiperazines at the nonpermissive temperature with use of a 24-h clonogenic assay described by Sehested et al. (1998). Cells expressing the L169A, L169V, L169F, and L169M mutants were all highly resistant to ICRF-154, ICRF-187, and ICRF-193. (Data for cells expressing L169F in which the sensitivity against ICRF-202 was also assessed are illustrated in Fig. 2, A through D; data for the L169A, L169V, and L169M mutants are not shown). Cells expressing the R162K mutation were considerably more sensitive toward all four analogs tested than were cells expressing the R162Q mutation (Fig. 2, A through D). These observations suggest that a positive charge at residue 162 may be important for bisdioxopiperazine sensitivity. This is most pronounced for ICRF-193, in which nearly wild-type sensitivity is observed (Fig. 2C). Interestingly, cells expressing the R162Q and L169I mutations displayed differential sensitivity toward different bisdioxopiperazine analogs. These cells were completely resistant toward ICRF-154 (Fig. 2A) while retaining a significant amount of sensitivity toward ICRF-193 (Fig. 2C) and ICRF-202 (Fig. 2D). In Fig. 2B, R162Q- and L169I-expressing cells seem to display some sensitivity toward ICRF-187, but the effect of the drug is limited because of the ICRF-187 concentration range used. We therefore performed additional clonogenic assays on R162Q- and L169I-expressing cells at very high concentrations of ICRF-187 and ICRF-154 (Fig. 2E). At these concentrations, it is evident that R162Q- and L169I-expressing cells are completely resistant toward ICRF-154 while retaining some sensitivity toward ICRF-187. In summary R162Q- and L169I-expressing cells are nonresponsive toward ICRF-154 while displaying some sensitivity toward the other bisdioxopiperazine analogs tested. To analyze the effects of the R162Q, R162K, L169F, and L169I mutations in vitro, the corresponding mutant proteins were purified and characterized.

Purification and Basal Catalytic Activity of Wild-Type and Mutant Human Topoisomerase IIα Proteins. Human topoisomerase II proteins were overexpressed in JELΔtop1 yeast cells from the galactose-inducible expression vector YepWOB6 under control of the GAL1 promoter. All of the purified human topoisomerase IIα proteins expressed from the YepWOB6 vector have the first 28 residues of human topoisomerase IIα replaced with the first five residues derived from yeast topoisomerase II (Wasserman et al., 1993). Because the effect of the studied single amino-acid substitutions on bisdioxopiperazine sensitivity is much more dramatic than the effect of substituting the 28 extreme N-terminal residues with the yeast sequence (which causes a 2-fold resistance toward ICRF-187 in decatenation assay), we can justify our use of this overexpression system, which has been used previously to probe the interaction of ICRF-193 with wild-type and mutant human topoisomerase IIα (Patel et al., 2000). Before assessing the effect of the different drugs on wild-type and mutant proteins, the specific activity expressed as U of activity/ng protein, in which 1 U is defined as the activity required for the complete decatenation of 200 ng of kDNA at 37°C in 20 min, was determined for the five purified proteins—wild-type, R162Q, R162K, L169I, and L169F—in three independent decatenation experiments. No significant difference in specific activity was observed between the five purified proteins ($n = 3$; Table 2), although the L169F protein tended to have a slightly increased specific activity.

Catalytic Inhibition of Purified Wild-Type and Mutant Human Topoisomerase IIα by Different Bisdioxopiperazine Analogs. We tested the ability of the four bisdioxopiperazines ICRF-154, ICRF-187, ICRF-193, and ICRF-202 (Fig. 1) to inhibit the strand-passage activity of wild-type and mutant human topoisomerase IIα using the decatenation assay (Fig. 3). In this strand-passage assay, the L169F protein was not inhibited by any of the analogs tested (Fig. 3, A through D), explaining the lack of bisdioxopiperazine sensitivity seen with L169F-expressing cells. The decatenation activity of the R162Q and L169I proteins was partially sensitive toward ICRF-187 (Fig. 3B), ICRF-193 (Fig. 3C), and ICRF-202 (Fig. 3D) while being completely resistant toward ICRF-154 (Fig. 3A), thus confirming the differential sensitivity pattern observed in clonogenic assay. Finally, the catalytic sensitivity of the R162K protein was closest to that of the wild-type protein for all analogs tested (Fig. 3, A through D), which also correlates well with the clonogenic data in which R162K-expressing cells can be killed by all four analogs tested. Generally, the inhibition of catalytic (strand-passage) activity in vitro correlates well with the clonogenic-sensitivity patterns for all combinations of mutations and bisdioxopiperazine analogs tested. However, there are some minor discrepancies from this general picture. Whereas for ICRF-154 and ICRF-193 the sensitivity pattern in decatenation assay correlates well with the clonogenic data presented in the previous section, the situation is less clear for ICRF-202 and especially for ICRF-187. Although ICRF-193 concentrations capable of inhibiting the decatenation activity of R162Q and L169I mutant proteins by 25 to 50% were also capable of killing cells expressing these mutant proteins, this was not the case for ICRF-187, in which almost no cytotoxicity is observed at concentrations significantly inhibiting the catalytic activity of purified R162Q and L169I proteins. The reason for this discrepancy remains unclear, but it may reflect differences in cellular uptake between the different bisdioxopiperazines. Drug accumulation studies using radioactively labeled compounds would be required to test this hypothesis. We do not believe that this difference between ICRF-187 and ICRF-193 is related to the different composition of the linker substituents in these compounds. However, these observations do not challenge the finding that R162Q and L169I both respond to ICRF-187, ICRF-193, and ICRF-202 but not to ICRF-154, which is the only bisdioxopiperazine analog with no linker substituents.

Mitindomide Interacts with Human Topoisomerase IIα at the Bisdioxopiperazine Interaction Site and Resembles ICRF-154 in Its Sensitivity Profile. We also assessed the effect of the "bisdioxopiperazine-like" drug mitindomide in the decatenation assay. All mutations tested, except for R162K, were highly cross-resistant toward mitin-

domide (Fig. 4). This demonstrates that mitindomide and the bisdioxopiperazines do have a common mechanism of action in the inhibition of topoisomerase II. We believe that mitindomide and the bisdioxopiperazines share the same interaction site on human topoisomerase IIα, including the Walker A nucleotide-binding site. It is interesting that the sensitivity

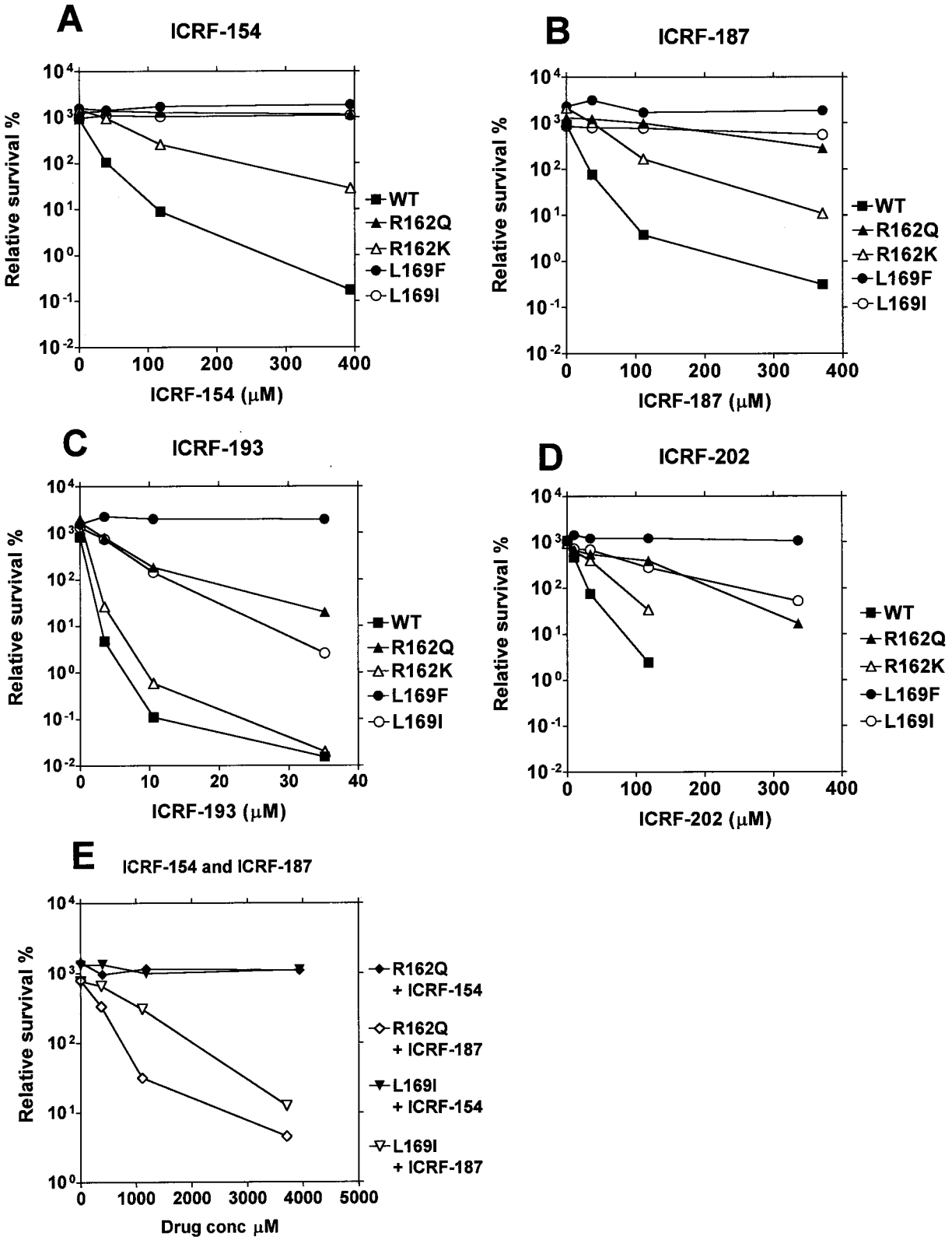

Fig. 2. Clonogenic sensitivity of JN394t2–4 cells conditionally expressing wild-type, R162K, R162Q, L169F, and L169I human topoisomerase IIα toward ICRF-154, ICRF-187, ICRF-193, and ICRF-202. Clonogenic assay was performed as described under *Materials and Methods*. Drug treatments are depicted on the figure. Figure 2 is representative of at least two independent experiments performed with similar results.

profile of the mutant panel toward mitindomide is almost identical with that of ICRF-154 (with the exception that mitindomide is capable of causing a slight inhibition of L169I catalytic activity) but is dissimilar to that of ICRF-187. This result indicates that mitindomide represents more closely the active conformer of ICRF-154 than that of ICRF-187. We do not present clonogenic data for mitindomide because the potency of this drug is too low to induce a cytotoxic response in the yeast cells.

Strand-Passage Activity of Mutant Topoisomerase IIα Proteins at Subsaturating ATP Levels. We next determined the ATP concentration resulting in half-maximal decatenation activity for wild-type and mutant human topoisomerase IIα proteins. The L169I mutant protein had exactly the same ATP requirement as the wild-type protein because half-maximal decatenation was achieved at 30 μM ATP. The other mutant proteins included in this study all displayed an increased requirement for ATP, because the ATP concentration resulting in half-maximal decatenation activity was approximately 5 times higher than for the wild-type and L169I proteins. All bisdioxopiperazine resistance-conferring mutations characterized up to now have displayed increased ATP requirements (Wessel et al., 1999, 2002; Jensen et al., 2000b; Patel et al., 2000; present study), indicating that these mutations decrease the protein's affinity for ATP. This result is not surprising because these mutations map to the nucleotide-binding Walker A motif. The finding that the L169I mutation has the same affinity for ATP as does the wild-type protein may be explained by the minor structural perturbation that the L169I mutation is likely to represent, which apparently does not affect the interaction with ATP. Comparing the ATP dependencies of the four mutant proteins with the bisdioxopiperazine resistance level, no correlation can be established because the R162K protein, which is only slightly resistant to bisdioxopiperazine compounds, displays exactly the same ATP dependence as the L169F mutation, which is completely insensitive to all analogs tested. The general conclusion of these ATP-affinity comparisons is that decreased ATP affinity and bisdioxopipera-

TABLE 2

Specific activity of purified wild-type and mutant human topoisomerase IIα

Values shown are mean ± S.D. of three independent determinations.

Amino-Acid Substitution	Specific Activity
	U activity/ng
None (wild-type)	0.17 ± 0.07
R162Q	0.20 ± 0.08
R162K	0.15 ± 0.07
L169F	0.40 ± 0.24
L169I	0.14 ± 0.06

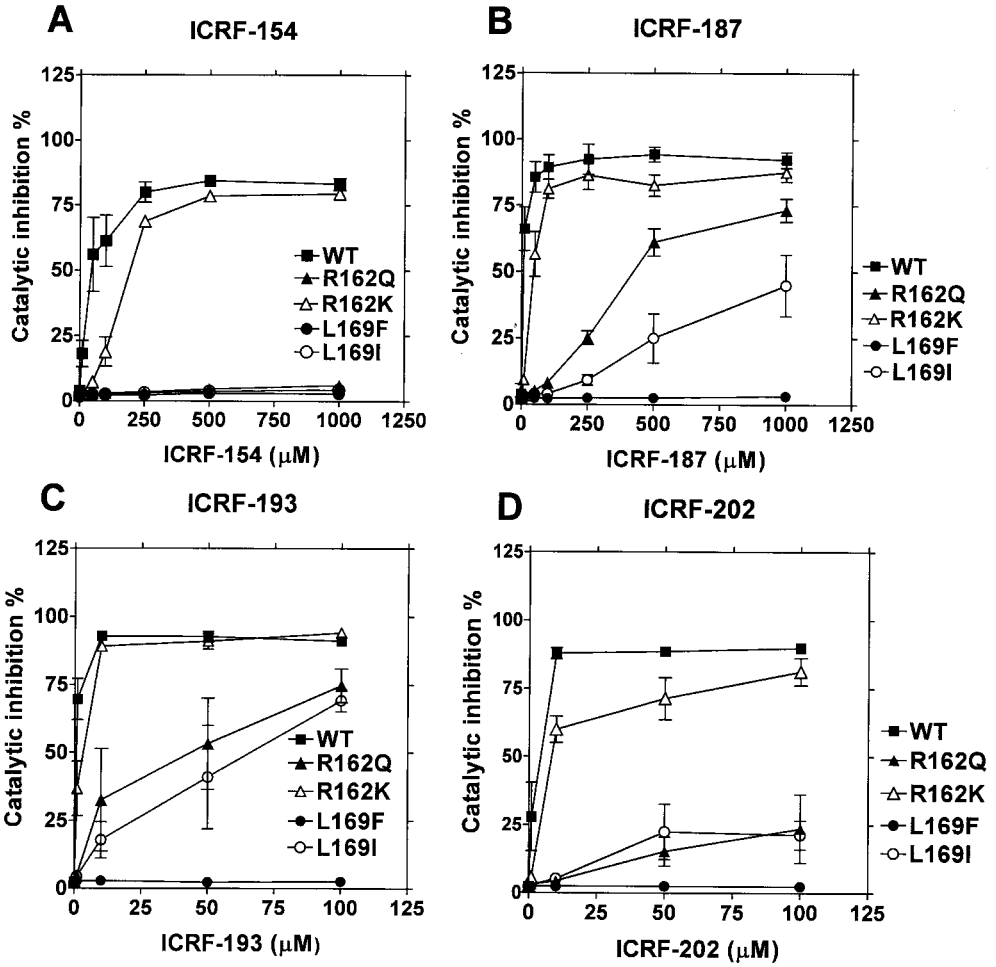

Fig. 3. Inhibition of the strand-passage activities of wild-type, R162K, R162Q, L169F, and L169I human topoisomerase IIα protein by ICRF-154, ICRF-187, ICRF-193, and ICRF-202 as determined in decatenation assay. Decatenation assay was carried out as described under *Materials and Methods*. Drug treatments are depicted on the figure. Error bars represent S.E.M. of two to six independent experiments.

zine resistance do clearly not correlate, meaning that bisdioxopiperazine resistance is not directly linked to decreased ATP affinity. This result indicates that the ATP and bisdioxopiperazine interaction sites are not identical, although they clearly overlap.

Determining the Stability of the Closed-Clamp Complex Intermediate Form of Wild-Type and Mutant Human Topoisomerase IIα on DNA. We next assessed the stability of the closed-clamp complex intermediate induced by ICRF-154, ICRF-187, and ICRF-193 using either the wild-type or the four mutant proteins. In this assay, the amount of DNA-bound topoisomerase II resistant to high salt (1 M KCl) was used as a measure of the stability of the closed-clamp intermediate (Renodon-Cornière et al., 2002). Before assessing the effect of different bisdioxopiperazine compounds, we first determined the level of the salt-stable complex formation induced by 1 mM of the nonhydrolyzable ATP analog AMP-PNP, which is known to lock type II topoisomerases as a closed clamp on circular DNA (Roca and Wang, 1992). Whereas the AMP-PNP–induced closed-clamp levels of the four mutant proteins were reduced compared with the wild-type protein, the four mutant proteins displayed only minor differences in their basal bisdioxopiperazine-independent closed-clamp stability (Table 3).

We next proceeded to determine bisdioxopiperazine-induced closed-clamp stability. Figure 6 depicts the results of these experiments. For the L169F, L169I, and R162Q proteins, an overall correlation between the level of salt-stable protein complex and inhibition of decatenation activity/clonogenic sensitivity exists. These mutants all display reduced closed-clamp formation and drug-resistant decatenation activity (compare Figs. 3 and 6). As in the other assays described above, the L169F mutant protein was totally insensitive to the action of all three analogs tested (ICRF-154, ICRF-187, and ICRF-193) and formed no detectable salt-stable complex on the DNA, supporting the notion that this mutation abolishes bisdioxopiperazine inhibition of the enzyme and possibly drug binding. We believe that the finding that the L169F mutant protein has a slightly elevated (not significant) basal catalytic activity is insufficient to explain the complete lack of response of all bisdioxopiperazine analogs in the three assays used. For the R162Q and L169I mutations, a correlation between the level of salt-stable complex and resistance toward ICRF-187– and ICRF-193–mediated inhibition of catalytic activity can be seen (compare Fig. 3, B and C, with Fig. 6, B and C) Here, a low level of salt-stable complex (L169I) correlates with a high level of resistance. Concerning ICRF-154, such a correlation cannot be established because the resistance toward catalytic inhibition by this drug is complete (there is no residual ICRF-154–mediated catalytic inhibition of R162Q and L169I enzymatic activity; Fig. 3A). The R162K mutation has the highest

TABLE 3
Closed-clamp activity of wild-type and mutant human topoisomerase IIα in the presence of 1 mM AMP-PNP
Values shown are mean ± S.D.

Amino-Acid Substitution	Closed Clamp Induced by 1 mM AMP-PNP	Reference
	%	
None (wild-type)	40.0 ± 2.0(n=5)	Renodon-Cornière et al. (2002)
R162Q	26.0 ± 0.5(n=2)	Present study
R162K	21.5 ± 0.5(n=2)	Present study
L169F	22.5 ± 4.0(n=3)	Renodon-Cornière et al. (2002)
L169I	24.5 ± 0.5(n=2)	Present study

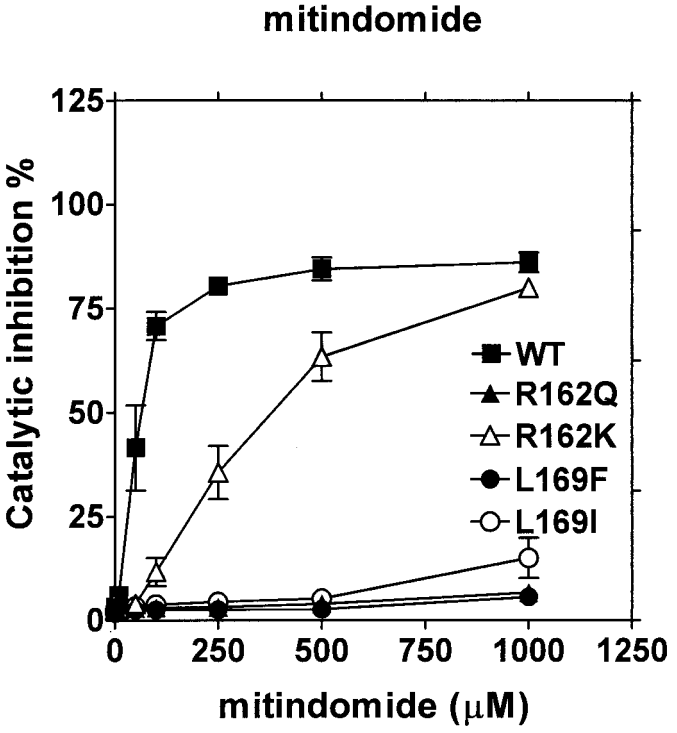

Fig. 4. Inhibition of the strand-passage activities of wild-type, R162K, R162Q, L169F, and L169I human topoisomerase IIα protein by mitindomide as determined in decatenation assay. Decatenation assay was carried out as described under *Materials and Methods*. Error bars represent S.E.M. of two to six independent experiments.

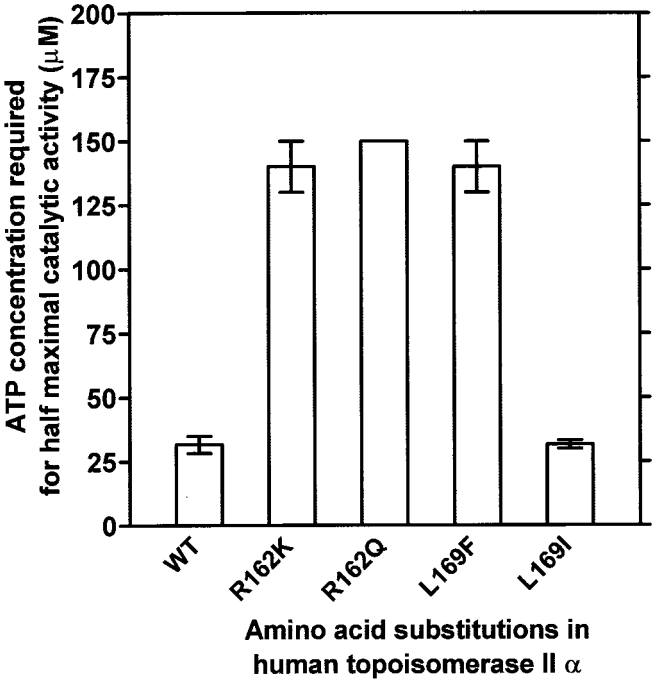

Fig. 5. Determination of the ATP concentration required for 50% DNA strand-passage activity by wild-type, R162K, R162Q, L169F, and L169I human topoisomerase IIα. Decatenation was carried out as in Fig. 3 except for the lowered ATP concentrations. Error bars represent S.E.M. of three independent experiments.

level of salt-stable complex and also displays the lowest level of drug resistance in decatenation assay (compare Figs. 3 and 6), confirming the pattern seen with the other mutants. What does not fit into the general correlation is that the R162K mutation displays a slightly increased level of salt-stable complex formation (compared with wild-type protein) while being slightly resistant in decatenation and clonogenic assay. This result suggests that qualitative differences may exist between AMP-PNP–stabilized and bisdioxopiperazine-stabilized closed-clamp complexes, as suggested by Renodon-Cornière et al. (2002).

Discussion

The exact site(s) of interaction of bisdioxopiperazines and eukaryotic topoisomerase II has not been determined. In the experiments described here, we constructed a set of mutations that change two residues in human topoisomerase IIα, R162 and L169 (Wessel et al., 1999; Jensen et al., 2000b; Patel et al., 2000), that seem to have important roles in determining bisdioxopiperazine sensitivity. These mutations belong to a small stretch of eight amino acids **RN**G**Y**GAK**L** (overlapping with the Walker A nucleotide binding site) that include four independently isolated mutations (Wessel et al., 1999, 2002; Jensen et al., 2000b; Patel et al., 2000; J. Nitiss and A. Renodon-Cornière, unpublished observations) associated with bisdioxopiperazine resistance (the residues associated with bisdioxopiperazine resistance are in bold). To test the hypothesis that this protein region is directly involved in bisdioxopiperazine action on human topoisomerase IIα, we carried out a structure/function analysis study that includes different mutant proteins (R162Q, R162K, L169F, and L169I) as well as different bisdioxopiperazine derivatives (ICRF-154, ICRF-187, ICRF-193, and ICRF-202). The effects of these agents were examined using clonogenic assays of yeast cells functionally expressing the human enzymes, by enzyme-activity assays (decatenation assay), and by assessing the salt-stable closed-clamp intermediate form of topoisomerase II on DNA. The levels of DNA-bound topoisomerase II in the closed-clamp form were determined using a recently developed SPR assay (Renodon-Cornière et al., 2002).

For two mutant alleles of human topoisomerase IIα, R162Q and L169I, we observed differential sensitivity toward closely related bisdioxopiperazine analogs, a result consistent with this region directly participating in bisdioxopiperazine binding. Furthermore, the overall structural difference between the wild-type protein with leucine at position 169 and an isoleucine substitution (L169I) is expected to be minor. Yet this small structural difference is sufficient to cause significant bisdioxopiperazine resistance, especially

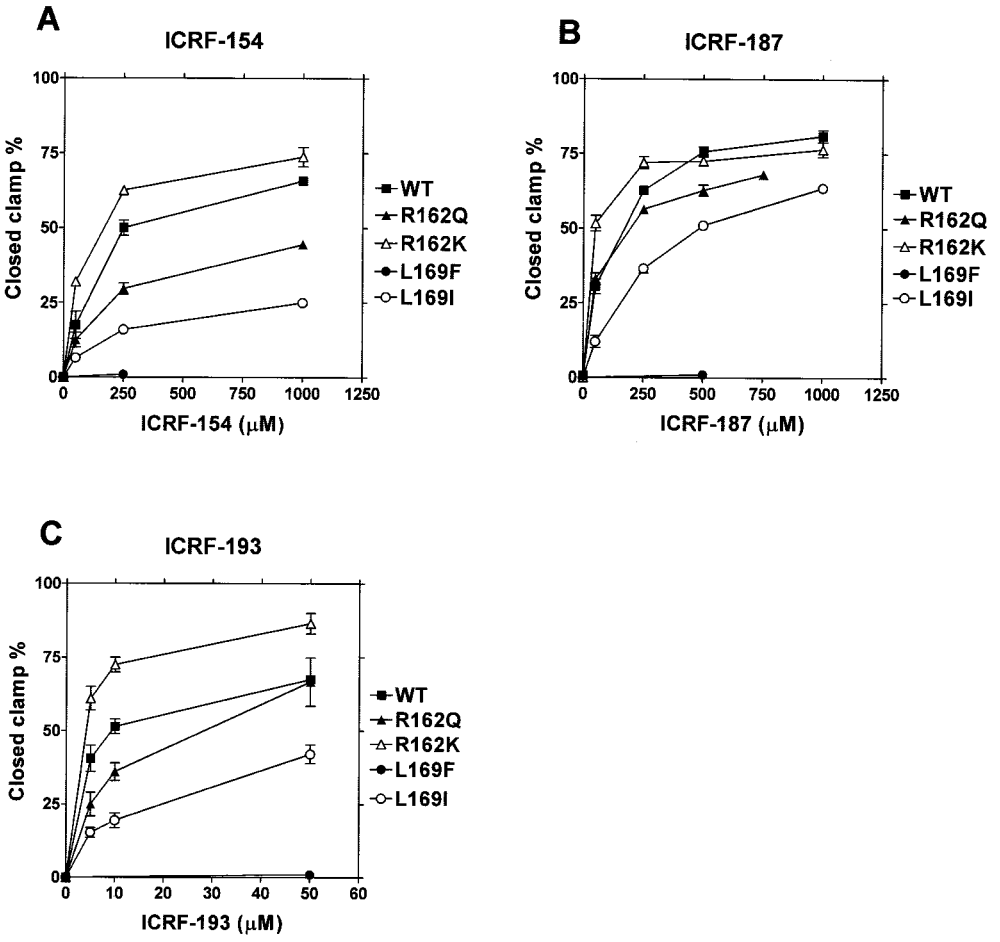

Fig. 6. Dose-dependent stabilization of wild-type, R162K, R162Q, L169F, and L169I human topoisomerase IIα salt-stable closed-clamp complex on circular DNA by ICRF-154, ICRF-187, and ICRF-193 as determined in SPR assay. SPR assays were carried out as described under *Materials and Methods*. Drug treatments are depicted on the figure. Error bars represent S.E.M. of three independent experiments.

toward ICRF-154, which also suggests that this protein region is directly involved in bisdioxopiperazine binding. The finding that the R162K mutation causes only slight resistance toward the different bisdioxopiperazines, whereas the R162Q mutation causes significant resistance toward these analogs, suggests that the positive charge at this position may be important for drug action.

Although the region defined by amino acids 162 to 169 clearly plays an important role in bisdioxopiperazine sensitivity, other work strongly suggests that other protein domains are also important. Thus, a Y50F mutation in human topoisomerase IIα completely abolishes sensitivity to bisdioxopiperazines in vivo and in vitro (Sehested at al., 1998). In addition, Chang et al. (1998) observed that a truncation mutant of topoisomerase II that completely lacks the ATPase domain could still form a stable closed clamp in the presence of bisdioxopiperazines, although at a greatly reduced level. A plausible explanation for these observations is that the bisdioxopiperazine-interacting region spans several different protein domains, including regions outside the ATP-binding domain of the enzyme.

The four mutant proteins examined in this work all displayed reduced AMP-PNP–induced closed-clamp stability compared with the wild-type protein. The ability of AMP-PNP to stabilize the closed clamp depends on several different factors, including the binding affinity of the enzyme for AMP-PNP and the degree of coupling of ATP hydrolysis to clamp reopening. Interestingly, one of the mutant proteins analyzed here, L169I, showed no reduction in enzymatic activity at subsaturating ATP levels, indicating that it has the same affinity for ATP as the wild-type protein. This result indicates that alterations in ATP use are not required for bisdioxopiperazine resistance and suggests that the coupling between ATP hydrolysis and reopening of the clamp may be impaired in the L169I mutant protein. In contrast to the results obtained with AMP-PNP, the four mutant proteins showed markedly different levels of closed-clamp stability with the different bisdioxopiperazines. This result strongly suggests that the mutations alter interactions with bisdioxopiperazines and that the observed bisdioxopiperazine resistance is caused by alterations in drug/protein interactions.

Concerning the inhibition of catalytic (strand-passage) activity and clonogenic sensitivity, the role of linker substituents in bisdioxopiperazine action is likely to be the stabilization of the protein/drug interaction. In these assays, the effect of the linker substituent-lacking compound ICRF-154 is clearly different from the other linker-containing analogs because no response toward the R162Q and L169I mutants could be detected, whereas the other analogs produced an inhibitory response, suggesting that the linker substituents are required for catalytic inhibition of the R162Q and L169I mutant proteins possibly by participating in specific interactions involving the substituted amino acids. If so, the linker substituents would also be expected to contact residues 162 and 169 in the wild-type protein, which is consistent with ICRF-154 being the least potent analog against wild-type protein. An alternative explanation for the observed resistance profile is that the mutations somehow change the overall three-dimensional structure of the N-terminal drug-binding site, thereby reducing its affinity for the bisdioxopiperazine compounds. At least for the L169I mutation, we find this highly unlikely because of the small structural change this substitution is likely to represent. We therefore favor the hypothesis that L169 and probably also R162 are involved directly in drug binding and that bisdioxopiperazine linker substituents play an important role in this interaction. We found that the sensitivity pattern of mitindomide in decatenation assay is almost identical with that of ICRF-154, with the only difference being that mitindomide causes a slight inhibition of L169I catalytic activity. However, the observed resistance pattern is quite dissimilar to that of ICRF-187, ICRF-193, and ICRF-202. Our data therefore indicate that mitindomide represents more closely the active conformer of ICRF-154 than that of ICRF-187, despite the fact that mitindomide has been suggested to represent the active conformer of ICRF-187 (Hasinoff et al., 1997).

Concerning closed-clamp formation, the role of the bisdioxopiperazine linker substituents is less clear. Although ICRF-154 is clearly less efficient in stabilizing the salt-stable complex of the R162Q and L169I mutant proteins on DNA than ICRF-187 and ICRF-193, some complex stabilization is still seen. Evidently, the interactions between the R162Q and L169I proteins and ICRF-154 are too weak to suppress catalytic activity while being strong enough to induce the salt-stable complex to some extent. The finding that all tested bisdioxopiperazines (ICRF-154, ICRF-187, and ICRF-193) display increased complex formation with the R162K mutant protein despite the fact that it is slightly drug-resistant precludes us from establishing a clear correlation between catalytic inhibition/cytotoxicity and closed-clamp formation, suggesting that factors other that drug/protein interactions may contribute to the stability of the closed-clamp intermediate complex on DNA in vitro. The R162K mutation could simply enhance the stability of the closed-clamp complex at 1 M KCl once it is formed. The observation that the R162K protein displays reduced closed-clamp stabilization by AMP-PNP does not preclude this hypothesis. The R162K protein has reduced affinity for ATP, and consequently its affinity for AMP-PNP is most likely to be reduced, which is expected to result in reduced AMP-PNP–induced closed-complex stabilization, per se. For the other mutants analyzed in the present work, reduced closed-clamp stabilization seems to correlate with reduced drug sensitivity in vitro and in vivo, suggesting that, at least for these mutants, protein/drug interaction is likely to determine the level of closed-clamp complex formed and that the R162K mutation represents an exception.

In summary, we have shown that mutations at residues 162 and 169 in human topoisomerase IIα are capable of conferring differential sensitivity toward closely related bisdioxopiperazines, emphasizing the highly specific interaction of these compounds with their target. Our results show that the region of topoisomerase II that includes these residues is critical for bisdioxopiperazine drug action and is probably required for drug/protein interaction.

Acknowledgments

The expert technical assistance of Sanne Christiansen in purifying kDNA is appreciated. We are much obliged to The Drug Synthesis Chemistry Branch, Development Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Maryland, for the gifts of mitindomide and ICRF-202.

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