Analysis of Bisdioxopiperazine Dexrazoxane Binding to Human DNA Topoisomerase II α: Decreased Binding as a Mechanism of Drug Resistance†

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ABSTRACT: Topoisomerase II is an ATP-operated clamp that effects topological changes by capturing a double stranded DNA segment and transporting it through another DNA molecule. Despite the extensive use of topoisomerase II-targeted drugs in cancer chemotherapy and the impact of drug resistance on the efficacy of treatment, much remains unknown concerning the interactions between these agents and topoisomerase II. To identify the interaction of the bisdioxopiperazine dexrazoxane (ICRF-187) with topoisomerase II, we developed a rapid gel-filtration assay and characterized the binding of (^{3}H)-dexrazoxane to human topoisomerase II α. Dexrazoxane binds to human topoisomerase II α in the presence of DNA and ATP with an apparent K_d of 23 μM and a stoichiometry of 1 drug molecule per enzyme dimer. Various N-terminal single amino acid substitutions in human topoisomerase II α that were previously shown to confer specific bisdioxopiperazine resistance either totally abolished drug binding or resulted in less efficient binding. The effect of the various mutations on drug binding correlated well with their effect on drug resistance in vivo and in vitro. Interestingly, an altered active site tyrosine mutant of human topoisomerase II α, which is incapable of carrying out DNA strand passage, was unable to bind dexrazoxane, which agrees with the drug's proposed mechanism of action late in the topoisomerase II catalytic cycle. The direct correlation between the level of drug binding and dexrazoxane resistance is consistent with a decreased drug binding mechanism of action for these dexrazoxane resistance conferring mutations.

DNA topoisomerases are nuclear enzymes that catalyze topological changes in DNA (*1*, *2*). These enzymes have been found in all cell types and are essential for cell viability. Topoisomerase II is a homodimer that passes one DNA duplex through a transient enzyme-bridged double strand break in a second DNA segment, changing the linking number of DNA in steps of two. Mammalian topoisomerase II exists as two isozymes: a 170 kDa α isoform that is preferentially expressed in proliferating cells and a 180 kDa β isoform found in both proliferating and nonproliferating cells.

Much of the current interest in DNA topoisomerases arises from their role as targets for anticancer therapeutics and their involvement in resistance to chemotherapy (*1*, *3*, *4*). In particular, topoisomerase II is the target of many widely used antitumor agents, including etoposide, teniposide, doxorubicin, mitoxantrone, and amsacrine (*5*). These drugs are referred to as topoisomerase II poisons because they kill cells in an unusual fashion. Rather than inhibiting the overall catalytic activity of the enzyme, they act by stabilizing the transient covalent DNA-topoisomerase II complexes, which eventually results in the generation of lethal DNA double strand breaks in treated cells. These agents thus convert this essential enzyme into a potent cellular toxin.

A second major class of drugs exists that does not stabilize the covalent complexes of topoisomerase II. These drugs, which inhibit the enzyme at other steps in the catalytic cycle, have been termed catalytic inhibitors (*6*, *7*). They include agents that competitively inhibit ATP binding such as novobiocin and coumermycin A1 (*8*), agents that abolish the binding of topoisomerase II to DNA such as the intercalative agent aclarubicin (*9*, *10*), and merbarone that does not block DNA binding but inhibits DNA cleavage (*11*, *12*). One of the most studied classes of topoisomerase II catalytic inhibitors are the bisdioxopiperazines, such as dexrazoxane (ICRF-187). Originally developed as an antitumor agent (*13*), dexrazoxane is now clinically used for the protection of cells against doxorubicin-induced cardiotoxicity (*14*) and is also a powerful nontoxic protector against etoposide-induced toxicity in vivo, thus enabling high-dose etoposide treatment of tumors in the central nervous system in a mouse model (*15*). This compound is likewise capable of protecting against necrosis induced by subcutaneous doxorubicin and daunorubicin injection in mice (*16*) and is currently in two phase three clinical trials as an antidote to accidental anthracycline extravasation. Several studies have indicated that bisdiox-

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opiperazines trap topoisomerase II as a salt-stable closed clamp intermediate on DNA in the presence of ATP, inhibiting the ATPase activity and catalytic turnover of the enzyme (*17*−*20*).

A high variability in the response of different patients and/or cancers to topoisomerase II-targeted drugs has been observed (*4*, *7*, *21*). Drug resistance obviously greatly affects the success of cancer chemotherapy. We have previously identified several functional mutations in human topoisomerase II α conferring bisdioxopiperazine resistance (*20*, *22*−*25*). These mutations are primarily located at or near the Walker A consensus ATP binding site of htopoIIα.[1] It has been unclear whether these mutations cause their drug-resistant phenotype by reducing the protein's affinity for the drug or by other mechanisms such as interfering with ATP binding and/or hydrolysis, changing the overall conformation of the enzyme, or by shifting the progression through the catalytic cycle to favor an open clamp form that may be less sensitive to the inhibition by the bisdioxopiperazine drugs.

To distinguish between these possibilities and to obtain a better understanding of the mechanism of bisdioxopiperazine resistance, the binding of dexrazoxane to htopoIIα was assessed by using a rapid gel-filtration assay with tritiated (^{3}H)-dexrazoxane. Our results show that dexrazoxane binds to the full-length protein only in the presence of both DNA and ATP. Interestingly, the bisdioxopiperazine-resistant htopoIIα mutants all displayed decreased affinity for dexrazoxane, correlating with their level of resistance both in vivo and in vitro. These results show that single amino acid substitutions in the N-terminal part of human topoisomerase II α are capable of determining the drug binding capability of the entire full-length protein demonstrating directly that the N-terminal ATPase clamp constitutes the principal drug binding site for dexrazoxane, and most likely for other bisdioxopiperazines as well, on human topoisomerase II α.

EXPERIMENTAL PROCEDURES

Drugs. Dexrazoxane (Cardioxane) was purchased from Chiron Corp. (Amsterdam, The Netherlands), dissolved in sterile distilled water, and stored at −80 °C. ^{3}H-dexrazoxane (500 mCi/mmol, 1.0 mCi/mL in ethanol/water (1:1)) was customer labeled by Moravek Biochemicals, Inc. (Brea, CA) and stored at − 20 °C.

Induction and Expression of wt and Mutant htopoIIα in Yeast Cells. For the expression and purification of htopoIIα, we used a modification of the protocol in (*26*). The htopoIIα cDNA was in the expression vector YEpWOB6 (*27*) or in pGALhTOP2 controlled by a galactose-inducible GAL promoter. Both wt and mutant YEpWOB6 and wt pGALhTOP2 were transformed to the protease-deficient yeast strain Jel1Δtop1 (*trp1*, *leu2*, *ura3-52*, *pbr1-1122*, *pep4-3*, Δ*his3::PGAL10-GAL4*, *top1::LEU2*).

Construction of Mutations in htopoIIα. The mutations Y50F, R162K, R162Q, Y165S, L169F, and L169I, which all confer specific resistance to bisdioxopiperazines, were reconstructed in the plasmid YEpWOB6 (*27*) by oligonucleotide-directed mutagenesis using a Quick change site-directed mutagenesis kit (Stratagene) as previously described (*23*). Primers used to construct the mutations are described in ref *24* for R162Q, in ref *22* for L169F, in ref *18* for Y50F and Y165S, and in ref *20* for R162K and L169I.

Preparation of pUC18. Negatively supercoiled pUC 18 plasmid was isolated from 500 mL of an overnight culture grown in LB medium suplemented with 100 mg/L ampicillin using a Jetstar medi plasmid purification kit (Genomed, Bad Oeynhausen, Germany) as decribed by the manufacturer.

Preparation of ^{3}H Labeled kDNA. Tritium labeled kDNA was isolated from *Crithidia fasciculata* as described in ref *28*.

Dexrazoxane Binding Assay. The binding of dexrazoxane to htopoIIα was measured by a modification of rapid gel filtration (*29*, *30*). G-50 sephadex fine (Sigma) was incubated at least 1 day in water and stored at 4 °C before use. Binding mixtures contained purified wt or mutant htopoIIα (at concentrations ranging from 20 to 100 nM) and (^{3}H)-dexrazoxane (at concentrations ranging from 4 to 120 μM) in 50 μL of USB buffer (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) in the presence of 500 ng of pUC18 and 1 mg/mL BSA. For the competitive experiments, binding mixtures contained 100 nM wt htopoIIα, 20 μM (^{3}H)-dexrazoxane, 500 ng of pUC18, and 1 mg/mL BSA in 50 μL of USB buffer (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) in the absence or the presence of increasing concentrations of nonradioactive dexrazoxane (at concentrations ranging from 1 to 100 μM). Samples were incubated at room temperature for 25−35 min. During this time, 1 mL columns of G-50 sephadex were prepared and centrifuged at 1500*g* for 4 min. Then, binding mixtures were rapidly loaded on the corresponding columns, which were centrifuged at 1500*g* for 4 min. The amount of (^{3}H)-dexrazoxane bound to htopoIIα was determined by scintillation counting in the excluded volumes. Bound (^{3}H)-dexrazoxane (B) was obtained after subtraction of (^{3}H)-dexrazoxane bound to boiled htopoIIα under the same conditions. F corresponds to the total amount of (^{3}H)-dexrazoxane in each sample.

Decatenation Assay. Topoisomerase II catalytic activity was measured by the kDNA decatenation assay as previously described in ref *20*. Briefly, 200 ng of (^{3}H)-kDNA isolated from *C. fasciculata* was incubated with increasing concentrations of drug in a 20 μL 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 II α for 20 min at 37 °C (where one U of activity is defined as the amount of enzyme required for complete decatenation in the absence of drug). After addition of stop buffer containing 5% sarkosyl, 0.0025% bromophenol blue, and 50% glycerol, the 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, where 100% inhibition corresponds to the radioactivity retained on the filter when no enzyme is added.

RESULTS

Characterization of the wt and Mutant Topoisomerase IIα (htopoIIα). Two wt htopoIIα were expressed for purification

[1] Abbreviations: AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); BSA, bovine serum albumin; htopoIIα, human topoisomerase II α; wt, wild type.

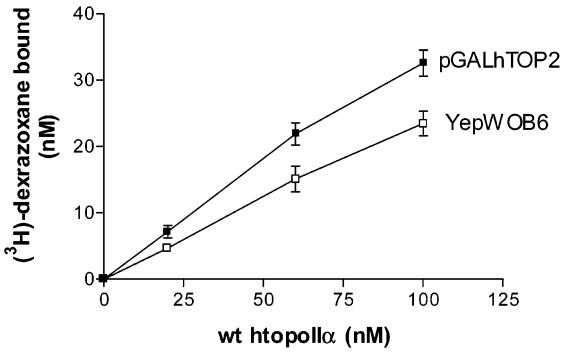

FIGURE 1: Binding of dexrazoxane to increasing concentration of pGALhTOP2- or YepWOB6-expressed wt htopoIIα. Twenty μM (^{3}H)-dexrazoxane was incubated with increasing concentrations of pGALhTOP2-wt htopoIIα (closed squares) or YepWOB6-wt htopoIIα (open squares) in the presence of 1 mM ATP and 500 ng of pUC18 as described in Experimental Procedures. Bound and free fractions of (^{3}H)-dexrazoxane were separated by filtration through 1 mL G-50 sephadex columns, and the flow-through was counted in scintillation liquid. Binding of (^{3}H)-dexrazoxane to heat inactivated wt htopoIIα was measured and was subtracted as background. Data represent the averages of four to seven independent experiments.

using two different galactose-inducible expression vectors YepWOB6 and pGALhTOP2. Both proteins were overexpressed in JELΔtop1 yeast cells (*31*). Induction and purification were performed by a modification of the protocol of ref *26* as previously described (*31*, *32*). The pGALhTOP2 vector carries the entire open reading frame of htopoIIα, while all the purified proteins expressed from the YepWOB6 vector have the first 28 residues substituted by the first five residues of yeast topoisomerase II (*31*). We used the full-length human protein for determining the protein's affinity for dexrazoxane and the yeast−human hybrid for determining the effect of single amino acid substitutions on drug binding. In these experiments, we used six previously described htopoIIα mutants known to confer specific resistance to this compound, Y50F (*23*), R162K, R162Q (*24*), Y165S (*25*), L169F (*22*), and L169I (*20*). Finally, we constructed a mutation changing the tyrosine involved in transesterification (Y805) to Phe to generate a mutant enzyme that was unable to cleave DNA. The enzymes were purified to >95% homogeneity (*18*). No significant difference in specific activity (except for the Y805F mutant, which has no activity) was observed between the different purified proteins (data not shown), indicating that the native catalytic activity of htopoIIα is not significantly altered by these mutations.

Binding of (^{3}H)-Dexrazoxane to wt htopoIIα. We first examined the interaction of (^{3}H)-dexrazoxane with the two different purified wt htopoIIα in the presence of 500 ng of pUC18 plasmid and 1 mM ATP. The amount of drug bound to the enzymes was determined for a range of protein concentrations using rapid gel filtration, which separated bound and free (^{3}H)-dexrazoxane by brief centrifugation on BSA-saturated G-50 sephadex columns. We found a linear increase of (^{3}H)-dexrazoxane bound to wt htopoIIα, as increasing amounts of proteins are used (Figure 1). Wt htopoIIα expressed from the pGALhTOP2 vector showed slightly higher levels of (^{3}H)-dexrazoxane bound as compared to wt htopoIIα expressed from the YepWOB6 vector under the same conditions (Figure 1). To identify the optimal conditions for drug binding measurements, we carried out a series of control experiments, in particular, regarding the concentrations of enzyme, drug, and BSA (data not shown). To achieve an appropriate signal-to-noise ratio for measurements, we used standard enzyme and drug concentrations of 100 nM and 20 μM, respectively. A range of enzyme concentrations was also examined, and dexrazoxane binding with an enzyme concentration under 20 nM was under the limit detection of the assay. An enzyme concentration above 100 nM results in binding saturation, which may be due to enzyme aggregation or increased nonspecific binding. To further minimize nonspecific binding of the enzyme to the G-50 sephadex gel, we included 1 mg/mL BSA to the incubation mixture.

It is important to note that in the absence of ATP, dexrazoxane binding to topoisomerase II was not significantly above background. This result was expected since dexrazoxane cannot trap topoisomerase II in a closed clamp in the absence of ATP. More surprisingly, dexrazoxane binding to topoisomerase II was also not observed if DNA was omitted from the reaction mixture. This result suggests that closed clamp formation in the presence of dexrazoxane may be much less efficient in the absence of DNA.

Characterization of the Binding of (^{3}H)-Dexrazoxane to wt htopoIIα. To characterize in detail the binding interactions between dexrazoxane and htopoIIα, we used the pGALhTOP2-wt htopoIIα, which showed the highest binding capacity and is identical to the primary structure of topoisomerase II expressed in human cells (Figure 1).

To determine the affinity and stoichiometry of the interaction between dexrazoxane and htopoIIα, increasing concentrations of (^{3}H)-dexrazoxane were incubated with wt htopoIIα in the presence of pUC18 and ATP. Determination of the stoichiometry of drug binding depends on quantitative elution of topoisomerase II. Under our assay conditions, recovery of topoisomerase II was typically greater than 80%, as determined by Western blotting (data not shown). Binding was concentration dependent and saturable. By fitting the binding curve to a one site binding equation (GraphPad Prism version 3.02 software), we calculated an apparent K_d of 23 ± 4 μM (Figure 2A). From the plateaus, the level of maximum drug bound was 90 ± 7 nM, and so, given an enzyme dimer concentration of 100 nM, the stoichiometry of dexrazoxane binding to htopoIIα dimer can be estimated as ∼1 (Figure 2A). A Scatchard plot analysis of the data yields a straight line, which suggests a monophasic affinity system (Figure 2B). In addition, this analysis yields similar results as the reciprocal of the slope indicated an apparent K_d of 29 μM and the *X* intercept a maximum drug bound level of 100 nM (Figure 2B).

If nonradioactive dexrazoxane was added in incubation mixtures, we observed a competition between (^{3}H)-dexrazoxane and nonradioactive dexrazoxane (Figure 3). The maximum displacement of (^{3}H)-dexrazoxane was achieved at 100 μM, and the concentration that displaces 50% of (^{3}H)-dexrazoxane is ∼5.5 μM (Figure 3).

Binding of (^{3}H)-Dexrazoxane to htopoIIα Mutants Conferring Dexrazoxane Resistance. To assess if htopoIIα mutants known to confer specific resistance to dexrazoxane exert their effects by simply decreasing the binding of the drug to the enzyme or by means of indirect mechanisms, we compared the binding of (^{3}H)-dexrazoxane to wt htopoIIα and to the different mutants that had been demonstrated to have dexrazoxane resistant catalytic activity (Y50F, R162K,

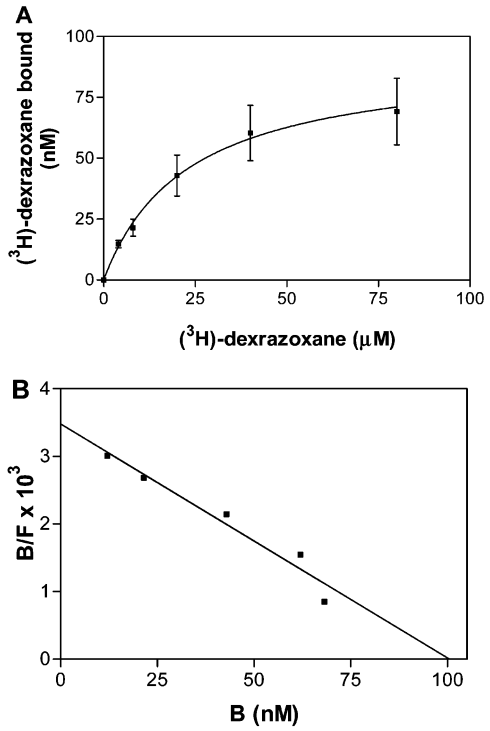

FIGURE 2: Binding of (^{3}H)-dexrazoxane to wt htopoIIα. (A) 100 nM pGALhTOP2-wt htopoIIα was incubated with increasing concentrations of (^{3}H)-dexrazoxane in the presence of 1 mM ATP and 500 ng of pUC18 as described under Experimental Procedures. Bound and free fractions of (^{3}H)-dexrazoxane were separated by filtration through 1 mL G-50 sephadex columns, and the flow-through was counted in scintillation liquid. Binding of (^{3}H)-dexrazoxane to heat inactivated wt htopoIIα was measured at each drug concentration and was subtracted as background. Data represent the averages of three to five independent experiments. (B) Scatchard plot of the (^{3}H)-dexrazoxane binding shown in panel A. B and F correspond to the concentration of bound and free drug molecules, respectively.

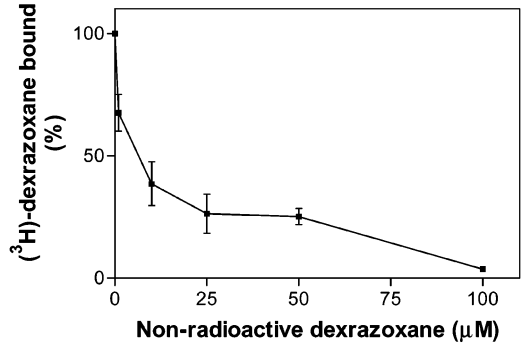

FIGURE 3: Displacement of (^{3}H)-dexrazoxane by nonradioactive drug. 100 nM pGALhTOP2-wt htopoIIα and 20 μM (^{3}H)-dexrazoxane were incubated with increasing concentrations of nonradioactive dexrazoxane in the presence of 1 mM ATP and 500 ng of pUC18 as described under Experimental Procedures. Bound and free fractions of (^{3}H)-dexrazoxane were separated by filtration through 1 mL G-50 sephadex columns, and the flow-through was counted in scintillation liquid. Binding of (^{3}H)-dexrazoxane to heat inactivated wt htopoIIα was subtracted as background. Data represent the averages of three to four independent experiments.

R162Q, Y165S, L169I, and L169F). Of these, R162K, R162Q, and L169I can be classified as partially resistant based on both in vitro catalytic inhibition and toxicity in yeast transformants, while the remaining mutants are highly resistant (*20*, *23*, *25*). Figure 4 shows that the level of (^{3}H)-dexrazoxane bound to the enzyme is proportional to the

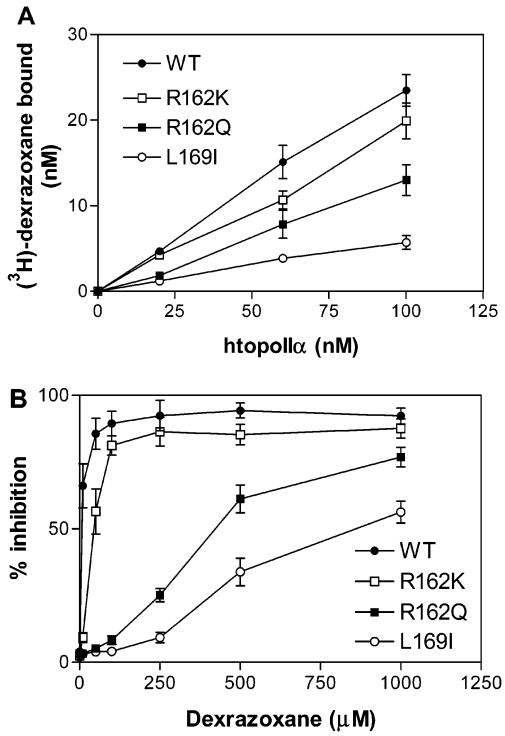

FIGURE 4: (A) Binding of (^{3}H)-dexrazoxane to increasing concentrations of wt and mutant htopoIIα and (B) comparison with the inhibition of their catalytic activities by dexrazoxane. (A) Drug binding assay was carried out as described in Experimental Procedures. Twenty μM (^{3}H)-dexrazoxane was incubated with increasing concentrations of YepWOB6-wt (closed circles) or mutants R162K (open squares), R162Q (closed squares), or L169I (open circles) htopoIIα in the presence of 1 mM ATP and 500 ng of pUC18. Bound and free fractions of (^{3}H)-dexrazoxane were separated by filtration through 1 mL G-50 sephadex columns, and the flow-through was counted in scintillation liquid. Binding of (^{3}H)-dexrazoxane to heat inactivated wt htopoIIα was measured and subtracted as background. Data represent the averages of four to eight independent experiments. (B) Decatenation assay was carried out as described in Experimental Procedures. 200 ng of (^{3}H)-kDNA was incubated with increasing concentrations of dexrazoxane in the presence of 1 mM ATP and 3 units of YepWOB6-wt (closed circles) or mutants R162K (open squares), R162Q (closed squares), or L169I (open circles) htopoIIα (*20*). 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. 100% inhibition corresponds to the radioactivity retained on the filter when no enzyme is added. Data represent the averages of three to six independent experiments.

concentration of wt and the partially resistant mutants, as increasing their concentration from 20 to 100 nM increases the amount of (^{3}H)-dexrazoxane bound to the enzyme. For all the mutant proteins, a reduced level of dexrazoxane binding was observed as compared to the level seen with wt htopoIIα. Interestingly, we found that this reduction is comparable to their level of drug resistance observed in vivo and in vitro (*20*, *23*, *25*). Thus, while no (^{3}H)-dexrazoxane binding was detected (data not shown) with the mutants Y50F, Y165S, and L169F (which were shown to be totally resistant to dexrazoxane inhibition (*22*, *23*, *25*)), the level of drug binding with the partially resistant mutants L169I, R162Q, and R162K correlates well with their level of drug resistance in vitro (compare Figure 4A and B).

Binding of (^{3}H)-Dexrazoxane to the Active Site Tyrosine Mutant of htopoIIα. We have previously reported that expression of an active site mutation of htopoIIα did not

result in bisdioxopiperazine sensitivity in yeast (*33*). Recently, we showed that mutating the active site tyrosine dramatically reduces the level of salt-stable closed clamp complex in the presence of dexrazoxane, although the level of salt-stable closed clamps was not significantly altered in the presence of AMPPNP (*18*). Therefore, it was of interest to assess the level of dexrazoxane binding to an active site mutant htopoIIα. We measured the binding of (^{3}H)-dexrazoxane with increasing concentrations of the active site tyrosine mutant of htopoIIα, Y805F, in the presence of 1 mM ATP and 500 ng of pUC18. No (^{3}H)-dexrazoxane binding was detected in our conditions (data not shown). This result indicates that binding of dexrazoxane is also disfavored in a mutant that is unable to cleave DNA.

DISCUSSION

The mechanism of action of the bisdioxopiperazine class of anti-cancer agents has been the subject of much interest since the original observation in ref *6* that these drugs act as catalytic inhibitors of DNA topoisomerase II. Thus, a large number of biochemical experiments has demonstrated that the bisdioxopiperazines such as dexrazoxane (ICRF-187) act on the topoisomerase II enzyme by trapping it in a closed clamp around DNA, similarly to what occurs when the enzyme binds to a non-hydrolyzable ATP analogue (*17*−*19*, *34*, *35*).

The precise interaction of dexrazoxane with topoisomerase II and its presumed pharmacophore still remains to be elucidated. One way of approaching this end is to study the binding of a radiolabeled drug to the purified enzyme under various conditions. Further, although the nature of the bisdioxopiperazine pharmacophore is still largely unknown, we have previously described mutations in htopoIIα that confer specific resistance to bisdioxopiperazines both in vitro and in vivo (*20*, *23*, *25*). These mutations could cause drug resistance by a number of ways, the simplest being by alterations that prevent drug binding. However, drug insensitivity need not involve changes in drug binding and could arise by an alteration of the overall three-dimensional structure of the drug interacting site, interference with ATP binding and hydrolysis, or shifting the progression through the enzyme catalytic cycle to steps that are unfavorable for bisdioxopiperazine binding. In this report, we have described a rapid gel-filtration assay using G-50 sephadex for bisdioxopiperazine binding to human topoisomerase II.

We first examined the binding of dexrazoxane to two different forms of purified wt htopoIIα. The YepWOB6 vector, which has the first 28 residues of htopoIIα replaced with the first five residues derived from yeast topoisomerase II, has been commonly used for studies with recombinant htopoIIα. A second vector, pGALhTOP2-wt htopoIIα, carries the entire open reading frame of the human enzyme, although expression levels of the protein are considerably reduced as compared to YepWOB6 (unpublished data). The level of dexrazoxane binding to pGALhTOP2-wt htopoIIα was slightly higher than the level observed with YepWOB6-wt htopoIIα (Figure 1) indicating that the first 28 residues of htopoIIα play a small role in drug binding. PGALhTOP2-wt htopoIIα and YepWOB6-wt htopoIIα have similar specific activities (data not shown) and also form similar amounts of salt-stable closed clamps in the presence of dexrazoxane or a nonhydrolyzable ATP analogue, AMPPNP (data not shown) (*18*). We therefore used the all human pGALhTOP2-wt htopoIIα in the first part of the study to characterize the interaction of dexrazoxane with wt enzyme (Figures 1−3) and the YepWOB6-wt htopoIIα for comparing wt and mutant enzymes (Figure 4).

In the presence of ATP and DNA, wt htopoIIα can bind dexrazoxane with an apparent K_d of 23 μM. This is to our knowledge the first time that a binding constant has been determined for the interaction of full-length human topoisomerase and bisdioxopiperazine. This K_d value is slightly higher than the IC_{50} for decatenation by the enzyme of 5.5 $\pm$ 1.0 μM under our standard conditions (Figure 4B). It is not surprising that there is a difference between the K_d for dexrazoxane and topoisomerase II and the IC_{50}, given that the enzyme can still carry out stoichiometric strand passage in the presence of drugs. Thus, the IC_{50} is a function of the initial enzyme and substrate concentrations.

We observed a stoichiometry of approximately one drug molecule bound per enzyme homodimer (Figure 2). Whether one dioxopiperazine ring binds to each monomer or whether the whole drug binds to one of the monomers is still unknown. A detailed kinetic study of yeast topoisomerase II trapped as a bisdioxopiperazine stabilized closed clamp on DNA concluded that the drug did not completely inhibit ATP hydrolysis even though clamp re-opening was inhibited (*34*). This may suggest that the drug can assume an asymmetrical binding mode, at least after the hydrolysis of one of the two bound ATP molecules. Such asymmetry is not surprising in light of the findings by Lindsley and colleagues that ATP hydrolysis by the holoenzyme occurs at two different points in the reaction cycle (*34*).

A further advantage of the drug binding system is its ability to easily compare the binding of dexrazoxane to different htopoIIα mutants, thus enabling a more detailed mechanistic examination of the enzyme. First, the Y805F mutant enzyme, which is incapable of carrying out strand passage, exhibits both a reduction in closed clamp formation (*18*) and a reduction in dexrazoxane binding. The Y805F mutant is not deficient in the formation of closed clamps in the presence of a nonhydrolyzable ATP analogue (*18*). These results suggest that there is likely to be a clear difference in the action of bisdioxopiperazines as compared to nonhydrolyzable ATP analogues. For example, strongest binding of bisdioxopiperazines may occur when only one of the two ATP binding sites is occupied, as suggested in the previous paragraph. It is also important to note that some stabilization of the closed clamp conformation does occur with the Y805F mutant (*18*), and the drug binding to this mutant may be below the detection level of our assay.

We next examined whether dexrazoxane was able to bind to the different htopoIIα mutants known to be resistant to bisdioxopiperazine. It is important to note that none of these mutants had any alteration in DNA binding as compared to the wt protein (*18*, *20*), suggesting that the mechanism by which the mutants become resistant to dexrazoxane does not directly involve the interaction of DNA with the enzyme. The mutants that lead to high level dexrazoxane resistance (Y50F, Y165S, and L169F) failed to form measurable levels of drug binding (data not shown). These results suggest that these mutants confer bisdioxopiperazine resistance in part because of a failure of the mutant protein to interact with

drug (*23*, *25*). For the R162K, R162Q, and L169I mutants, which also show a specific resistance to bisdioxopiperazines (*20*), albeit at a lower level, we observed reduced levels of dexrazoxane binding (Figure 4A). Moreover, the differential binding profile in the drug binding assay correlates well with their sensitivity profile in the catalytic assay (compare Figure 4A and B) and also with their cytotoxicity in yeast (*20*). Taken together, these results strongly suggest that the resistance of these htopoIIα mutants to bisdioxopiperazines is caused simply by decreasing drug binding.

The results reported in this manuscript are consistent with bisdioxopiperazine binding at the extreme amino terminus of the enzyme. This hypothesis is supported by the large number of mutants that have been isolated in the amino terminus of human topoisomerase II α that confer resistance to bisdioxopiperazines and is consistent with the biochemical effects of bisdioxopiperazines on topoisomerase II. However, Hsieh and colleagues recently showed that bisdioxopiperazines are able to trap a closed clamp of a topoisomerase II completely lacking the N-terminal domain of the enzyme, including the ATPase domain of the enzyme (*17*). It should be noted however, that the efficiency of trapping a closed clamp conformation was substantially reduced as compared to a protein with an intact ATPase domain. This result may suggest that the bisdioxopiperazine binding site may extend beyond the N-terminal domain of the protein. Hsieh et al. also found closed clamp formation in a protein lacking the DNA cleavage domain of topoisomerase II, which also supports drug localization within the amino terminal portion of the protein (*17*).

The structure of the N-terminal domain of eukaryotic topoisomerase II has not yet been reported. However, the present results that reduced drug binding correlates with reduced drug sensitivity in a number of assays may facilitate computational docking studies on the putative bisdioxopiperazine pharmacophore using the crystal structure of prokaryotic enzymes. Recent results suggest that there may be significant conservation in the ATP binding domains of topoisomerases across different kingdoms (*36*).

In summary, results of the present study provide novel information regarding the interactions of topoisomerase II with the catalytic inhibitor dexrazoxane. They indicate that dexrazoxane requires the presence of both ATP and DNA to bind htopoIIα and a stoichiometry of one drug molecule per one enzyme homodimer. This method allowed us to compare the binding of wt and mutant enzymes to dexrazoxane, and we demonstrated that alterations in drug/protein interactions correlate with bisdioxopiperazine resistance in vivo and in vitro. Taken together, the present results suggest a direct correlation between drug−enzyme binding and drug resistance.

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