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Biochemical and Proteomics Approaches to Characterize Topoisomerase IIα Cysteines and DNA as Targets Responsible for Cisplatin-Induced Inhibition of Topoisomerase IIα

Brian B. Hasinoff, Xing Wu, Oleg V. Krokhin, Werner Ens, Kenneth G. Standing, John L. Nitiss, Tejomoortula Sivaram, Angela Giorgianni, Shaohua Yang, Yu Jiang, and Jack C. Yalowich

Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada (B.B.H., X.W.); Department of Physics and Astronomy, University of Manitoba and Manitoba Centre for Proteomics, Winnipeg, Manitoba, Canada (O.V.K., W.E., K.G.S.); Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, Tennessee (J.L.N.); and Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania (T.S., A.G., S.Y., Y.J., J.C.Y.)

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ABSTRACT

Cisplatin was shown to strongly inhibit the decatenation and relaxation activity of isolated human DNA topoisomerase IIα. This inhibition was not accompanied by stabilization of a covalent topoisomerase IIα-DNA intermediate. Pretreatment of kinetoplast plasmid DNA (kDNA) or pBR322 DNA with submicromolar concentrations of cisplatin quickly rendered these substrates incompetent in the topoisomerase IIα catalytic assay. Cisplatin nearly equally inhibited growth of a parental K562 and an etoposide-resistant K/VP.5 cell line that contained decreased topoisomerase IIα levels, a result consistent with isolated enzyme experiments demonstrating that cisplatin was not a topoisomerase IIα poison. Because cisplatin is known to react with protein sulfhydryl groups, the 13 cysteine groups in the topoisomerase IIα monomer were evaluated by mass spectrometry to determine which cysteines were free and disulfide-bonded to identify possible sites of cisplatin adduction. High-pressure liquid chromatography-matrix-assisted laser desorption ionization mass spectrometry showed that topoisomerase IIα contained at least five free cysteines (170, 216, 300, 392, and 405) and two disulfide-bonded cysteine pairs (427–455 and 997-1008). Cysteine 733 was also disulfide-bonded, but its partner cysteine could not be identified. Cisplatin antagonized the formation of a fluorescence adduct between topoisomerase IIα and the sulfhydryl-reactive maleimide reagent 10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-2-carboxylic acid methyl ester (ThioGlo-1). Dithiothreitol, which was shown by spectrophotometry to react rapidly with cisplatin (6-min half-time), diminished the capacity of cisplatin to interfere with ThioGlo-1 binding to topoisomerase IIα. The results of this study suggest that cisplatin may exert some of its cell growth inhibitory and antitumor activity by inhibition of topoisomerase IIα through reaction with critical enzyme sulfhydryl groups and/or by forming DNA adducts that render the DNA substrate refractory to topoisomerase IIα.

Cisplatin is widely used for the treatment of cancer and is thought to act by forming intrastrand and interstrand cross-links with DNA (Waud, 1995; Reedijk, 1996). A variety of other biomolecules have also been shown to react with cisplatin because of its electrophilicity toward sulfhydryl, methionine, histidine, and other amino acids with nitrogen-containing side chains (Waud, 1995; Reedijk, 1996; Ivanov et al., 1998; Hagrman et al., 2003). In a study of the topoisomerase II inhibitory effects of a platinum(II) complex of the catalytic topoisomerase II inhibitor dexrazoxane, we showed that cisplatin strongly inhibited the decatenation activity of topoisomerase II (Hasinoff et al., 2004). Topoisomerase II alters DNA topology by catalyzing the passing of an intact DNA double helix through a transient double-stranded break made in a second helix and is critical for relieving torsional

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ABBREVIATIONS: MALDI, matrix-assisted laser desorption ionization; kDNA, kinetoplast plasmid DNA; ThioGlo-1, 10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-2-carboxylic acid methyl ester; μ-HPLC, micro-high-pressure liquid chromatography; HPLC, high-pressure liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TOF, time-of-flight.

stress that occurs during replication and transcription and for daughter strand separation during mitosis (Fortune and Osheroff, 2000; Li and Liu, 2001). Several widely used anticancer agents, including doxorubicin and other anthracyclines, amsacrine, etoposide, and mitoxantrone, also target topoisomerase II and are thought to be cytotoxic by virtue of their ability to stabilize a covalent topoisomerase II-DNA intermediate (the cleavable complex) and are so-called topoisomerase II poisons (Fortune and Osheroff, 2000; Li and Liu, 2001).

The fact that bacterial topoisomerase II, DNA gyrase, can be inhibited by cisplatin (Neumann et al., 1996) suggested that mammalian topoisomerase II might also be susceptible to inhibition by cisplatin. There has also been a report that a cisplatin-resistant leukemia cell line exhibits increased topoisomerase II activity, a result that is consistent with topoisomerase II being a target of cisplatin (Barret et al., 1994). Mouse cancer cells transfected with topoisomerase IIα showed increased resistance to cisplatin, again consistent with a role for topoisomerase IIα as a determinant of cisplatin activity (Eder et al., 1995). Novobiocin-mediated inhibition of topoisomerase II activity was also shown to result in increased cytotoxicity to cisplatin (Ali-Osman et al., 1993), a result also consistent with topoisomerase II contributing to cisplatin action. Together, these studies are consistent with a model in which a portion of cisplatin activity is at the level of topoisomerase II. It has also recently been shown (van Waardenburg et al., 2004) that cisplatin causes Pt-1,3-d(GTG) poisoning of topoisomerase I in vitro and that persistent platinated-DNA adducts correlate with increased covalent topoisomerase I-DNA complexes in cells. These results indicate that the cytotoxic activity of cisplatin is due, in part, to poisoning of topoisomerase I and are consonant with a related mechanism for inhibition of topoisomerase II. Because cisplatin can react with sulfhydryl groups (Ivanov et al., 1998; Sadowitz et al., 2002; Hagrman et al., 2003) and several studies have shown that sulfhydryl-reactive agents, including maleimide (Jensen et al., 2002), quinones (Wang et al., 2001), selenium compounds (Zhou et al., 2003) and etoposide orthoquinone (Gantchev and Hunting, 1998) inhibit topoisomerase IIα, we investigated the inhibitory activity of cisplatin toward topoisomerase IIα further, with a view to determining whether this inhibition contributes to the cell growth inhibitory and antitumor effects of cisplatin. Free and disulfide-bonded cysteine groups on topoisomerase IIα were determined using MALDI mass spectrometry to identify possible targets of cisplatin. We hypothesized that this inhibition might occur by binding directly with free sulfhydryl, or other reactive groups on topoisomerase IIα critical for activity and/or by binding to the DNA substrate in such a way that topoisomerase IIα is unable to process it.

Materials and Methods

Materials. pBR322 plasmid DNA was obtained from MBI Fermentas (Burlington, ON, Canada) and kDNA was from TopoGEN (Columbus, OH). Trypsin (excision grade, bovine pancreas) and Thio-Glo-1 were from Calbiochem (San Diego, CA). HindIII was from Invitrogen (Burlington, ON, Canada). Unless indicated, other chemicals were from Sigma-Aldrich(Oakville, ON, Canada). Except where indicated, the errors quoted are standard errors from nonlinear least-squares analysis (SigmaPlot; SPSS Inc., Chicago, IL).

Kinetics of the Reaction of Cisplatin with Dithiothreitol. The reaction of dithiothreitol with cisplatin was followed spectrophotometrically by spectral scanning and at a fixed wavelength of 260 nm on a Cary 1 double beam spectrometer (Varian, Mississauga, ON, Canada) with a cell compartment at 37°C. The reactions were initiated by adding a small volume of stock dithiothreitol solution to 1 ml of freshly prepared 100 μM cisplatin in 20 mM Tris-HCl, pH 8.0, in a 1-cm silica cell. The final dimethyl sulfoxide concentration was 0.5% (v/v).

MALDI Mass Spectrometry. To confirm the identity of human topoisomerase IIα, preliminary peptide mapping studies were carried out. The topoisomerase IIα samples (3.5–7.5 μg in 30–50 μl) were reduced (10 mM dithiothreitol, 30 min, 57°C), alkylated (50 mM iodoacetamide, 30 min in the dark at room temperature), dialyzed against 100 mM NH_4HCO_3 [6 h, 7000-Da cut-off), and digested overnight with trypsin at a 1:100 (w/w) enzyme to substrate ratio for 12 h at 37°C]. The digests were then lyophilized, resuspended in 5 μl of 0.5% (v/v) trifluoroacetic acid, and finally subjected to offline μ-HPLC-single mass spectrometry (MS) and tandem mass spectrometry (MS/MS) analysis.

For the detection of free cysteines on topoisomerase IIα, the protein was first alkylated (20 mM iodoacetamide, 30 min in the dark at room temperature), dialyzed to remove the excess of iodoacetamide, fully reduced with dithiothreitol, alkylated with iodoacetic acid, and then processed as described above. After these treatments, all free cysteine residues should be found alkylated with iodoacetamide, whereas those found in disulfide bonds should be alkylated with iodoacetic acid, which gives a +0.984-Da mass shift in the mass spectrum compared with the iodoacetamide alkylation.

For the assignment of disulfide bonds, a topoisomerase IIα sample (7.5 μg) was dialyzed against 100 mM NH_4HCO_3 and digested with trypsin [1:50 (w/w) enzyme to substrate ratio]. Half of this sample was fully reduced and alkylated with iodoacetamide, and the other half was neither reduced nor alkylated. Tryptic fragments with disulfide bonds that disappeared after the reduction/alkylation treatment were identified by comparison of the two μ-HPLC-MS runs (with and without reduction/alkylation). The identity of the disulfide-containing tryptic fragments was then confirmed by MS/MS.

Chromatographic separations were performed using a micro-Agilent 1100 Series system (Agilent Technologies, Wilmington, DE). Deionized water and HPLC-grade acetonitrile were used for the preparation of eluents. The 5-μl samples were injected onto a 150-μm $\times$ 150-mm column (Vydac 218 TP C18, 5 μm; Grace Vydac, Hesperia, CA) and eluted with a linear gradient of 1 to 80% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid over 60 min. The column effluent (4 μl/min) was mixed on-line with a dihydroxybenzoic acid matrix solution [150 mg/ml in 1:1 (v/v) acetonitrile/water, 0.5 μl/min] and deposited by a computer-controlled robot onto a movable gold target at 1-min intervals (Krokhin et al., 2002). It was necessary to collect only 40 fractions, because almost all of the tryptic peptides were eluted in 40 min under the chromatographic conditions used. The fractions were air dried and subjected to MALDI MS analysis.

The chromatographic fractions were analyzed both by MS with a mass range of 570 to 5000 Da and by MS/MS on the Manitoba/Sciex prototype quadrupole/TOF mass spectrometer (commercial model sold as QSTAR by Applied Biosystems, Foster City, CA) (Loboda et al., 2000). In this instrument, ions are produced by irradiation of the sample with photon pulses from a 20-Hz nitrogen laser (VCL 337ND; Spectra Physics, Mountain View, CA) with 300-mJ energy per pulse. Orthogonal injection of ions from the quadrupole into the TOF section normally produces a mass resolving power of ~10,000 (full-width-half-maximum) and an accuracy of a few millidaltons in the TOF spectra in both MS and MS/MS modes. The program "m/z" was used to pick peaks with a signal-to-noise ratio >2.5 and the program "ProFound" (both from the Manitoba Centre for Proteomics, www.proteome.ca) was used for peptide mass fingerprint analysis. All masses cited are MH^+ monoisotopic masses.

Cell Culture and Growth Inhibition Assays. Human leukemia K562 cells, obtained from the American Type Culture Collection (Manassas, VA), and K/VP.5 cells (a 26-fold etoposide-resistant K562-derived cell line with decreased levels of topoisomerase IIα protein and mRNA) (Ritke and Yalowich, 1993; Ritke et al., 1994a,b; Fattman et al., 1996) were maintained as suspension cultures in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum and 2 mM L-glutamine. Exponentially growing cells were plated in 24-well plates at a concentration of 1.5 to 1.7 × 10^5 cells/ml and incubated with various concentrations of cisplatin for 48 h, after which cells were counted on a model ZBF Coulter counter (Beckman Coulter, Fullerton, CA). The IC_{50} growth-inhibitory concentration for each cell line was calculated from a nonlinear least-squares fit to a four-parameter logistic equation. The IC_{50} values from four separate experiments using both cell lines were compared using a paired *t* test.

Topoisomerase IIα Preparation. A high copy yeast expression vector for production and purification of human topoisomerase IIα in yeast was constructed. A 2-μm plasmid pEG(KT) bearing the *GAL* promoter, the URA3 marker for selection, and the *leu2-d* allele was used for plasmid amplification of a high copy yeast expression vector. Beginning with a centromeric plasmid, pYX113, containing full-length human topoisomerase IIα cDNA, a SacI restriction site 5′ to the ATG start site of topoisomerase IIα was engineered to facilitate excision of full-length topoisomerase IIα using a single restriction enzyme because a SacI site is present 1 kilobase downstream of the topoisomerase IIα coding sequence. SacI digestion of the engineered pYX113 plasmid liberated topoisomerase IIα that was ligated into the SacI site of pEG(KT) downstream of the *GAL* promoter to yield the pSY3 topoisomerase IIα expression plasmid. Transformation of pSY3 into the protease-deficient topoisomerase I-negative yeast strain JelΔTop1 (*Mat* a, *trp1, leu2, ura-52, pBR322 DNA-1122, pep4-3, his3*::*PGAL10-GAL4, TOP1*::*LEU*2) made auxotrophic for leucine by inserting the *his3* gene in the LEU2 locus was followed by selection under URA^- conditions. Yeast was grown in leucine-free media to promote plasmid amplification followed by addition of galactose for induction. Full-length human topoisomerase IIα was extracted and purified as described previously (Sehested et al., 1998). In addition, JelΔTop1 transformed by the vector YepWOB6 containing the human topoisomerase IIα sequences was used for expression and purification of enzyme for mass spectrometry studies. YepWOB6 (Wasserman et al., 1993) codes for the first five amino acids of yeast topoisomerase II substituted for the first 28 amino acids of topoisomerase IIα.

kDNA Decatenation Assays and pBR322 DNA Relaxation and Cleavage Assays. A modified and improved spectrofluorometric decatenation assay was used to determine the inhibition of topoisomerase IIα by cisplatin (Barnabé and Hasinoff, 2001; Hasinoff et al., 2004). kDNA consists of highly catenated networks of circular DNA. Topoisomerase IIα decatenates kDNA in an ATP-dependent reaction to yield individual minicircles of DNA. The 20-μl reaction mixture contained dithiothreitol or not, as indicated, 0.5 mM ATP, 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 30 μg/ml bovine serum albumin, 40 ng of kDNA, cisplatin (0.5 μl in dimethyl sulfoxide), and 10 ng of topoisomerase IIα protein (the amount that gave approximately 80% decatenation). The final dimethyl sulfoxide concentration of 2.5% (v/v) has been shown in controls not to affect the activity of topoisomerase IIα. The assay incubation was carried out at 37°C for 20 min and was terminated by the addition of 12 μl of 250 mM Na_2EDTA. Samples were centrifuged at 8000*g* at 25°C for 15 min, and 20 μl of the supernatant was added to 180 μl of 600-fold diluted PicoGreen dye (Molecular Probes, Eugene, OR) in a 96-well plate. The fluorescence, which was proportional to the amount of kDNA, was measured in a BMG Fluostar Galaxy (Durham, NC) fluorescence plate reader using an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

An ethidium bromide agarose gel assay as described previously (Hasinoff et al., 1997) was also used to determine whether cisplatin-treated kDNA was a substrate for, or inhibited the decatenation activity of topoisomerase IIα, when mixed with untreated kDNA. The kDNA decatenation products from the reaction mixture described above were resolved on a 1.2% agarose gel in TAE buffer (4 mM Tris base/glacial acetic acid [0.11% (v/v)]/2 mM Na_2EDTA) containing 0.5 μg/ml ethidium bromide at 8 V/cm for 2 h. The DNA in the gel was imaged by its fluorescence on an Alpha Innotech (San Leandro, CA) Fluorochem 8900 imaging system equipped with a 365-nm UV illuminator and a charge-coupled device camera.

The cisplatin-treated kDNA for the gel electrophoresis experiments was prepared by treating 2 μg of kDNA in 50 μl of 1 mM Tris-HCl, pH 7.5, with 300 μM cisplatin for 1 h at 37°C. Free cisplatin was removed from the kDNA by centrifuging the sample at 12,000*g* for 15 min, removing the supernatant, and resuspending the kDNA pellet in 50 μl. This procedure was repeated two more times. The washed kDNA concentration was determined using the PicoGreen assay described above. The concentration of free cisplatin remaining in the assay buffer was calculated to be less than 0.3 μM at the highest concentration of cisplatin-treated kDNA used. The final dimethyl sulfoxide concentration in the assay buffer was 0.002% (v/v). The decatenation gel assay on the washed cisplatin-kDNA and kDNA mixtures were carried out as described above, except with the amounts of cisplatin-kDNA, kDNA, and topoisomerase II protein specified in the figure.

Topoisomerase II-cleaved DNA complexes produced by anticancer drugs may be trapped by rapidly denaturing the complexed enzyme with SDS (Burden et al., 2001). The cleavage of double-stranded closed circular pBR322 DNA to form linear DNA was followed by separating the SDS-treated reaction products using ethidium bromide gel electrophoresis as described previously (Burden et al., 2001). The 20-μl cleavage assay reaction mixture contained 200 ng of topoisomerase IIα protein, 80 ng of pBR322 plasmid DNA (MBI Fermentas), 0.5 mM ATP in assay buffer [10 mM Tris-HCl, 50 mM KCl, 50 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5% (v/v) glycerol, pH 8.0, and cisplatin (0.5 μl in dimethyl sulfoxide)]. The order of addition was assay buffer, DNA, cisplatin, and then topoisomerase IIα. The reaction mixture was incubated at 37°C for 10 min and quenched with 1% (v/v) SDS/25 mM Na_2EDTA. The reaction mixture was treated with 0.25 mg/ml proteinase K (Sigma-Aldrich) at 55°C for 30 min to digest the protein. The linear pBR322 DNA cleaved by topoisomerase IIα was separated by electrophoresis (2 h at 8 V/cm) on a TAE ethidium bromide (0.5 μg/ml) agarose gel [1.2% (w/v)].

The topoisomerase IIα-catalyzed relaxation of cisplatin-treated and untreated pBR322 DNA and mixtures of the two was determined using the cleavage assay conditions as described above except that a smaller amount of topoisomerase IIα (100 ng) was used. The assay mixture also contained 100 ng of pBR322 DNA and 0, 25, 50, or 100 ng of cisplatin-treated pBR322 DNA as indicated. The cisplatin-treated pBR322 DNA was prepared by treating 1 μg of pBR322 DNA in 50 μl of 1 mM Tris-HCl/0.1 mM EDTA buffer, pH 7.6, with 200 μM cisplatin for 1 h at 37°C. Free cisplatin was removed from the pBR322 DNA solution using a Wizard SV Gel and PCR Cleanup minicolumn (Promega, Madison, WI).

ThioGlo-1 Determination of Cisplatin Adducts with Cysteine Sulfhydryl Groups on Topoisomerase IIα. ThioGlo-1 (200 μM) was incubated for 1 h at 37°C with 1.1 μg of human topoisomerase IIα in a final reaction volume of 20 μl containing 41 mM sodium phosphate, pH 7.0, and 1% SDS. Cisplatin or *N*-ethylmaleimide used alone or in combination with simultaneously added dithiothreitol was incubated with topoisomerase IIα 30 min before addition of ThioGlo-1. Electrophoresis on a 7% polyacrylamide gel was followed by UV illumination and fluorescence signal capture using a Stratagene Eagle Eye II fitted with a SYBR Green filter. Protein bands were then stained with Coomassie Blue, and the gels were imaged in white light on the Eagle Eye II. Quantitation of the fluorescence and protein intensities was performed with the use of Kodak ID software (Eastman Kodak, Rochester, NY). Drug-induced inhibition of ThioGlo-1-topoisomerase IIα adduct formation was cal-

culated by taking the ratio of the fluorescence and protein signals for each experimental condition and comparing this ratio with the ratio observed under control conditions where only ThioGlo-1 and topoisomerase IIα were present in reaction mixtures.

Results

Reaction of Cisplatin with Dithiothreitol. Because it has been shown previously that cisplatin can react with free (Ishikawa and Ali-Osman, 1993; Dabrowiak et al., 2002) or protein sulfhydryl groups (Ivanov et al., 1998; Sadowitz et al., 2002; Hagrman et al., 2003) and because our enzyme preparation contained dithiothreitol, we first investigated the reaction of cisplatin with dithiothreitol. As shown in Fig. 1A, the reaction of cisplatin with dithiothreitol resulted in spectral changes similar to those seen with the reaction of other small thiols such as glutathione, which reacts with cisplatin with a 2:1 stoichiometry (Ishikawa and Ali-Osman, 1993). The absorbance-time traces shown in Fig. 1B seemed to initially level off at a 1:1 ratio of dithiothreitol to cisplatin, suggesting the initial formation of a 1:1 complex for the dithiol. However, at longer times and higher ratios, the absorbance continued to increase and after several hours a white precipitate was produced, suggesting that the initial complex formed underwent further reaction. The half-time for the reaction at 100 μM of each reactant was approximately 6 min and as such is faster than its reaction with glutathione or other thiols (Ishikawa and Ali-Osman, 1993; Dabrowiak et al., 2002). Initial velocities were measured over the first 1 min of the reaction (Fig. 1C), and the plot shows a small amount of saturation behavior that could be caused by initial cisplatin outer sphere complex formation before inner sphere coordination through the sulfhydryl group.

Cisplatin-Treated kDNA Is Not a Competent Substrate for Topoisomerase IIα. As shown in Fig. 2A, when kDNA was pretreated with cisplatin for various times the ability of topoisomerase IIα to decatenate kDNA rapidly decreased. Treatment with 0.02 μM cisplatin resulted in a reduction of about 40% in the amount of decatenated kDNA produced, whereas treatment with 0.2 μM cisplatin resulted in complete inhibition of decatenation. This result suggests that after rapid adduction of cisplatin to kDNA, the product formed was no longer able to function as a substrate for topoisomerase IIα. After addition of 0.2 μM cisplatin to the assay mixture, further addition of 0.5 μM dithiothreitol was unable to reverse the cisplatin-induced inhibition of decatenation (data not shown). It should be noted that the free cisplatin remaining in the pretreatment mixture would be available to react with topoisomerase II and inhibit it during the course of the assay itself.

Fig. 2B shows the effect of pretreating kDNA for 40 min with different concentrations of cisplatin on the topoisomerase IIα-catalyzed decatenation reaction. Cisplatin pretreatment potently inhibited the formation of decatenation product with IC_{50} values of 0.018 and 0.34 μM without or with added dithiothreitol (40 μM), respectively. These results suggest that dithiothreitol does not completely protect kDNA, probably because cisplatin preincubated with kDNA inhibited decatenation activity faster (half-time ~1 min; Fig. 2A) than dithiothreitol reacted with cisplatin (half-time ~6 min; Fig. 1A).

From the IC_{50} of 0.018 μM obtained in the absence of added dithiothreitol (Fig. 2B), it is possible to calculate the mole ratio of kDNA to cisplatin in the assay mixture. Assuming an average kDNA base pair molecular weight of 660 g/mol, the reaction mixture contained one cisplatin per 162 kDNA base pairs. However, the calculated value of one cisplatin per 162 kDNA base pairs is an upper limit on the

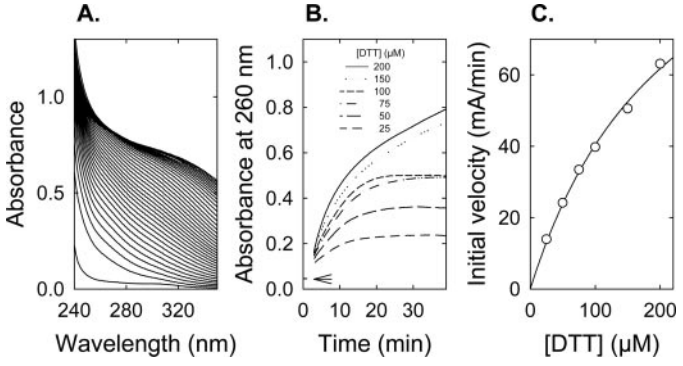

Fig. 1. Cisplatin reacts with dithiothreitol. A, spectral changes occurring upon the addition of 100 μM dithiothreitol to 100 μM cisplatin. Spectra were recorded every 2 min, and the absorbances continuously increased with time at all wavelengths. B, increases in absorbance with time at 260 nm after the addition of various concentrations of dithiothreitol to 100 μM cisplatin. The arrow in the bottom left indicates the initial absorbance recorded before addition of dithiothreitol. C, initial rate of absorbance change at 260 nm upon the addition of various concentrations of dithiothreitol to 100 μM cisplatin. The solid line is a fit of the initial velocity data to a Michaelis-Menten saturation-type equation. The reactions were followed in 20 mM Tris buffer, pH 8.0, at 37°C.

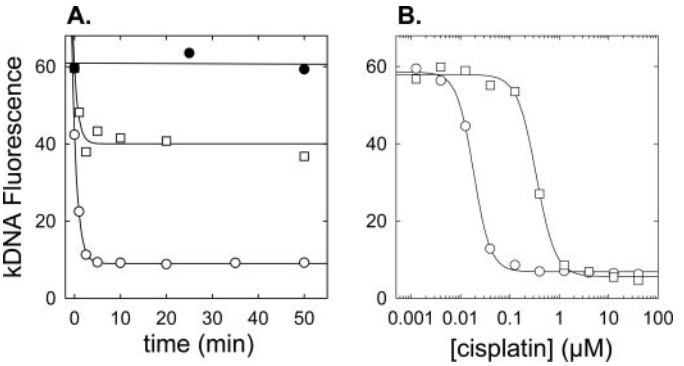

Fig. 2. Pretreating kDNA with cisplatin inhibits kDNA decatenation by topoisomerase IIα. A, effect on decatenation reaction of pretreating kDNA with cisplatin for various times. The kDNA (2 μg/ml, ~3 μM DNA base pair equivalents) was pretreated with either 0 (●), 0.02 (□), or 0.20 μM (○) cisplatin for the times indicated at 37°C. The cisplatin-kDNA mixture was then diluted 4-fold into the decatenation assay buffer containing topoisomerase IIα for 20 min. Incubation in the absence of added cisplatin had no effect on the amount of decatenated kDNA product produced, whereas treatment with 0.02 and 0.20 μM cisplatin ultimately resulted in 40 and 100% loss of decatenated product, respectively. Both these processes were rapid (half-time of 1.1 and 0.7 min, respectively). The 0.20 μM cisplatin treatment reduced the kDNA-PicoGreen complex fluorescence to background levels. The curved solid lines are nonlinear least-squares calculated fits of the fluorescence-concentration data to a three-parameter exponential decay equation. The straight line is linear-least-squares calculated. B, effect on the topoisomerase IIα-catalyzed deca-tenation reaction of pretreating kDNA with various concentrations of cisplatin for 40 min without (○) and with (□) added 40 μM dithiothreitol. Preincubation of kDNA with cisplatin inhibited topoisomerase IIα decatenation of kDNA with IC_{50} values of 0.018 ± 0.001 and 0.34 ± 0.02 μM, respectively. The curved solid lines are nonlinear least-squares calculated fits of the kDNA fluorescence-concentration data to a four-parameter logistic equation. Dithiothreitol partially protected the kDNA from cisplatin. The results shown are from a single experiment but are typical of two separate experiments. All concentrations are final concentrations in the 20-μl assay mixture.

amount of cisplatin that could have adducted to the DNA because it is unknown what fraction of the cisplatin reacted with kDNA. Given the relatively short incubation times (1 h or less) relative to the slow aquation (half-time of 8 h) of cisplatin that precedes binding to DNA (Davies et al., 2000), the amount of platination of the DNA is probably very small. Therefore, our results suggest that when even a small fraction of the kDNA reacted with cisplatin, the kDNA was no longer a competent substrate for topoisomerase IIα. It should be noted that in the clinical setting, the number of cisplatin adducts per base pair is probably very low. Although we have not directly measured platinated-DNA adducts, our experimental conditions make it likely that only a small fraction of kDNA reacted with cisplatin consistent with our in vitro results having potential relevance to a clinical or cellular setting.

Cisplatin Inhibits the Catalytic Activity of Topoisomerase IIα. In the experiments shown in Fig. 3A, various concentrations of cisplatin were first incubated for 40 min at 37°C in the absence or presence of 40 μM dithiothreitol. Topoisomerase IIα was then added to each of these solutions for 40 min on ice (to preserve its activity), followed by addition of kDNA to start the reaction. In the absence of added dithiothreitol, the decatenation activity was potently inhibited with an IC_{50} value of 0.11 μM. The purified topoisomerase IIα preparation contained dithiothreitol resulting in a final concentration of 0.12 μM in the assay mixture. Therefore, the IC_{50} value observed in the absence of any added dithiothreitol would probably be even lower. The IC_{50} value was 6.0 μM in the presence of 40 μM dithiothreitol. These results suggest that dithiothreitol partially protected the topoisomerase IIα from inactivation with dithiothreitol by acting as a sacrificial scavenger of the cisplatin by protecting sulfhydryl or other reactive protein groups. On the other hand, the cisplatin-dithiothreitol adduct may itself also have some potential to inhibit topoisomerase IIα.

The experiments shown in Fig. 3B were carried out as described above for Fig. 3A except that the topoisomerase IIα incubation time with cisplatin was reduced to 1 min before addition of kDNA to start the decatenation reaction. The 11-fold increase in the IC_{50} value to 1.2 μM in the absence of added dithiothreitol (compared with Fig. 3A results) shows that the cisplatin-mediated inhibition depended upon the incubation time. In the presence of added dithiothreitol, the IC_{50} value was essentially unchanged at 8.3 μM, compared with the incubation of cisplatin with dithiothreitol shown in Fig. 3A (IC_{50} of 6.0 μM). In other words, when dithiothreitol and cisplatin were preincubated the inactivation of topoisomerase IIα was independent of whether the enzyme was subsequently incubated (in the cold) with cisplatin/dithiothreitol for 1 min or 40 min before starting the decatenation reaction. These results are consistent with the spectrophotometric results of Fig. 1A, which showed that by 40 min cisplatin would have largely reacted with dithiothreitol and largely prevented its ability to inhibit topoisomerase IIα. It should be noted in these experiments that the free cisplatin may also react with the kDNA during the course of the assay.

Topoisomerase IIα Does Not Decatenate Cisplatin-Treated kDNA and Cisplatin-Treated kDNA Does Not Inhibit the Decatenation Activity of Topoisomerase IIα. Although the results of Fig. 2 showed that addition of cisplatin to kDNA resulted in the inhibition of kDNA decatenation, these results do not distinguish whether cisplatin acted by inhibiting topoisomerase IIα, through reaction with the kDNA substrate, or both. Thus, studies were also undertaken to determine whether kDNA pretreated with cisplatin was a competent substrate for the topoisomerase IIα decatenation reaction. As can be seen from the results in Fig. 4,

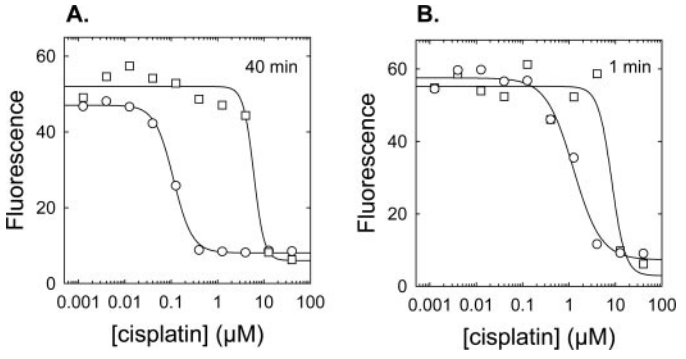

Fig. 3. Cisplatin inhibits the decatenation activity of topoisomerase IIα. A, effect of cisplatin on the catalytic activity of topoisomerase IIα. Various concentrations of cisplatin were preincubated for 40 min without (○) and with (□) added 40 μM dithiothreitol at 37°C. Topoisomerase IIα was then added and a further incubation at 0°C was carried out for 40 min. The treated topoisomerase IIα was then added to the assay mixture which contained kDNA. Preincubation of topoisomerase IIα with cisplatin in these experiments resulted in inhibition of the decatenation of kDNA with IC_{50} values of 0.11 ± 0.06 and 6.0 ± 1.5 μM, without and with added 40 μM dithiothreitol, respectively. The concentration of dithiothreitol in the assay mixture contributed from the topoisomerase IIα stock solution was 0.12 μM. The concentration of topoisomerase II was 1.5 nM in the assay mixture. The results shown are from a single experiment but are typical of two separate experiments. B, treatment was as described above, except that various concentrations of cisplatin were preincubated with topoisomerase IIα at 0°C for 1 min rather than 40 min. Preincubation of topoisomerase IIα with cisplatin in these experiments resulted in inhibition of the decatenation of kDNA with IC_{50} values of 1.2 ± 0.2 and 8.3 ± 2.3 μM, without and with added 40 μM dithiothreitol, respectively. The concentration of dithiothreitol in the assay mixture from the topoisomerase IIα stock was 0.12 μM. All concentrations are final concentrations in the 20-μl assay mixture. The curved solid lines are nonlinear least-squares calculated fits of the fluorescence-concentration data to a four-parameter logistic equation.

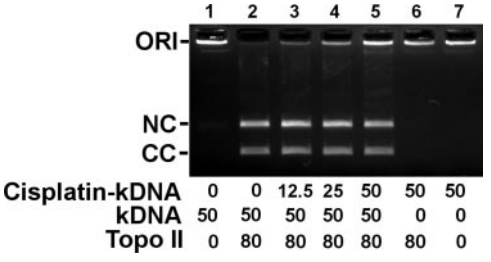

Fig. 4. Topoisomerase IIα does not decatenate cisplatin-treated kDNA and cisplatin-treated kDNA does not inhibit the decatenation activity of topoisomerase IIα. In the fluorescent image of the ethidium bromide gel, the numbers below each lane are the amount of each component in the reaction mixture in nanograms. The unlabeled slower running bands are intermediate size catenanes. As shown in lane 1, the high molecular weight kDNA did not move from the loading well origin (ORI). As shown in lane 2, topoisomerase II completely decatenated the kDNA to produce a combination of nicked open circular (NC) kDNA and closed circular (CC) decatenated kDNA. As shown in lane 6, topoisomerase IIα had no detectable decatenation activity toward cisplatin-treated kDNA. Lanes 3 to 5 show that the addition of 12.5, 25, or 50 ng of cisplatin-treated kDNA to the reaction mixture did not affect the ability of topoisomerase IIα to decatenate untreated kDNA. The values below each lane of the gel are the amounts in nanograms of DNA in the cisplatin-treated kDNA, of untreated kDNA, and of topoisomerase II protein, respectively, in the 20-μl assay mixture. Other replicates (not shown) were run with authentic marker decatenated kDNA (TopoGEN). Topo II, topoisomerase IIα.

lanes 1, 6, and 7, cisplatin-treated kDNA was not a substrate for topoisomerase IIα because cisplatin-treated kDNA did not move from the origin, either in the presence or absence of topoisomerase IIα (lanes 6 and 7, respectively), similar to the untreated control in the absence of topoisomerase IIα (lane 1).

Experiments were also carried out to determine whether cisplatin-treated kDNA inhibited the ability of topoisomerase IIα to decatenate untreated kDNA. As shown in Fig. 4, this was done by adding various amounts of cisplatin-treated kDNA (0, 12.5, 25, or 50) to the reaction mixture that contained 80 ng of untreated kDNA. As shown in lanes 3 to 5, the addition of cisplatin-treated kDNA to the reaction mixture did not inhibit the ability of topoisomerase IIα to decatenate untreated kDNA. This result also indicates that cisplatin bound to the cisplatin-treated kDNA did not exchange with untreated kDNA or with topoisomerase IIα to inhibit their activity, at least over the time course of this assay.

Topoisomerase IIα Does Not Relax Cisplatin-Treated Supercoiled pBR322 DNA. To determine whether the inability of topoisomerase IIα to decatenate cisplatin-treated kDNA (Fig. 4) was because kDNA was a high molecular weight, highly networked substrate, experiments similar to those described above with cisplatin-treated kDNA were carried out to determine whether topoisomerase IIα relaxed supercoiled cisplatin-treated pBR322 plasmid DNA. As shown in Fig. 5A, lane 2, topoisomerase IIα completely relaxed pBR322 DNA, whereas the results of lane 6, compared with lane 7, showed that topoisomerase IIα had no detectable relaxation activity toward cisplatin-treated pBR322 DNA. As shown in lanes 3 to 5, the addition of various amounts of cisplatin-treated pBR322 DNA (25, 50, or 100 ng) to the reaction mixture did affect the ability of topoisomerase IIα to relax untreated pBR322 DNA. Thus, these results differ from the cisplatin-kDNA results of Fig. 4. Whereas these results demonstrate that cisplatin-treated pBR322 DNA is not a competent substrate for topoisomerase IIα, they also show that cisplatin-treated pBR322 DNA can inhibit the ability of topoisomerase IIα to relax untreated pBR322 DNA.

Inhibition of Topoisomerase IIα Activity by Cisplatin Is Not Accompanied by Stabilization of the Cleavable Complex. Several widely used anticancer agents, including etoposide, are thought to be cytotoxic by virtue of their ability to stabilize a covalent topoisomerase II-DNA intermediate (the cleavable complex) (Fortune and Osheroff, 2000; Li and Liu, 2001). Thus, cleavage assay experiments as described previously (Burden et al., 2001) were carried out using etoposide as a control to see whether cisplatin stabilized the cleavable complex. As shown in Fig. 5B, lane 3, the addition of etoposide to the experimental mix containing topoisomerase IIα and supercoiled pBR322 DNA induced formation of linear pBR322 DNA. Linear DNA was identified by comparison with linear pBR322 DNA produced by action of the restriction enzyme HindIII acting on a single site on pBR322 DNA (data not shown). However, as shown in lanes 4 to 7, the addition of 2.5, 5, 10, or 25 μM cisplatin to the reaction mixture induced little or no detectable formation of cleaved linear pBR322 DNA. The results of Fig. 5B, lanes 4 to 7, also show that the addition of 2.5, 5, 10, or 25 μM cisplatin to the reaction mixture progressively inhibited the strand passing activity of topoisomerase IIα, as indicated by the progressive loss of relaxed pBR322 DNA relative to supercoiled pBR322, thus demonstrating inhibition of relaxation. This result is consistent with cisplatin-induced inhibition of decatenation where kDNA was the substrate (Fig. 2).

Comparison of the Effects of Cisplatin on the Growth of a K562 Cell Line with the K/VP.5 Cell Line with a Decreased Level of Topoisomerase IIα. One method by which cancer cells increase their resistance to topoisomerase II poisons is by lowering their level or activity of topoisomerase II (Ritke et al., 1994a; Fortune and Osheroff, 2000). With less topoisomerase II in the cell, cells produce fewer DNA strand breaks, and topoisomerase II poisons are less lethal to cells. These cell lines provide a convenient way to test whether a drug that inhibits topoisomerase II acts as a topoisomerase II poison. On the other hand, a lack of change in sensitivity of a putative topoisomerase II poison to a cell line with a lowered topoisomerase II level can be taken to indicate that poisoning of topoisomerase II is not a significant mechanism for this particular agent. We previously showed that the K/VP.5 cell line with acquired resistance to etoposide contained one-fifth the topoisomerase IIα content of the parental K562 cells (Ritke and Yalowich, 1993; Ritke et al., 1994a,b; Fattman et al., 1996). The averaged data for the cisplatin-mediated inhibition growth of the K562 and K/VP.5

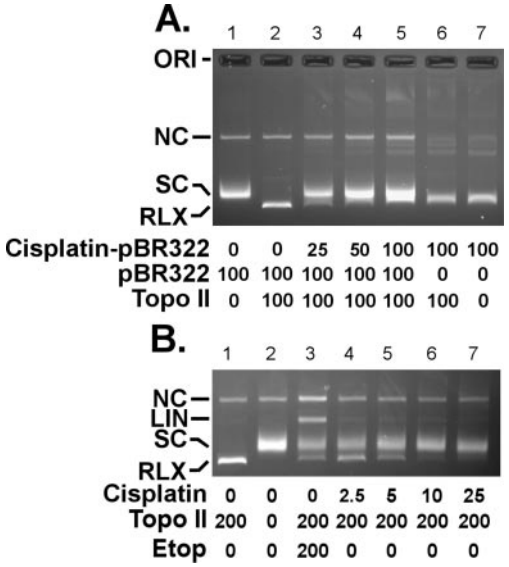

Fig. 5. Effect of cisplatin- and cisplatin-treated DNA on the topoisomerase IIα-mediated relaxation and cleavage of pBR322 DNA. A, this fluorescent image of the ethidium bromide-stained gel shows that topoisomerase IIα did not relax cisplatin-treated supercoiled (SC) pBR322 plasmid DNA. The numbers below each lane are the amount of each component in the reaction mixture in nanograms. As shown in lane 2, topoisomerase IIα completely relaxed pBR322 DNA (RLX). As shown in lane 6, topoisomerase IIα had no detectable relaxation activity toward cisplatin-treated pBR322 DNA. As shown in lane 7, cisplatin-treated pBR322 DNA had a slightly greater electrophoretic mobility than untreated pBR322 DNA and also increased the mobility of nicked circular pBR322 DNA. Lanes 3 to 5 show that the addition of 25, 50, or 100 ng of cisplatin-treated pBR322 DNA to the reaction mixture did affect the ability of topoisomerase IIα to relax untreated pBR322 DNA. B, this gel shows that the addition of cisplatin to the reaction mixture induced little formation of linear DNA (LIN), whereas treatment with etoposide produced a much greater amount of linear DNA (lane 3). The numbers below each lane are the amount of topoisomerase IIα protein in nanograms and the cisplatin and etoposide concentrations in micromolar. As shown in lane 3, etoposide (Etop) induced the formation of linear DNA. Lanes 4 to 7 show that the addition of 2.5, 5, 10, or 25 μM cisplatin to the reaction mixture progressively inhibited the relaxation activity of topoisomerase IIα. Topo II, topoisomerase IIα.

cell lines are shown in Fig. 6. IC_{50} values of 1.65 ± 0.13 and 2.23 ± 0.17 μM (n = 4) were determined for the cisplatin-mediated inhibition growth of the K562 and K/VP.5 cells, respectively. This decrease in sensitivity to cisplatin displayed by the K/VP.5 cell line barely achieved significance (p = 0.04, paired t test). Thus, it can be concluded that cisplatin does not exert its cellular activity by poisoning topoisomerase IIα. These results are consistent with the lack of cisplatin-induced poisoning of isolated topoisomerase IIα to form double-stranded linearized pBR322 DNA (Fig. 5B).

Cisplatin Forms Adducts with Cysteine Sulfhydryl Groups on Topoisomerase IIα. Maleimide compounds have previously been suggested to bind to topoisomerase IIα sulfhydryl groups to inhibit catalytic activity of the enzyme (Jensen et al., 2002). In addition, the sulfhydryl-reactive fluorescent maleimide compound ThioGlo-1 has been used previously to demonstrate that sulfhydryl-reactive compounds react with sulfhydryl groups on proteins and topoisomerase IIα (Fabisiak et al., 2002; Yalowich et al., 2004) and with glutathione in cells (Kagan et al., 2001). As shown in Fig. 7A, lane 1, a 1-h incubation of 200 μM ThioGlo-1 with 1.1 μg of topoisomerase IIα in the presence of 1% SDS resulted in a prominent fluorescence band associated with topoisomerase IIα. Incubation of topoisomerase IIα for 30 min with 100 μM *N*-ethylmaleimide before addition of ThioGlo-1 prevented the formation of the fluorescent ThioGlo-1-topoisomerase IIα band (lane 3). In addition, preincubation of 50 μM dithiothreitol with *N*-ethylmaleimide prevented *N*-ethylmaleimide inhibition of the topoisomerase IIα-ThioGlo-1 adduct, (compare lanes 3 and 4). Together, these results indicate that ThioGlo-1 bound specifically to free cysteine sulfhydryl groups on topoisomerase IIα. A 30-min incubation of 20 to 100 μM cisplatin with topoisomerase IIα before ThioGlo-1 addition resulted in a concentration-dependent decrease in the amount of topoisomerase IIα-ThioGlo-1 fluorescence adduct produced (lanes 5, 7, and 9). The cisplatin-mediated inhibition of the formation of the topoisomerase IIα-ThioGlo-1 adduct is quantified in Fig. 7B. Treatment of topoisomerase IIα with 100 μM cisplatin resulted in a significant increase (p = 0.015) in the inhibition of the topoisomerase IIα-ThioGlo-1 adduct compared with treatment with 20 μM cisplatin. The treatment of topoisomerase IIα with cisplatin was also carried out in the presence of 50 μM dithiothreitol to determine whether this sulfhydryl compound could antagonize the inhibition of the formation of the topoisomerase IIα-ThioGlo-1 adduct. Dithiothreitol would be expected to react with cisplatin as was shown in Fig. 1 and compete for binding to topoisomerase IIα sulfhydryl groups. The results of Fig. 7A indicate that 50 μM dithiothreitol alone did not interfere with 200 μM ThioGlo-1 binding to topoisomerase IIα (compare lanes 1 and 2). Dithiothreitol preincubation with cisplatin partially protected against cisplatin binding to sulfhydryl groups on topoisomerase IIα (compare lanes 5 and 6, lanes 7 and 8, and lanes 9 and 10). As shown in Fig. 7B, 50 μM dithiothreitol significantly (p = 0.011) antagonized 100 μM cisplatin inhibition of the formation of the topoisomerase IIα-ThioGlo-1 adduct. Together, these results suggest that cisplatin may form adducts with free cysteine sulfhydryl groups on topoisomerase IIα. However, these results do not exclude the possibility that cisplatin also reacted with other critical reactive groups on topoisomerase IIα.

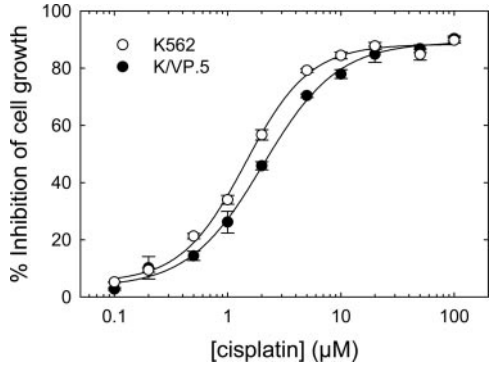

Fig. 6. Inhibition of growth of K562 (○) and K/VP.5 (●) leukemia cells by cisplatin. Cells were treated with cisplatin for 48 h before assessment of growth inhibition by counting cells. The extent of growth in drug-treated versus control cells was expressed as percentage of inhibition of control growth. The curved solid lines are nonlinear least-squares fits to a four-parameter logistic equation using the averaged percentage of growth inhibition values from four separate experiments. Because not all cisplatin concentrations were used in all experiments, error bars represent S.E.M. (at 50, 20, 10, 5, 2, and 1 μM cisplatin) or the range (at 100, 0.5, 0.2, and 0.1 μM cisplatin).

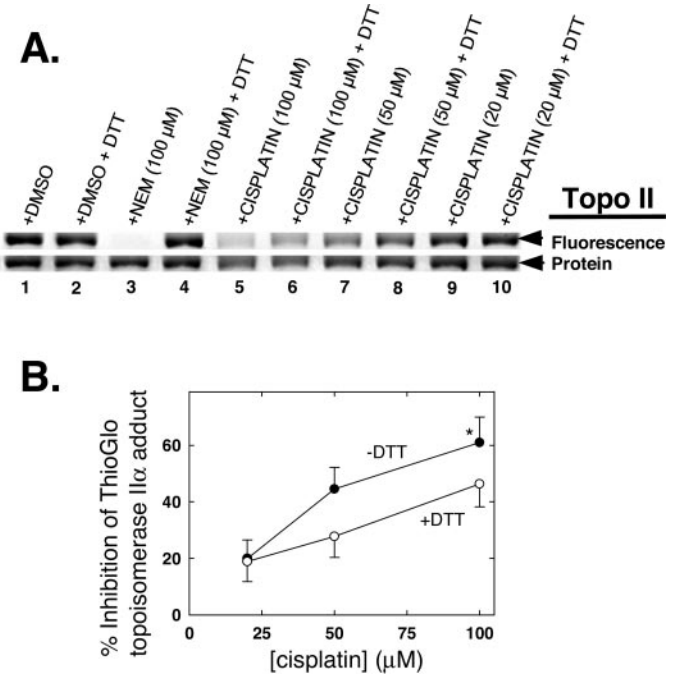

Fig. 7. Cisplatin inhibits formation of topoisomerase IIα adducts with the fluorescent sulfhydryl-reactive reagent ThioGlo-1. A, top band, negative image of the 170-kDa topoisomerase IIα-ThioGlo-1-fluorescence adduct in the electrophoresis gel. Bottom band, white light-positive image of the same bands stained with the protein stain Coomassie Blue. The sulfhydryl-reactive compound *N*-ethylmaleimide (NEM) completely inhibited formation of the ThioGlo-1-topoisomerase IIα fluorescence adduct. This adduct formation was antagonized by the addition of dithiothreitol (DTT) to the reaction mixture. Cisplatin also inhibited formation of the fluorescent ThioGlo-1-topoisomerase IIα adduct in a concentration-dependent manner. Dithiothreitol antagonized the action of cisplatin. B, cisplatin-mediated inhibition of the ThioGlo-1-topoisomerase IIα adduct. The inhibitory effects of cisplatin in the absence (●) or presence (○) of 50 μM dithiothreitol on ThioGlo-1-topoisomerase IIα fluorescence adduct formation was quantified after assessing the ratio of the fluorescence versus protein signal for each experimental condition. Results shown are the mean ± S.E.M. from six to eight separate experiments. The * indicates a significance of p = 0.011 compared with the dithiothreitol treatment. DMSO, control containing dimethyl sulfoxide. Topo II, topoisomerase IIα.

The MALDI Mass Spectrometry Determination of Free and Disulfide-Bonded Cysteine Sulfhydryl Groups on Topoisomerase IIα. MALDI MS analysis of the topoisomerase IIα digest that was fully reduced and alkylated with iodoacetamide resulted in a very complex mass spectrum. However, μ-HPLC fractionation significantly simplified the data interpretation and simultaneously improved the signal-to-noise ratio. Preliminary peptide mapping studies were carried out to confirm the identity of human topoisomerase IIα. Mass lists from the spectra of all 40 fractions were combined and submitted to a ProFound search and allowed a confident identification of human DNA topoisomerase IIα (SwissProt accession number P11388). A sequence coverage of 65 to 70% was obtained for different samples (two missed cleavages, 20 ppm mass tolerance). At this point, four of the 13 cysteines of the topoisomerase IIα monomer remained unidentified because they were either large hydrophobic peptides or peptides that were too small to be detected. Eight tryptic fragments containing nine cysteine residues (170, 216, 300, 392, 405, 455, 733, 997, and 1008) were recovered from this digest (Table 1). These peptides were the subject of the following detailed study that aimed to detect free and disulfide-bonded cysteines on topoisomerase IIα.

After the first alkylation of topoisomerase IIα with iodoacetamide, the excess iodoacetamide was removed. The topoisomerase IIα was then fully reduced and alkylated with iodoacetic acid. Fragments that underwent alkylation with iodoacetic acid (e.g., peptides containing disulfide bonds) displayed a mass shift 0.984 Da greater than those alkylated with iodoacetamide. The relative intensities of the peaks corresponding to either iodoacetamide or iodoacetic acid alkylation provided a semiquantitative estimate (Table 1) of the degree of cysteine reduction of topoisomerase IIα. Figure 8A illustrates this approach for the peptide SFGSTCQLSEK (387–397), which was found completely alkylated with iodoacetamide after such a treatment. The peptide NSTECTLILTEGDSAK (451–166) was found in two consecutive HPLC fractions (Fig. 8, B and C) mostly alkylated with iodoacetic acid, which indicated its involvement in a disulfide bond. Table 1 that summarizes these results shows that at least five cysteine residues were detected mainly in a free sulfhydryl state (cysteines 170, 216, 300, 392, and 405).

An attempt was also made to confirm the findings mentioned above by identification of disulfide-containing fragments in the digest of the nonreduced topoisomerase IIα. This is a typical approach for MS assignment of disulfide bonds and involves comparing masses of nonreduced and reduced (alkylated) digests of protein that had been digested with intact disulfide bonds (Gorman et al., 2002). The masses of all fragments that disappeared after reduction were first inspected to see whether they corresponded to the combined masses of cysteine-containing fragments in any combination (Krokhin et al., 2003) and were analyzed by MS/MS afterward. Two disulfide bonds (Cys427-Cys455 and Cys997-Cys1008) were detected, indicating a correct assignment from the previous experiment had been made; this also showed that these cysteines were mostly oxidized on topoisomerase IIα. It is noteworthy that the small peptide CSAVK (427–431) was not detected in any sample preparation involved reduction and alkylation. However, it was found bonded to peptide NSTECTLILTEGDSAK (451–466) in a larger disulfide-linked fragment. Peptide VLFTCFK (Table 1) containing Cys733 was found alkylated after iodoacetic acid treatment, indicating that it was disulfide-bonded. However, its corresponding disulfide fragment was not located and thus the cysteine to which it was bonded could not be identified. The remaining possible candidates for the disulfide partner for Cys733 are Cys104, Cys862, and Cys1145. Of these, Cys862 is considered to be the best partner candidate because of its proximity to Cys733 in the primary sequence.

Discussion

Our studies showed that when kDNA or pBR322 DNA was treated with cisplatin, neither DNA was able to function as a

TABLE 1

Assignment of free cysteines and disulfide bonds for the 13 cysteine residues on topoisomerase IIα

Topoisomerase IIα was completely reduced, alkylated with iodoacetamide, and digested with trypsin for complete peptide mapping. Free cysteines were first alkylated with iodoacetamide and those in disulfide bonds were then alkylated with iodoacetic acid after complete reduction.

Predicted Tryptic Peptide Fragment (sequence number range)	MH^+ Calc. for Iodoacetamide Alkylation	MH^+ Exp. for Iodoacetamide Alkylation	MH^+ Exp. for Iodoacetamide/Iodoacetic Acid Alkylation	Found as Free Cysteine	MH^+ Exp. for Disulfide Fragment
	Da	*Da*	*Da*	*%*	*Da*
MSCIR (102–106)	666.307	N.F.	N.F.		N.F.
LCNIFSTK (169–176)	982.503	982.503	982.497 983.492	~80	N.F.
AGEMELKPFNGEDYTCITFQPDLSK[a] (201–225)	2890.328	2891.317	2891.313	~100	N.F.
WEVCLTMSEK (297–306)	1282.581	1282.599	1282.586	~100	N.F.
SFGSTCQLSEK (387–397)	1243.563	1243.565	1243.557	~100	N.F.
AAIGCGIVESILNWVK (401–416)	1729.931	1729.933	1729.935 1730.920	~90	N.F.
CSAVK (427–431)	564.282	N.F.	N.F.		
NSTECTLILTEGDSAK (451–466)	1738.817	1738.809	1738.816 1739.808	~10	2186.019
VLFTCFK (729–735)	914.481	914.485	915.473	~0	N.F.
VEPEWYIPIIPMVLINGAEGIGTGWSCK (836–863)	3129.579	N.F.	N.F.		N.F.
LQTSLTCNSMVLFDHVGCLK (991–1010)	2323.152	2323.135	2325.107	~0	2207.048
DELCR (1142–1146)	692.304	N.F.	N.F.		N.F.

N.F., not found.

[a] Underlined asparagine residue found converted into aspartic acid due to deamidation.

competent substrate for topoisomerase IIα. The loss of DNA substrate competency occurred at low nanomolar cisplatin concentrations and could be partially antagonized by dithiothreitol in the pretreatment incubation mixture (Fig. 2). Although low nanomolar cisplatin was reacted with nanomolar kDNA, there was potential for supraclinical DNA-adduct level formation to occur. However, the 1-h or less incubations, relative to the slow process of cisplatin aquation and subsequent DNA platination (Davies et al., 2000), suggest that very low levels of cisplatin-kDNA adduct formation had occurred, leading to the loss of the ability topoisomerase IIα to decatenate this substrate. Thus, this result is consistent with a mechanism by which cisplatin can perturb topoisomerase II function in situ. The ability of dithiothreitol to partially antagonize these effects can be explained from our spectrophotometric kinetic studies that showed that cisplatin reacted (half-time of 6 min) with dithiothreitol to form a complex similar to other sulfhydryl compounds (Ishikawa and Ali-Osman, 1993; Dabrowiak et al., 2002; Sadowitz et al., 2002).

Using mixtures of untreated and cisplatin-treated kDNA (Fig. 4), we also showed that topoisomerase IIα maintained its catalytic activity in the presence of cisplatin-treated kDNA. We suggest that cisplatin bound to kDNA did not exchange either with the untreated kDNA or with reactive groups on topoisomerase IIα to inhibit it. We further conclude that the cisplatin-kDNA adduct was itself not an inhibitor of topoisomerase IIα decatenation. However, the cisplatin-pBR322 DNA complex did partially inhibit topoisomerase IIα relaxation (Fig. 5A). The reason for the difference in the two types of DNA is unknown, but it may be because of the reaction of cisplatin-pBR322 DNA with reactive groups on topoisomerase IIα or with untreated DNA. This difference might also arise because kDNA consists of networked high molecular weight interlinked plasmid DNA compared with the pBR322 DNA minicircles. These two types of DNA could thus differ in their reactivity. Cisplatin binds at two neighboring G-N^7 sites on DNA to form primarily an intrastrand bidentate adduct, but small amounts of interstrand bidentate (Reedijk, 1996) and even monodentate adducts can also form (Eder et al., 1995). It is likely that these types of adducts could impair the ability of topoisomerase IIα to process cisplatin-treated DNA either by preventing double-strand breaks and/or separation required for strand passage, or by preventing the cisplatin-treated DNA from binding to topoisomerase IIα. Thus, our results indicating that cisplatin-treated DNA is not a competent substrate for topoisomerase IIα suggest an additional mechanism by which cisplatin may exert its growth inhibitory effects.

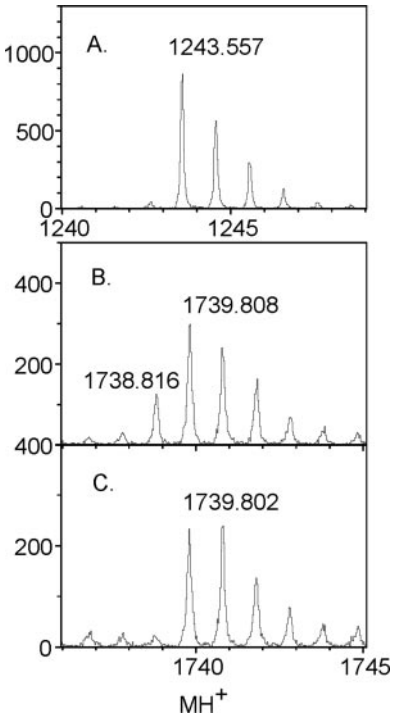

Fig. 8. Mass spectrometric detection of free and disulfide-bonded cysteines on topoisomerase IIα. A, mass spectrum of the tryptic peptide SFGSTCQLSEK (387–397) fragment found in HPLC fraction 16. This fragment was completely alkylated with iodoacetamide with a mass of 1243.557 Da (1243.563 calculated), indicating that it is found as a free cysteine on intact topoisomerase IIα. B and C, mass spectrum of the tryptic peptide NSTECTLILTEGDSAK (451–166) found in two consecutive fractions (20 and 21). This fragment was mainly alkylated with iodoacetic acid (1739.808 Da), indicating that it is disulfide-bonded.

When topoisomerase IIα was cisplatin-treated it lost its catalytic activity in a time-dependent manner, and this inhibitory activity could be partly, but not totally, antagonized by the sulfhydryl reagent dithiothreitol (Fig. 3). Given that dithiothreitol reacts with cisplatin (Fig. 1), it was not surprising that dithiothreitol antagonized cisplatin-mediated topoisomerase IIα inhibition. The fact that cisplatin that had reacted with dithiothreitol did not completely antagonize topoisomerase IIα decatenation activity might indicate that the cisplatin-dithiothreitol adduct still has the potential to inhibit topoisomerase IIα or alternatively that there are other nonsulfhydryl reactive groups on topoisomerase IIα. The cisplatin-dithiothreitol adduct may undergo secondary reactions similar to the cisplatin reaction with human albumin, in which binding to methionine sulfur results in release of NH_3 because of a *trans*-bond weakening effect, which opens up a site on platinum for further coordination (Ivanov et al., 1998). Thus, results indicating that cisplatin inhibits topoisomerase IIα provide yet another potential mechanism by which cisplatin may exert its cell growth inhibitory effects.

The cisplatin-mediated inhibition of topoisomerase IIα was not accompanied by stabilization of a covalent topoisomerase IIα-DNA intermediate (Fig. 5B). Thus, cisplatin does not act as a topoisomerase IIα poison as does etoposide and several other anticancer drugs (Fortune and Osheroff, 2000; Li and Liu, 2001). The conclusion that cisplatin is not a topoisomerase II poison is also supported by the results of Fig. 6 in which it was shown that the K/VP.5 cell line that has a 5-fold lower level of topoisomerase IIα (Ritke and Yalowich, 1993; Ritke et al., 1994a) displayed minimal decreased sensitivity to cisplatin. It should be noted that because K/VP.5 cells were selected for resistance to etoposide (Ritke et al., 1994b), the adaptation of these cells may include events other than reduction of topoisomerase IIα levels, such as reduced topoisomerase IIα phosphorylation (Ritke et al., 1994a), altered stability of drug-induced topoisomerase IIα-DNA covalent complexes (Ritke et al., 1994b), and other undocumented genetic changes that may influence the resistance patterns to cisplatin.

A study that showed that topoisomerase IIα-transfected Chinese hamster ovary cells were 10-fold resistant to the growth inhibitory effects of cisplatin (Eder et al., 1995) are not consistent with the results of Fig. 6. In that study, how-

ever, heterologous expression of Chinese hamster ovary topoisomerase IIα into mouse mammary carcinoma resulted in elevated expression of topoisomerase IIα in all phases of the cell cycle, a situation that does not exist in the K562 and K/VP.5 cells used in the present work that contain varying levels of topoisomerase IIα. Therefore, it is not clear that results presented in that article and in the present work are comparable.

Several studies have shown that topoisomerase IIα is sensitive to thiol-reactive agents such as maleimide (Jensen et al., 2002), etoposide orthoquinone (Gantchev and Hunting, 1998), a variety of other quinones, *N*-ethylmaleimide, and organic disulfides (Wang et al., 2001; Lindsey et al., 2004), and selenium compounds (Zhou et al., 2003). The potency of quinone-induced topoisomerase IIα-mediated DNA damage paralleled the rate of electrophilic addition by glutathione (Wang et al., 2001). In addition, quinone-induced DNA cleavage was abolished in a cysteine-less yeast topoisomerase II (Wang et al., 2001). These results support the concept that cysteine residues are potential targets for topoisomerase IIα inhibition. Whereas maleimide is a catalytic inhibitor of topoisomerase IIα (Jensen et al., 2002), other sulfhydryl-reactive agents such as *N*-ethylmaleimide, organic disulfides (Wang et al., 2001), selenium compounds (Zhou et al., 2003), and etoposide orthoquinone (Gantchev and Hunting, 1998) are reported to act as topoisomerase IIα poisons, at least in isolated enzyme systems. These results suggest that agents that target protein sulfhydryl groups can act as either catalytic inhibitors or poisons of topoisomerase IIα. Because cisplatin is so reactive with free protein sulfhydryl groups (Ivanov et al., 1998; Dabrowiak et al., 2002; Sadowitz et al., 2002; Hagrman et al., 2003), we considered topoisomerase IIα cysteines as possible sites responsible for the inhibition of the catalytic activity of topoisomerase IIα observed in the presence of cisplatin. Our results using the fluorescent sulfhydryl-reactive reagent, ThioGlo-1 strongly suggested that cisplatin reacted with topoisomerase IIα sulfhydryl groups (Fig. 7). Despite these in vitro results and previous literature reports of the sensitivity of topoisomerase IIα sulfhydryl groups, it is not clear that the extent of reactivity of cisplatin with topoisomerase IIα relative to other sulfhydryl containing proteins will allow for selective topoisomerase II-targeted antitumor effects of cisplatin in a cellular context.

Because the topoisomerase IIα monomer contains 13 cysteine residues as possible targets for cisplatin, we also analyzed isolated enzyme to identify which cysteines were free or disulfide-bonded. Using μ-HPLC-MALDI MS analysis with differential alkylation of free and disulfide-bonded cysteines with iodoacetamide and iodoacetic acid, respectively, we found that at least five cysteine residues (170, 216, 300, 392, and 405) were present mainly as reduced free sulfhydryl groups on topoisomerase IIα. Five cysteines (427, 455, 733, 997, and 1008) were found to be disulfide-bonded. For four of them, their disulfide partners were assigned. Thus, a Cys997-Cys1008 bond was detected on a single tryptic peptide (991–1010), whereas Cys427 formed a disulfide bond with Cys455. Cys733 was also identified as being disulfide-bonded, but its partner cysteine could not be identified. Overall these results indicate the subset of cysteine sulfhydryl groups present as free cysteines that are the likely targets of cisplatin adduction to topoisomerase IIα that result in its inactivation. Further studies are underway to establish the specific cysteines that are targets for interaction with cisplatin.

Overall, our studies set the stage for further characterization of the cellular consequences of cisplatin effects at the level of topoisomerase II used alone or in combination with topoisomerase II poisons. The combination of cisplatin and etoposide has long provided the backbone for effective therapy of testicular cancers (Einhorn, 2002). In addition, cisplatin-mediated inhibition of topoisomerase II activity may contribute to the demonstrated enhancement of cytotoxicity when cisplatin is combined with a variety of other anticancer agents (Crul et al., 2002). In conclusion, the results of this study have shown that cisplatin-treated plasmid DNA is not a competent substrate for topoisomerase IIα and that cisplatin inhibits topoisomerase IIα catalytic activity, possibly through reaction with critical free cysteine sulfhydryl groups on the enzyme. Overall the results obtained provide a topoisomerase IIα-based mechanism that may partially contribute to cisplatin-induced cell growth inhibition and antitumor activity.

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Address correspondence to: Dr. Brian Hasinoff, Faculty of Pharmacy, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. E-mail: b_hasinoff@umanitoba.ca