1,4-Benzoquinone Is a Topoisomerase II Poison†

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ABSTRACT: Benzene is a human carcinogen that induces hematopoietic malignancies. It is believed that benzene does not initiate leukemias directly, but rather generates DNA damage through a series of phenolic metabolites, especially 1,4-benzoquinone. The cellular consequences of 1,4-benzoquinone are consistent with those of topoisomerase II-targeted drugs. Therefore, it has been proposed that the compound initiates specific leukemias by acting as a topoisomerase II poison. This hypothesis, however, has not been supported by in vitro studies. While 1,4-benzoquinone has been shown to inhibit topoisomerase II catalysis, increases in enzyme-mediated DNA cleavage have not been reported. Because of the potential involvement of topoisomerase II in benzene-induced leukemias, we re-examined the effects of the compound on DNA cleavage mediated by human topoisomerase IIα. In contrast to previous reports, we found that 1,4-benzoquinone was a strong topoisomerase II poison and was more potent in vitro than the anticancer drug etoposide. DNA cleavage enhancement probably was unseen in previous studies due to the presence of reducing agents in reaction buffers and the incubation of 1,4-benzoquinone with the enzyme prior to the addition of DNA. 1,4-Benzoquinone increased topoisomerase II-mediated DNA cleavage primarily by enhancing the forward rate of scission. In vitro, the compound induced cleavage at DNA sites proximal to a defined leukemic chromosomal breakpoint and displayed a sequence specificity that differed from that of etoposide. Finally, 1,4-benzoquinone stimulated DNA cleavage by topoisomerase IIα in cultured human cells. The present findings are consistent with the hypothesis that topoisomerase IIα plays a role in the initiation of specific leukemias induced by benzene and its metabolites.

Benzene is one of the top 20 production chemicals in the United States (*1*, *2*). The compound is used in the manufacture of plastics and gasoline and is a combustion product of cigarette smoke (*3*−*5*). Benzene is clastogenic and carcinogenic in humans (*6*−*11*). Although exposure to the chemical induces a variety of malignancies in mice and rats, it causes primarily hematopoietic cancers in humans (*6*, *7*, *9*−*16*). The two most frequent human malignancies associated with benzene are acute myelogenous leukemia (AML)[1] and acute non-lymphocytic leukemia (*6*, *7*, *9*−*11*, *13*−*15*).

The mechanism by which benzene induces leukemias has not been fully elucidated. However, it is believed that benzene does not trigger DNA damage directly. Rather, it acts through a series of phenolic metabolites. The parent compound is metabolized to benzene oxide in the liver by cytochrome P450 2E1 (*14*, *16*−*18*) (Figure 1). While most of the oxide is cleared by conjugation to glutathione, a small proportion is converted to phenol by a nonenzymatic rearrangement. Phenol is further metabolized by cytochrome P450 2E1 to 1,4-hydroquinone, which is carried throughout the body in the bloodstream. When transported to the bone marrow, 1,4-hydroquinone ultimately is converted to 1,4-benzoquinone by the high concentration (∼3% of dry weight) of endogenous myeloperoxidase in the marrow. 1,4-Benzoquinone is metabolized back to 1,4-hydroquinone by NAD(P)H:quinone oxidoreductase 1 (NQO1) (*19*−*23*).

1,4-Benzoquinone is thought to be a critical leukemogenic metabolite of benzene (*24*, *25*). Exposure of mammalian cells to 1,4-benzoquinone generates DNA mutations, insertions, deletions, and strand breaks (*10*, *24*, *26*−*28*). In addition, the compound induces DNA recombination, sister chromatid exchange, and apoptosis (*10*, *24*, *29*−*31*). The basis for the genotoxic effects of 1,4-benzoquinone is not well defined. Since the compound triggers oxidative stress and forms adducts with both proteins and DNA, multiple pathways may be involved (*10*, *16*, *24*, *32*−*34*). It also has been suggested that 1,4-benzoquinone acts (at least in part) through an effect on topoisomerase II (*21*, *22*, *24*, *25*, *35*−*37*).

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[1] Abbreviations: AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; NQO1, NAD(P)H:quinone oxidoreductase 1; DTT, dithiothreitol.

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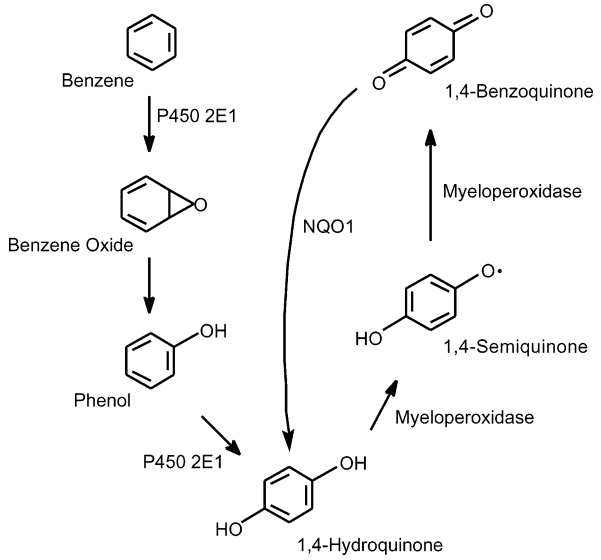

FIGURE 1: Metabolism of benzene to 1,4-benzoquinone. Benzene is metabolized to benzene oxide in the liver by cytochrome P450 2E1. A proportion of this compound is then converted to phenol by a nonenzymatic rearrangement. Phenol is further metabolized to 1,4-hydroquinone by cytochrome P450 2E1. 1,4-Hydroquinone ultimately is converted (through a semiquinone) to 1,4-benzoquinone by endogenous myeloperoxidase (*MPO*) in the bone marrow. 1,4-Benzoquinone is metabolized back to 1,4-hydroquinone by NAD(P)H:quinone oxidoreductase 1 (*NQO1*). Other pathways that clear benzene from the cell or result in the formation of other metabolites exist, but were omitted for simplicity.

Topoisomerase II is essential for proper chromosome structure and segregation and removes knots and tangles from the genetic material (*38*−*44*). The enzyme acts by passing an intact double helix through a transient double-stranded break that it creates in a separate segment of DNA (*38*−*40*, *42*, *44*, *45*). Vertebrates contain two isoforms of topoisomerase II, α and β (*46*−*53*). Topoisomerase IIα levels increase dramatically during periods of cell growth, and this isoform appears to be primarily responsible for the required roles of the enzyme during mitosis (*41*, *43*, *54*−*57*).

To maintain genomic integrity during its catalytic cycle, topoisomerase II forms covalent bonds between active-site tyrosyl residues and the 5′-DNA termini created by cleavage of the double helix (*38*−*40*, *42*, *44*, *45*, *58*−*60*). Normally, these covalent topoisomerase II-cleaved DNA complexes (known as *cleavage complexes*) are fleeting intermediates and are tolerated by the cell (*38*−*40*, *42*, *44*, *45*). However, when the concentration or longevity of cleavage complexes increases significantly, DNA strand breaks and many of the genotoxic events described above occur (*39*, *42*, *44*, *61*−*65*).

A variety of widely prescribed anticancer drugs such as etoposide kill cells by increasing physiological levels of topoisomerase II−DNA cleavage complexes (*39*, *42*, *44*, *66*−*70*). These drugs are referred to as *topoisomerase II poisons* (to distinguish them from *catalytic inhibitors* of the enzyme) because they convert this essential enzyme to a potent cellular toxin (*39*, *42*, *44*, *66*, *68*, *70*, *71*).

Although topoisomerase II is an important target for cancer chemotherapy, mounting evidence suggests that DNA cleavage mediated by the enzyme also plays a role in the generation of specific forms of leukemia (*62*, *63*, *72*−*78*). Therapy-related AMLs with characteristic translocations involving the *MLL* gene at chromosomal band 11q23 have been identified in a small proportion of cancer patients who were treated with regimens that included topoisomerase II-targeted drugs (*62*, *63*, *72*, *73*, *75*, *77*−*79*). In addition, infant AMLs with 11q23 chromosomal translocations have been linked to the maternal consumption (during pregnancy) of foods that contain high concentrations of naturally occurring topoisomerase II poisons (*74*, *76*, *80*, *81*).

It has been proposed that 1,4-benzoquinone induces leukemia by acting as a topoisomerase II poison (*21*, *22*, *24*, *25*, *36*, *37*). However, attempts to demonstrate stimulation of topoisomerase II-mediated DNA cleavage by the compound have proven unsuccessful (*25*, *36*, *37*). In most studies, only inhibition of enzyme catalysis was observed (*35*−*37*).

Two recent findings prompted us to re-examine the effects of 1,4-benzoquinone on human topoisomerase IIα. First, individuals who are heterozygous or homozygous for the *C609T* polymorphism of the *NQO1* gene display an increasingly higher risk for leukemias with 11q23 chromosomal translocations (*19*−*23*). Especially prominent are ALLs in which the *MLL* gene becomes fused to the *AF-4* gene on chromosome 4 (*22*). The *C609T* polymorphism encodes an inactive form of NQO1, the enzyme that metabolizes 1,4-benzoquinone to the less reactive 1,4-hydroquinone (*19*−*23*). Second, some sulfhydryl-reactive chemicals, such as quinones, have been shown to increase levels of DNA cleavage mediated by human topoisomerase IIα (*82*−*85*).

Therefore, the present study assessed the effects of 1,4-benzoquinone on the DNA cleavage activity of human topoisomerase IIα in vitro and in cultured CEM leukemia cells. In contrast to previous reports, results indicate that 1,4-benzoquinone is in fact a potent topoisomerase II poison. Evidence suggests that stimulation of enzyme-mediated DNA scission is dependent on the sulfhydryl-reactive properties of 1,4-benzoquinone. DNA cleavage enhancement may not have been observed in earlier studies because the unique characteristics of sulfhydryl-reactive topoisomerase II poisons had not yet been established.

EXPERIMENTAL PROCEDURES

Enzymes and Materials. Human topoisomerase IIα was expressed in *Saccharomyces cerevisiae* (*86*) and purified as described previously (*87*, *88*), except that 0.1 mM DTT, instead of 0.5 mM, was used in the purification and storage buffers. For all of the procedures described below, residual levels of DTT in reaction mixtures never exceeded 0.4 μM. Negatively supercoiled pBR322 DNA was prepared using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. 1,4-Benzoquinone was purchased from Sigma, prepared as a 20 mM stock in deionized water, and stored at −20 °C. Etoposide (Sigma) was prepared as a 20 mM stock in 100% DMSO and stored at 4 °C. All other chemicals were analytical reagent grade.

DNA Cleavage of Plasmid Substrates. DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (*89*). Assay mixtures contained 135 nM topoisomerase IIα and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of cleavage buffer [10 mM Tris-HCl (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM NaEDTA, and 2.5% glycerol] and were assembled on ice. 1,4-Benzoquinone or etoposide (0−50 μM) was added to reaction mixtures last. DNA cleavage was initiated by

shifting samples to 37 °C, followed by incubation for 6 min. Assays that examined the effects of reducing agents on 1,4-benzoquinone contained 100 or 250 μM DTT, or 500 μM glutathione. Topoisomerase II−DNA cleavage complexes were trapped by adding 2 μL of 5% SDS, followed by 1 μL of 375 mM EDTA (pH 8.0). Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reactions were incubated for 30 min at 45 °C to digest the topoisomerase IIα. Samples were mixed with 2 μL of 60% sucrose in 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF, heated for 3 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3), 2 mM EDTA that contained 0.5 μg/mL ethidium bromide. DNA cleavage was monitored by the conversion of negatively supercoiled plasmid DNA to linear molecules. DNA bands were visualized by ultraviolet light and quantified using an Alpha Innotech digital imaging system.

To determine the reversibility of DNA cleavage that was induced by 1,4-benzoquinone, 1 μL of 375 mM EDTA, 1 μL of 5M NaCl, 2 μL of 2.5 mM DTT, or 2 μL of 5 mM glutathioine was added to assay mixtures prior to treatment with SDS. To determine whether cleaved DNA was protein-linked, proteinase K treatment was omitted.

Order-of-addition experiments were carried out to assess the effects of 1,4-benzoquinone on human topoisomerase IIα in the absence of DNA. In these experiments, the compound (or an equivalent amount of H_2O) was incubated with the enzyme for 0−3 min at 37 °C in 10 μL of cleavage buffer. Cleavage reactions were initiated by adding negatively supercoiled pBR322 DNA in 10 μL of cleavage buffer. The concentrations of topoisomerase IIα, plasmid molecules, and 1,4-benzoquinone in the final reaction mixtures were 135 nM, 5 nM, and 25 μM, respectively.

Site-Specific DNA Cleavage Induced by 1,4-Benzoquinone. DNA sites cleaved by human topoisomerase IIα in long linear DNA fragments were mapped as described by O'Reilly and Kreuzer (*90*). A linear 4330-bp fragment (*Hin*dIII/*Eco*RI) of pBR322 plasmid DNA singly labeled with [γ-^{32}P]-phosphate on the 5′-terminus of the *Hin*dIII site was used as the cleavage substrate. Reaction mixtures contained 0.35 nM DNA fragments and 60 nM human topoisomerase IIα in 50 μL of cleavage buffer. Assays were carried out in the absence of compound or in the presence of 50 or 100 μM 1,4-benzoquinone, or 100 μM etoposide. Reaction mixtures were incubated for 10 min at 37 °C. Cleavage intermediates were trapped by adding 5 μL of 10% SDS, followed by 5 μL of 250 mM NaEDTA (pH 8.0), and topoisomerase IIα was digested with proteinase K (5 μL of a 0.8 mg/mL solution) for 30 min at 45 °C. Reaction products were precipitated twice in 100% ethanol, dried, and resuspended in 40% formamide, 8.4 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanole FF. Samples were subjected to electrophoresis in a 6% acrylamide gel, which was then fixed in 10% methanol/10% acetic acid for 5 min and dried. DNA cleavage products were visualized on a Bio-Rad Molecular Imager FX.

DNA sites cleaved by human topoisomerase IIα in oligonucleotide substrates were determined as described previously (*91*). Two independent double-stranded substrates were prepared on an Applied Biosystems DNA synthesizer. The first was a 63-bp oligonucleotide corresponding to residues 2565−2627 of the *MLL* gene and its complement. This substrate spans a previously mapped leukemic breakpoint at position 2595 (*92*). The sequences of the top and bottom strands were 5′-AAGAGGAAATCAGCAC-CAACTGGGGGAATGAATAAGAACTCCCAT-TAGCAGGTGGGTTTAGCG-3′ and 5′-CGCTAAACCCA-CCTGCTAATGGGAGTTCTTATTCATTCCCCCAGTTGGT-GCTGATTTCCTCTT-3′, respectively. The second substrate was a 47-bp oligonucleotide corresponding to residues 80−126 of pBR322 and its complement (*93*−*95*). The sequences of the top and bottom strands were 5′-CCGTGTATGAAATC-TAACAATG↓CGCTCATCGTCATCCTCGGCACCGT-3′ and 5′-ACGGTGCCGAGGATGACGATG↓AGCGCATT-GTTAGATTTCATACACGG-3′, respectively. This substrate contains a single strong cleavage site for topoisomerase II that has been well characterized (*93*−*95*). Points of scission are denoted by arrows. Oligonucleotides were labeled on the 5′-termini of the top strands with [γ-^{32}P]phosphate and purified as described previously. In all cases, double-stranded DNA substrates were generated by annealing equimolar amounts of complementary oligonucleotides at 70 °C for 10 min and cooling to 25 °C.

Reaction mixtures contained 135 nM human topoisomerase IIα and 10 nM double-stranded oligonucleotide in 20 μL of cleavage buffer in 0−50 μM 1,4-benzoquinone or etoposide. Reactions were incubated at 37 °C for 15 min. DNA cleavage products were trapped by the addition of 2 μL of 10% SDS, followed by 1 μL of 375 mM EDTA (pH 8.0). Samples were digested with proteinase K, ethanol precipitated, and resolved by electrophoresis in 7 M urea, 14% polyacrylamide gels in 100 mM Tris−borate (pH 8.3), 2 mM EDTA. DNA cleavage products were visualized and quantified on a Bio-Rad Molecular Imager FX.

DNA Ligation. Two different assay systems were used to characterize the effects of 1,4-benzoquinone on DNA ligation mediated by human topoisomerase IIα. The first monitored the ability of the enzyme to reseal DNA strand breaks that it generated in negatively supercoiled pBR322 plasmid DNA (*96*). DNA cleavage/religation equilibria were established as described above under "DNA Cleavage of Plasmid Substrates" in the absence of compound or in the presence of 25 μM 1,4-benzoquinone, or 50 μM etoposide (these concentrations induce equivalent levels of enzyme-mediated DNA cleavage). Religation was initiated by shifting reaction mixtures from 37 to 0 °C, and reactions were stopped at time points up to 30 s by the addition of 2 μL of 5% SDS. One microliter of 375 mM NaEDTA (pH 8.0) was added, and samples were processed and analyzed as described for topoisomerase IIα cleavage reactions that utilized plasmid substrates.

The second assay monitored the ability of human topoisomerase IIα to ligate a DNA nick whose 5′-terminal phosphate was activated by covalent attachment to *p*-nitrophenol (*91*). The presence of this activating group mimics the covalent bond between the DNA and the active-site tyrosine that is formed during the scission event (*91*, *97*). DNA ligation reactions were carried out according to Bromberg et al. (*91*). The substrate for this assay was based on the 47-mer pBR322 substrate described in the previous section with the following exceptions. An oligonucleotide spanning the 5′-terminus to the point of topoisomerase II scission on the top strand was synthesized, labeled on its 5′-termini with [γ-^{32}P]phosphate, and purified as above. An

oligonucleotide extending from the point of scission to the 3′-terminus of the top strand was synthesized and 5′-activated with *p*-nitrophenol (*91*). Equimolar amounts of these oligonucleotides were annealed to the intact complementary bottom strand by incubating at 70 °C for 10 min and cooling to 25 °C.

Briefly, ligation assays contained 135 nM topoisomerase IIα and 10 nM activated nicked double-stranded oligonucleotide in a total of 20 μL of 10 mM Tris-HCl (pH 7.9), 135 mM KCl, 7.5 mM $CaCl_2$, 0.1 mM EDTA, and 2.5% glycerol. Reactions were carried out in the presence of 0−500 μM 1,4-benzoquinone or etoposide. Reaction mixtures were incubated at 37 °C for 48 h, and ligation was stopped by the addition of 2 μL of 10% SDS, followed by 1 μL of 375 mM EDTA (pH 8.0). Samples were processed, resolved in denaturing polyacrylamide gels, and analyzed as described above for oligonucleotide cleavage reactions.

DNA Relaxation. DNA relaxation reactions were carried out as described by Fortune and Osheroff (*89*). Assay mixtures contained 135 nM topoisomerase IIα and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of 10 mM Tris-HCl (pH 7.9), 135 mM KCl, 5 mM $MgCl_2$, 0.1 mM NaEDTA, 2.5% glycerol, and 250 μM ATP. 1,4-Benzoquinone (0−50 μM) was added to mixtures last. DNA relaxation was initiated by shifting samples to 37 °C, followed by incubation for 30 min. Reactions were stopped by the addition of 1 μL of 375 mM EDTA (pH 8.0), followed by the addition of 2 μL of 0.5% SDS and 77 mM EDTA. Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reactions were incubated for 30 min at 45 °C to digest the enzyme. Samples were subjected to electrophoresis in 1% agarose gels 100 mM Tris−borate (pH 8.3), 2 mM EDTA. Gels were stained for 30 min with 0.5 μg/mL ethidium bromide and DNA bands were quantified as described above.

Topoisomerase II-Mediated DNA Cleavage in Human CEM Leukemia Cells. Human CEM leukemia cells were cultured under 5% CO_2 at 37 °C in RPMI 1640 medium (Cellgro by Mediatech, Inc.), containing 10% heat-inactivated fetal calf serum (Hyclone) and 2 mM glutamine (Cellgro by Mediatech, Inc.). The *i*n vivo *c*omplex of *e*nzyme (ICE) bioassay (*98*, *99*) (as modified on the TopoGEN, Inc. website) was used to determine the effects of 1,4-benzoquinone on topoisomerase IIα-mediated DNA breaks in treated CEM cells. Exponentially growing cultures were treated with 10 μM 1,4-benzoquinone for 4 or 8 h, or with 25 μM etoposide for 1 h for comparison. Cells ($\sim 5 \times 10^6$) were harvested by centrifugation and lysed by the immediate addition of 3 mL of 1% sarkosyl. Following gentle douncing, cell lysates were layered onto a 2-mL cushion of CsCl (1.5 g/mL) and centrifuged at 80 000 rpm for 5.5 h at 20 °C. DNA pellets were isolated, resuspended in 5 mM Tris-HCl (pH 8.0) and 0.5 mM EDTA, and blotted onto nitrocellulose membranes using a Schleicher and Schuell slot blot apparatus. Covalent complexes formed between topoisomerase IIα and DNA were detected using a polyclonal antibody directed against human topoisomerase IIα (Kiamaya Biochemical Co.) at a 1:4000 dilution.

RESULTS

Although it has been proposed that 1,4-benzoquinone induces specific leukemias (at least in part) by acting as a topoisomerase II poison (*21*, *22*, *24*, *25*, *36*, *37*), enhancement of enzyme-mediated DNA cleavage has not been observed in previous studies (*25*, *36*, *37*). Only inhibition of topoisomerase II catalytic activity by the compound has been reported (*35*−*37*).

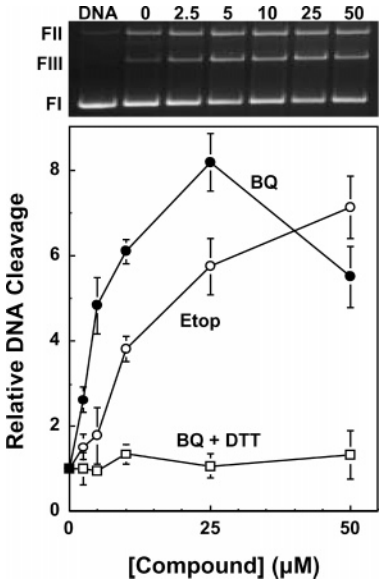

FIGURE 2: 1,4-Benzoquinone stimulates DNA cleavage mediated by human topoisomerase IIα. An ethidium bromide-stained agarose gel of DNA cleavage reactions carried out in the presence of 0−50 μM 1,4-benzoquinone is shown at the top. The mobilities of negatively supercoiled DNA (form I, FI), nicked circular plasmid (form II, FII), and linear molecules (form III, FIII) are indicated. Levels of DNA cleavage were quantified and expressed as a fold-enhancement over reactions that were carried out in the absence of 1,4-benzoquinone. Assays were carried out in the presence of 1,4-benzoquinone (BQ, ●), 1,4-benzoquinone in the presence of 100 μM DTT (BQ + DTT, □), or etoposide (Etop, ○). Error bars represent the standard deviation of three independent experiments.

The *C609T* polymorphism of the *NQO1* gene recently was correlated with an increased propensity for pediatric de novo leukemias with 11q23 chromosomal translocations (*19*−*23*). Although AMLs were observed, the highest odds ratio was found for infant ALLs in which the *MLL* gene became fused to the *AF-4* gene on chromosome 4 (*22*). This polymorphism encodes an inactive form of NQO1, the enzyme that metabolizes 1,4-benzoquinone (*19*−*23*). Since 11q23 translocations are characteristic of leukemias induced by topoisomerase II poisons (*62*, *63*, *72*, *73*, *75*, *77*−*79*), we felt that it was appropriate to re-examine the effects of 1,4-benzoquinone on topoisomerase II-mediated DNA cleavage.

1,4-Benzoquinone Enhances DNA Cleavage Mediated by Human Topoisomerase IIα. In contrast to results from previous studies (*25*, *36*, *37*), 1,4-benzoquinone enhanced DNA scission mediated by human topoisomerase IIα in a concentration-dependent manner (Figure 2). The quinone was more potent than the commonly used anticancer drug etoposide, and increased DNA cleavage ∼8-fold at a concentration of 25 μM.

These results raise the question of why DNA cleavage enhancement was not observed previously. We believe that the answer is two-fold. First, DTT and other reducing agents react with 1,4-benzoquinone and impair its ability to modify sulfhydryl residues on proteins (*35*). DTT concentrations used in earlier studies ranged from 75 to 500 μM, and in one case, the reducing agent was supplemented with 15 mM β-mer-

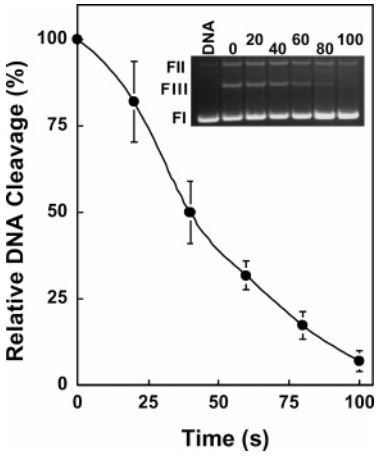

FIGURE 3: 1,4-Benzoquinone rapidly inactivates human topoisomerase IIα in the absence of DNA. Topoisomerase IIα was incubated with 1,4-benzoquinone for 0−100 s prior to the addition of DNA. Levels of DNA cleavage in reaction mixtures that were not preincubated (i.e., time zero) were set to 100%. Error bars represent the standard deviation of three independent experiments. A representative ethidium bromide-stained agarose gel of a reaction in which the enzyme was incubated with 1,4-benzoquinone for 0−100 s is shown in the inset. Supercoiled (FI), nicked circular (FII), and linear (FIII) DNA are labeled as in Figure 2.

captoethanol (*25*, *36*, *37*). The residual DTT concentration present in reaction mixtures used in this study was <0.4 μM.[2] As shown in Figure 2, the addition of 100 μM DTT to reaction mixtures blocked the ability of 1,4-benzoquinone to stimulate topoisomerase II-mediated DNA scission.

Second, while the addition of sulfhydryl-reactive topoisomerase II poisons to enzyme−DNA complexes enhances nucleic acid cleavage, incubation of these agents with the enzyme prior to the addition of DNA dramatically decreases all of the catalytic functions of topoisomerase II (*82*, *84*). In some of the previous studies, 1,4-benzoquinone was incubated with the human enzyme for up to 15 min prior to the addition of DNA (*25*). In the present study, the compound always was added to the enzyme−DNA complex. As shown in Figure 3, when human topoisomerase IIα was exposed to 1,4-benzoquinone for as little as 100 s prior to the addition of plasmid, levels of enzyme-mediated DNA cleavage dropped >90%.

Several controls were carried out to confirm that the enhanced DNA cleavage induced by 1,4-benzoquinone was mediated by topoisomerase IIα (Figure 4). First, no linear DNA was observed in reactions that contained 25 μM 1,4-benzoquinone but lacked enzyme. Second, the electrophoretic mobility of the cleaved DNA (i.e., the linear band) was dramatically reduced in the absence of proteinase K treatment, indicating that all of the cleaved plasmid molecules were covalently attached to topoisomerase IIα. Third, scission was reversed when EDTA or NaCl was added to reaction mixtures before cleavage complexes were trapped by the addition of SDS. This reversibility is inconsistent with an inorganic reaction. Taken together, the above findings provide strong evidence that 1,4-benzoquinone is indeed a strong topoisomerase II poison.

[2] The residual DTT in the reaction mixture comes from the final step of the topoisomerase IIα purification protocol. This compound is used as a general reducing agent to help preserve the enzyme during preparation and storage.

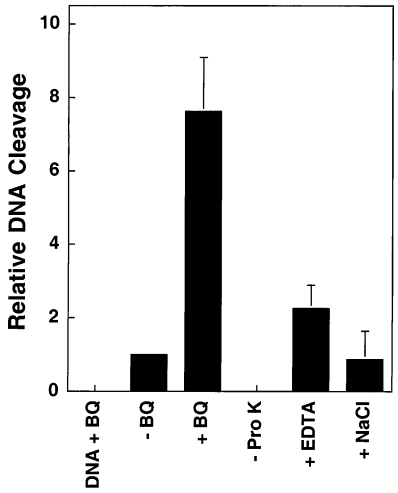

FIGURE 4: DNA cleavage in the presence of 1,4-benzoquinone is mediated by topoisomerase IIα. Levels of DNA cleavage were quantified and expressed as a fold-enhancement over reactions that were carried out in the absence of 1,4-benzoquinone. Data for reactions containing DNA and 1,4-benzoquinone (DNA + BQ) in the absence of enzyme are shown. DNA cleavage mediated by human topoisomerase IIα in the absence (−BQ) or presence (+BQ) of 25 μM 1,4-benzoquinone was examined. To determine whether the DNA cleavage observed in the presence of 1,4-benzoquinone was protein-linked, proteinase K treatment was omitted (−Pro K). Reversibility of reactions containing 1,4-benzoquinone was examined by adding EDTA or NaCl prior to SDS treatment. Error bars represent the standard deviation of three independent experiments.

1,4-Benzoquinone can undergo redox cycling to generate free radicals that can damage nucleic acids. In addition, 1,4-benzoquinone and other biologically relevant quinones can form adducts with DNA (*32*, *100*, *101*). It has been proposed that catechol estrogen-3,4-quinones initiate cancers by forming depurinating adducts with the N7 group of guanines (*100*). Since abasic sites and other DNA adducts have been shown to poison human topoisomerase IIα (*88*, *102*−*105*), it is possible that the enhancement of DNA scission by the quinone is due to a modification of the plasmid substrate. This possibility is supported by the observation that the DNA cleavage activity of etoposide quinone (a potent metabolite of etoposide), but not the parent compound, was reduced ~2-fold when guanine residues proximal to cleavage sites were replaced with N7-deazaguanine (*83*).

To address this important issue, 25 μM 1,4-benzoquinone was incubated with pBR322 for 10 min, but removed by gel filtration prior to DNA cleavage assays. If the compound enhances topoisomerase II-mediated scission primarily by forming DNA adducts or by inducing depurination, prior incubation of the plasmid with the quinone should stimulate DNA cleavage, even though there is no free 1,4-benzoquinone in the final reaction mixture. As shown in Figure 5, this was not the case. Levels of DNA cleavage observed with plasmid that had been incubated with 1,4-benzoquinone prior to assays (lane 5) were similar to those seen in parallel plasmid samples that were not exposed to the quinone (lane 2). This lack of cleavage enhancement was not related to the filtration procedure, because the back addition of 1,4-benzoquinone to these latter samples following filtration stimulated topoisomerase II-mediated DNA scission (lanes 3 and 6).

It has been suggested that some quinones stimulate topoisomerase II-mediated DNA cleavage by covalently

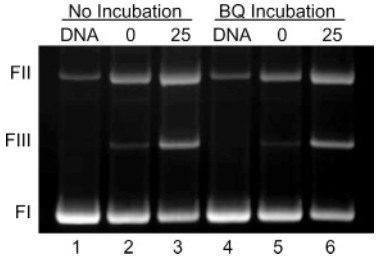

FIGURE 5: Incubation of plasmid DNA with 1,4-benzoquinone does not stimulate DNA cleavage mediated by human topoisomerase IIα. An ethidium bromide-stained agarose gel is shown. Supercoiled (FI), nicked circular (FII), and linear (FIII) DNA molecules are labeled as in Figure 2. All of the plasmid DNA samples were filtered through Bio-Spin 6 chromotography columns (Biorad) before being used in DNA cleavage assays. DNA substrates are shown as controls in lanes 1 and 4. Lanes 2−3 and 5−6 contained human topoisomerase IIα. Lanes 1−3 contained DNA that was not exposed to the quinone prior to filtration (No Incubation). Lanes 4−6 contained DNA that was incubated with 25 μM 1,4-benzoquinone for 10 min prior to filtration (BQ Incubation). Assays in lanes 2 and 5 contained no 1,4-benzoquinone in the final reaction mixtures. Assays in lanes 3 and 6 contained 25 μM 1,4-benzoquinone in the final reaction mixtures.

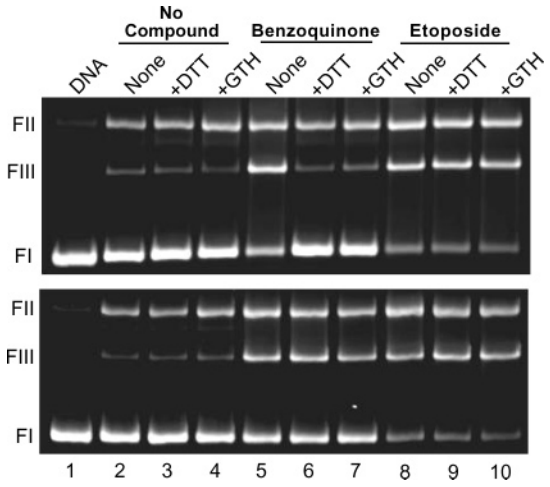

FIGURE 6: Effects of reducing agents on the ability of 1,4-benzoquinone to enhance DNA cleavage mediated by human topoisomerase IIα. Ethidium bromide-stained agarose gels are shown. Supercoiled (FI), nicked circular (FII), and linear (FIII) DNA molecules are labeled as in Figure 2. The top gel shows reactions in which reducing agents were incubated with compounds prior to their addition to cleavage reactions. The bottom gel shows reactions in which reducing agents were added to reaction mixtures after topoisomerase II−DNA cleavage complexes were established in the absence of compound or in the presence of 1,4-benzoquinone or etoposide. The DNA substrate is shown as a control in lane 1. Reactions contained no compound (lanes 2−4), 25 μM 1,4-benzoquinone (lanes 5−7), or 100 μM etoposide (lanes 8−10). Reactions contained no reducing agent (None, lanes 2, 5, and 8), 250 μM DTT (lanes 3, 6, and 9), or 500 μM glutathione (lanes 4, 7, and 10). Results are representative of three independent assays.

modifying the enzyme (presumably on a sulfhydryl residue) (*82*). If this is the case for 1,4-benzoquinone, once the quinone has modified topoisomerase IIα, it should not be able to dissociate from the enzyme. Therefore, order-of-addition experiments were carried out to address the effects of reducing agents on the actions of 1,4-benzoquinone (Figure 6). Consistent with the data shown in Figure 2, no enhancement of enzyme-mediated DNA cleavage was observed when 250 μM DTT was incubated with 1,4-benzoquinone before the quinone was added to the topoisomerase II−DNA complex (Figure 6, top). A similar result was seen when 500 μM glutathione was incubated with the compound. In contrast, once a topoisomerase II−DNA cleavage complex was established in the presence of 1,4-benzoquinone, neither reducing agent had any effect on the enhancement of DNA scission (Figure 6, bottom). In control experiments, DTT and glutathione did not significantly alter levels of DNA cleavage mediated by topoisomerase IIα in the absence of the quinone or in the presence of etoposide (Figure 6).

Site Specificity of DNA Cleavage Mediated by Human Topoisomerase IIα in the Presence of 1,4-Benzoquinone. As a first step toward determining the effects of 1,4-benzoquinone on the DNA cleavage site specificity of topoisomerase II, a singly end-labeled fragment of pBR322 was used as a substrate (*90*). This 4330-bp linear DNA allows cleavage to be monitored at the site-specific level. As shown in Figure 7, 1,4-benzoquinone increased DNA scission at a number of sequences. Furthermore, the pattern of cleavage-site utilization observed in the presence of the quinone differed significantly from that generated in reactions that contained etoposide. A number of distinct sites were seen for both compounds.

To further assess the DNA cleavage specificity of 1,4-benzoquinone, the ability of this compound to stimulate topoisomerase II-mediated DNA cleavage in a short double-stranded oligonucleotide containing a previously mapped leukemic chromosomal translocation breakpoint was determined and compared to that of etoposide (Figure 8). This breakpoint, at position 2595 of the *MLL* gene at chromosomal band 11q23, was identified in a patient who presented with infant AML at 20 months of age (*92*). 1,4-Benzoquinone enhanced DNA scission mediated by human topoisomerase IIα at several sites proximal to the translocation breakpoint. Furthermore, there were obvious differences between the DNA cleavage pattern generated in the presence of the quinone and etoposide.

Therefore, to further compare the DNA cleavage specificities of 1,4-benzoquinone and etoposide, a double-stranded 47-mer derived from plasmid pBR322 (residues 80−126) that contained a single, well-characterized cleavage site for human topoisomerase IIα was used as a substrate (*93*−*95*) (Figure 9). In the absence of a topoisomerase II poison, the enzyme preferentially cleaves this sequence when it contains a guanine residue immediately 5′ to the scissile bond (i.e., at the −1 position) (*91*). Consistent with a number of previous reports, etoposide dramatically enhanced topoisomerase II-mediated DNA cleavage when this sequence contained a cytosine residue at the −1 position (*91*, *106*, *107*). In contrast, 1,4-benzoquinone displayed no significant preference for any specific residue at this position. Thus, it appears that 1,4-benzoquinone enhances topoisomerase II-mediated DNA cleavage at an array of sites that differs from that utilized by the enzyme in the presence of etoposide.

Effects of 1,4-Benzoquinone on DNA Ligation Mediated by Human Topoisomerase IIα. Topoisomerase II poisons increase levels of enzyme-mediated DNA breaks by two nonmutually exclusive mechanisms (*39*, *42*, *44*). While drugs such as etoposide and TAS-103 act primarily by inhibiting the ability of topoisomerase II to ligate DNA breaks (*96*, *108*, *109*), poisons such as quinolones and genistein have little effect on strand rejoining and appear to act primarily by enhancing the forward rate of DNA scission (*110*−*112*).

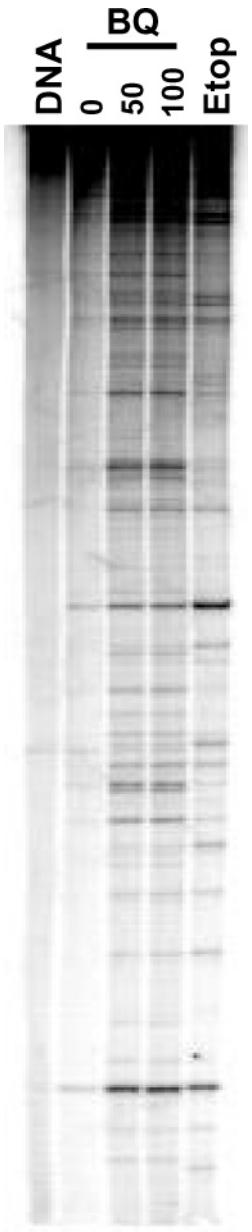

FIGURE 7: DNA cleavage site utilization by human topoisomerase IIα on a long linear fragment in the presence of 1,4-benzoquinone. The 4330-bp sequence was derived from plasmid pBR322. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions contained 0, 50, or 100 μM 1,4-benzoquinone (BQ), or 100 μM etoposide (Etop). A DNA control is shown in the far left lane. Data are representative of three independent assays.

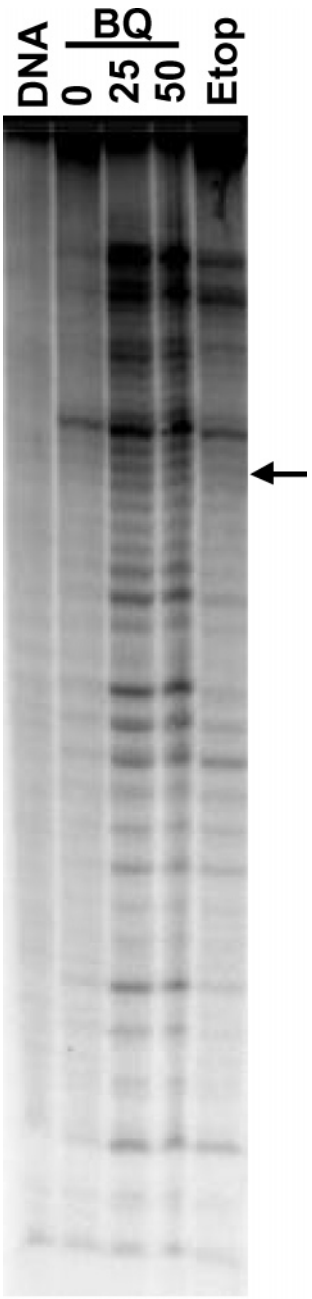

FIGURE 8: DNA cleavage site utilization by human topoisomerase IIα on an oligonucleotide substrate in the presence of 1,4-benzoquinone. The sequence was derived from the human *MLL* gene and contained a leukemic chromosomal translocation breakpoint at position 2595 (indicated by the arrow). An autoradiogram of a polyacrylamide gel is shown for DNA cleavage reactions carried out in the presence of 0, 25, or 50 μM 1,4-benzoquinone (BQ), or 50 μM etoposide (Etop). A DNA control is shown in the far left lane. Data are representative of three independent assays.

To determine the mechanistic basis for the actions of 1,4-benzoquinone against human topoisomerase IIα, the effects of the compound on enzyme-mediated DNA ligation were examined and compared to those of etoposide. Two different assay systems were used for these studies. The first assay monitored the rate at which the human enzyme religated DNA strand breaks that it had generated in negatively supercoiled plasmid molecules (*96*) (Figure 10, left panel). This was accomplished by shifting topoisomerase II−DNA cleavage complexes established at 37 °C to the suboptimal temperature of 0 °C. At this latter temperature, the enzyme rejoins strand breaks but cannot efficiently cleave DNA (*96*). The concentrations of 1,4-benzoquinone and etoposide (25 and 50 μM, respectively) employed induced similar levels of enzyme-mediated DNA cleavage in plasmid-based assays (see Figure 2).

The apparent first-order rate of religation in the presence of 1,4-benzoquinone (0.046 s^{-1}) was only 27% slower than that observed in the absence of the compound (0.063 s^{-1}) (Figure 10, left panel). This inhibition is considerably lower than that seen in the presence of etoposide. At the 30 s time point [which is ~3 times longer than the $t_{1/2}$ for religation in the absence of drug (~11 s)], the enzyme resealed <15%

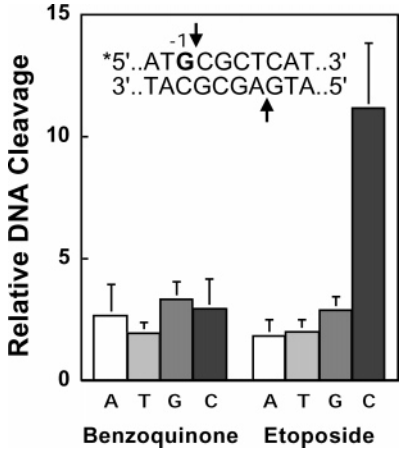

FIGURE 9: DNA cleavage specificity of human topoisomerase IIα in the presence of 1,4-benzoquinone. The substrate was a double-stranded 47-mer oligonucleotide derived from pBR322 that contained a single, well-defined cleavage site for the enzyme. The central sequence of this substrate is shown in the inset. Scissle bonds are indicated by the arrows and the base at the −1 position (relative to the scissile bond) of the top strand is in bold type. The position of the radiolabel is represented by an asterisk. Levels of DNA cleavage of the top strand are relative to those observed in the absence of topoisomerase II poisons. Results are shown for substrates that contained the indicated residues at the −1 position (the residue on the complementary strand also was changed to maintain base pairing) in the presence of 50 μM 1,4-benzoquinone or 50 μM etoposide. Error bars represent the standard deviation of three independent experiments.

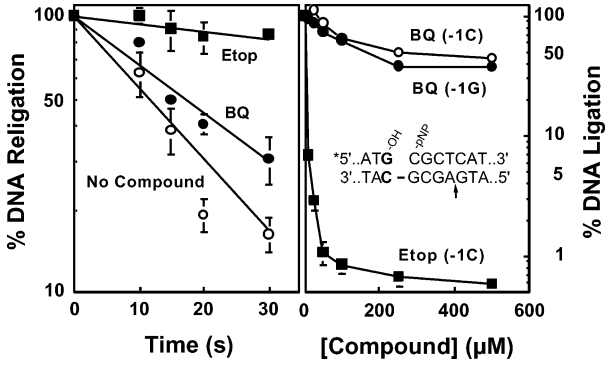

FIGURE 10: 1,4-Benzoquinone does not significantly inhibit DNA ligation mediated by human topoisomerase IIα. The left panel shows a time course for religation of cleaved plasmid DNA in the absence of compound (○) or in the presence of 25 μM 1,4-benzoquinone (BQ, ●) or 50 μM etopside (Etop, ■). These concentrations induced similar levels of enzyme-mediated DNA cleavage. The right panel shows the effects of 1,4-benzoquinone and etoposide on the ability of human topoisomerase IIα to ligate an activated substrate in a cleavage-independent assay. The substrate was the same pBR322-derived oligonucleotide used in Figure 8, except that the top strand contained a nick at the point of scission whose 5′-terminus was activated by covalent attachment to a *p*-nitrophenyl moiety (pNP). The central sequence of this substrate is shown in the inset. The scissle bond on the bottom strand is indicated by the arrow and the base pair that contained the residue at the −1 position (relative to the scissile bond) of the top strand is in bold type. (When the −1 G was changed to a C, the residue on the complementary strand was changed to a G to maintain base pairing.) The position of the radiolabel is denoted by an asterisk. Levels of DNA ligation of the top strand are relative to those determined in the absence of topoisomerase II poisons (set to 100%). Results are shown for the ligation of substrates that contained a −1 G or C in the presence of 0−500 μM 1,4-benzoquinone (● or ○, respectively) or a substrate that contained a −1 C (the preferred sequence for etoposide) in the presence of 0−500 μM etoposide (■). Error bars represent the standard deviation of three independent experiments.

of the cleaved plasmid molecules in reactions that contained etoposide.

The second assay monitored the ability of human topoisomerase IIα to ligate a DNA nick whose 5′-terminal phosphate had been activated by covalent attachment to *p*-nitrophenol (*91*, *109*) (Figure 10, right panel). The presence of this activating group mimics the covalent bond between the DNA substrate and the active-site tyrosine that is formed during the scission event (*97*). The ligation reaction occurs by the direct attack of the 3′-OH at the nick on the activated 5′-phosphate within the active site of topoisomerase IIα and proceeds without a covalent enzyme−DNA intermediate (*91*). The oligonucleotide substrate used for this assay contained the same sequence that was employed for the DNA cleavage site specificity experiments. The activated nick was located at the scissile bond of the top strand of the oligonucleotide.

Compared to etoposide, 1,4-benzoquinone had a marginal effect on rates of ligation (Figure 10, right panel). The IC_{50} values for inhibition of ligation by the quinone were 170 and 250 μM, respectively, for substrates that contained either a guanine residue (the wild-type sequence) or a cytosine residue (the preferred sequence for etoposide) at the −1 position relative to the scissile bond. In marked contrast, the IC_{50} value for etoposide with its preferred sequence (−1 cytosine residue) was 0.8 μM (*109*).

The above results strongly suggest that 1,4-benzoquinone does not have a major effect on the rate of DNA ligation mediated by human topoisomerase IIα. Therefore, it is proposed that the quinone increases levels of DNA breaks primarily by enhancing the forward rate of scission.

Inhibition of Topoisomerase II Catalytic Activity by 1,4-Benzoquinone. Previous studies reported that 1,4-benzoquinone inhibits the overall catalytic activity of human topoisomerase IIα (*35*−*37*). However, these earlier works examined quinone effects on the enzyme under conditions that undermined DNA cleavage enhancement (i.e., the presence of DTT or incubation of the compound with the enzyme prior to the addition of DNA). Therefore, a DNA relaxation assay was used to determine the effects of 1,4-benzoquinone on the overall catalytic activity of human topoisomerase IIα. Conditions employed were conducive to stimulation of DNA scission.

As shown in Figure 11, 1,4-benzoquinone was a strong inhibitor of topoisomerase II catalytic activity ($IC_{50} \approx 2.5$ μM). As determined by a DNA unwinding assay (*89*), inhibition was not due to a change in the topological state of the negatively supercoiled substrate (not shown). These results confirm that the quinone inhibits the ability of the human type II enzyme to carry out its catalytic DNA strand passage reaction.

1,4-Benzoquinone Increases Levels of DNA Cleavage Mediated by Topoisomerase IIα in Cultured Human Cells. The ICE bioassay (*98*, *99*) was employed to determine whether 1,4-benzoquinone increases levels of DNA cleavage mediated by topoisomerase IIα in human CEM leukemia cells. In this assay, cultured cells are lysed with an ionic detergent, and proteins that are covalently attached to genomic DNA are separated from free proteins by sedimentation through a CsCl cushion. The pelleted DNA from

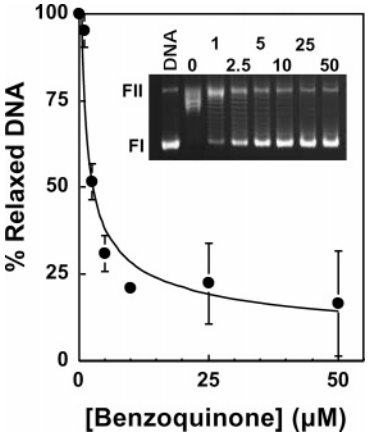

FIGURE 11: 1,4-Benzoquinone inhibits the overall catalytic activity of topoisomerase IIα. DNA relaxation reactions catalyzed by topoisomerase IIα were carried out for 30 min in the presence of 0−50 μM 1,4-benzoquinone. DNA relaxation was quantified by disappearance of the negatively supercoiled DNA substrate. Error bars represent the standard deviation of three independent experiments. The inset shows an ethidium bromide-stained agarose gel of DNA relaxation reactions carried out in the presence of 0−50 μM 1,4-benzoquinone. Negatively supercoiled DNA (FI) and nicked circular DNA (FII) are indicated as in Figure 2.

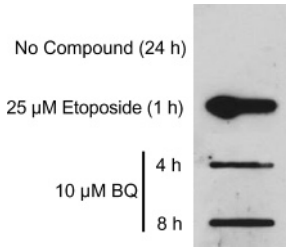

FIGURE 12: 1,4-Benzoquinone enhances DNA cleavage mediated by human topoisomerase IIα in treated human CEM cells. The ICE bioassay was used to monitor the level of cleavage complexes in cells treated with 1,4-benzoquinone. DNA (10 μg) from cultures grown in the absence of compound for 24 h, or in the presence of 10 μM 1,4-benzoquinone (BQ) for 4 or 8 h, or 25 μM etoposide for 1 h was blotted onto a nitrocellulose membrane. Blots were probed with a polyclonal antibody directed against human topoisomerase IIα. Results are representative of three independent experiments.

cultures treated with no drug for 24 h or 10 μM 1,4-benzoquinone for 4 or 8 h was blotted and probed with a polyclonal antibody specific for human topoisomerase IIα. (This reduced quinone concentration was used because it induced less than 20% cell death over the course of the assay. Cell death at higher concentrations was in excess of 50%.) Results from cells treated with 25 μM etoposide for 1 h are shown for comparison. As shown in Figure 12, levels of topoisomerase IIα that were covalently attached to DNA following treatment with 1,4-benzoquinone increased several-fold over the course of the experiment.

It should be noted that some cleavage-independent topoisomerase IIα−DNA cross-linking was observed in vitro in DNA cleavage assays that contained 1,4-benzoquinone. Therefore, a portion of the covalent topoisomerase IIα−DNA complex observed in quinone-treated CEM cells may reflect protein−DNA cross-linking rather than enzyme-mediated DNA scission. However, the topoisomerase IIα:DNA base pair ratio (∼1:325) used in the in vitro cleavage assays was >20 times higher than estimated (∼1:7500) for CEM cells. When in vitro experiments were carried out using topoisomerase IIα:DNA base pair ratios that approximated the cellular condition, no significant protein:DNA cross-linking was observed (not shown). Therefore, it is concluded that 1,4-benzoquinone is a topoisomerase II poison in cultured human cells.

DISCUSSION

Benzene is a human carcinogen that induces primarily hematopoietic malignancies (*6*−*11*, *13*−*16*). It is believed that the parent compound does not trigger leukemic chromosomal translocations directly. Rather, benzene generates DNA damage through a series of phenolic metabolites, especially 1,4-benzoquinone (*14*, *16*−*18*, *24*).

The mechanism by which 1,4-benzoquinone initiates leukemias has not been elucidated. The compound induces DNA strand breaks, homologous recombination, and sister chromatid exchange in treated cells (*10*, *24*, *27*, *29*, *31*). In addition, decreased levels of NQO1 (the enzyme that detoxifies 1,4-benzoquinone) correlate with a higher risk of AMLs and ALLs that are characterized by 11q23 chromosomal translocations (*19*−*23*). Since these findings are consistent with the actions of topoisomerase II-targeted drugs, it has been proposed that 1,4-benzoquinone initiates leukemias (at least in part) by acting as a topoisomerase II poison (*21*, *22*, *24*, *25*, *36*, *37*).

Cellular effects notwithstanding, this hypothesis has not been supported by in vitro studies (*25*, *36*, *37*). While previous work demonstrated inhibition of topoisomerase II catalysis by 1,4-benzoquinone, increases in enzyme-mediated DNA cleavage were never observed (*25*, *36*, *37*).

Recently, several quinones were found to poison human topoisomerase IIα (*82*, *83*, *85*). Some quinones, as well as other sulfhydryl-reactive compounds, appear to act in a manner that differs from most "traditional" topoisomerase II poisons (*82*, *84*, *85*). While topoisomerase II-active agents typically interact with the enzyme in a noncovalent fashion, it has been proposed that sulfhydryl-reactive compounds increase DNA cleavage by modifying the enzyme (*82*). These "sulfhydryl-reactive" poisons display two characteristics that distinguish them from other topoisomerase II-targeted agents: their activity is destroyed by exposure to reducing agents such as DTT (*82*, *84*), and incubation of the enzyme with these compounds prior to the addition of DNA renders topoisomerase II catalytically inert (*82*, *84*, *85*).

Previous in vitro work on 1,4-benzoquinone and the human type II enzyme was carried out before the properties of sulfhydryl-reactive topoisomerase II poisons had been reported. All of these studies were performed in the presence of significant concentrations of reducing agents (*25*, *36*, *37*). Furthermore, in some cases, 1,4-benzoquinone was incubated directly with topoisomerase IIα in an effort to maximize interactions with the enzyme (*25*). Consequently, earlier studies were not in a position to draw valid conclusions regarding the potential of 1,4-benzoquinone to act as a topoisomerase II poison.

In light of the recent observations regarding the actions of sulfhydryl-active topoisomerase II poisons, we examined the effects of 1,4-benzoquinone on the DNA cleavage activity of human topoisomerase IIα at low concentrations of DTT (<0.4 μM). In addition, the quinone was added to the enzyme−DNA complex rather than the enzyme alone.

Results demonstrate that 1,4-benzoquinone is a strong topoisomerase II poison that is more potent than the anticancer drug etoposide in vitro. Furthermore, the quinone induces the formation of topoisomerase IIα−DNA cleavage complexes in cultured human cells.

1,4-Benzoquinone displays all of the hallmarks of sulfhydryl-reactive topoisomerase II poisons (*82*, *84*, *85*). Although it has not been demonstrated directly, the ability of the compound to stimulate topoisomerase II-mediated DNA cleavage is consistent with a covalent modification of the human enzyme. The mechanism by which this proposed modification increases levels of cleavage is not known. The fact that DNA scission can be reversed by the addition of salt or EDTA, and that 1,4-benzoquinone has little effect on rates of DNA ligation, rules out the simplistic interpretation that the quinone modifies a sulfhydryl residue that is exposed in the cleavage complex and destroys enzyme activity.

It is notable that polymorphisms in two enzymes that are involved in quinone metabolism increase the risk of developing leukemias with 11q23 chromosomal translocations. In addition to the NQO1 polymorphism discussed above (*19*−*23*), the cytochrome P450 3A4 genotype (a promoter variant) decreases the risk of therapy-related leukemias in patients treated with etoposide and related epipodophyllotoxins (*113*). Cytochrome P450 3A converts etoposide to a catechol metabolite, which is further oxidized to etoposide quinone (*114*). Etoposide quinone is approximately twice as active as etoposide against human topoisomerase IIα (*83*).

In summary, it is likely that the genotoxicity/cytotoxicity of 1,4-benzoquinone is mediated by multiple cellular events. However, the present findings suggest that the chromosomal breaks induced by exposure to 1,4-benzoquinone are generated to some extent by topoisomerase IIα. Furthermore, these findings are consistent with the hypothesis that topoisomerase II-mediated DNA cleavage contributes to the development of specific leukemias that are induced by benzene and its metabolites.

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