Polychlorinated Biphenyl Quinone Metabolites Poison Human Topoisomerase IIα: Altering Enzyme Function by Blocking the *N*-Terminal Protein Gate†

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ABSTRACT: Polychlorinated biphenyls (PCBs) are associated with a broad spectrum of human health problems and cause cancer in rodents. In addition, these compounds cause chromosomal aberrations in humans and treated human cells. Although the underlying basis for the chromosomal damage induced by PCBs is not understood, it is believed that these compounds act through a series of phenolic and quinone-based metabolites. Recent studies indicate that several quinones that promote chromosomal damage also act as topoisomerase II poisons. Therefore, the effects of PCB quinone metabolites (including mono and dichlorinated compounds and *p*- and *o*-quinones) on the activity of human topoisomerase IIα were examined. Results indicate that these compounds are potent topoisomerase IIα poisons in vitro and act by adducting the enzyme. They also increase DNA cleavage by topoisomerase IIα in cultured human cells. In contrast, incubation of topoisomerase IIα with PCB metabolites in the absence of DNA leads to a rapid loss of enzyme activity. On the basis of (1) the differential ability of quinone-treated enzyme to bind circular and linear DNA molecules and (2) the generation of salt−stable noncovalent complexes between topoisomerase IIα and circular plasmids in the presence of PCB quinones, it appears that these compounds alter enzyme function (at least in part) by blocking the *N*-terminal gate of the protein. Finally, exposure to quinones generates a protein species with a molecular mass approximately twice that of a monomeric topoisomerase IIα protomer. This finding suggests that PCB quinones block the *N*-terminal gate by cross-linking the protomer subunits of topoisomerase IIα.

Polychlorinated biphenyls (PCBs[1]) represent a class of compounds with two aromatic six-member rings that contain from 1 to 10 chlorine atoms (*1*, *2*). These chemicals were employed in a variety of large-scale industrial applications from the 1930s to the 1970s (*1*−*3*). For example, PCBs were used as organic diluents, lubricants, and cooling fluids and were commonly utilized in the production of adhesives, paper, pesticides, and flame retardants. One of the properties that made PCBs so attractive for industrial purposes was their high stability and low biodegradability (*1*, *3*). As a result, however, these compounds accumulated in the environment. Due to human health concerns, most countries banned the production and use of PCBs in the 1970s (*1*, *3*). Three decades later, these compounds remain persistent environmental contaminants (*1*, *3*−*5*).

Exposure to PCBs is associated with a broad spectrum of human health problems, including neurotoxicity, hepatotoxicity, hypothyroidism, and immunodeficiency (*1*, *3*, *6*−*8*). PCBs are complete carcinogens in rodents and display genotoxic activity in vitro and in vivo (*9*, *10*). For example, in laboratory studies, these compounds induce chromosomal aberrations and sister chromatid exchanges in cultured human lympocytes (*11*). Similar chromosomal abnormalities have been found in the peripheral lympocytes of workers who were occupationally exposed to PCBs, with longer exposures correlating with greater DNA damage (*12*, *13*). Finally, PCBs cause liver cancer in mice and rats and are listed by the Environmental Protection Agency as probable human carcinogens (*1*, *7*, *14*).

The underlying basis for the chromosomal damage induced by PCBs is not understood. However, it is believed that these compounds do not trigger DNA damage directly. Rather, they act through a series of phenolic and quinone-based metabolites (*15*−*19*). It has been proposed that the generation of reactive oxygen species by redox cycling, the depletion of glutathione by adduction, or the generation of DNA lesions by oxidation may play a role in chromosome damage (*15*−*19*). In addition, PCB quinone metabolites form protein

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[1] Abbreviations: PCB, polychlorinated biphenyl; DTT, dithiothreitol; (2′Cl-2,5pQ), 2-(2-chloro-phenyl)-[1,4]benzoquinone; (3′Cl-2,5pQ), 2-(3-chloro-phenyl)-[1,4]benzoquinone; (4′Cl-2,5pQ), 2-(4-chloro-phenyl)-[1,4]benzoquinone; (3′,5′Cl-2,5pQ), 2-(3,5-dichloro-phenyl)-[1,4]-benzoquinone; (4′Cl-2,5HQ), 4′-Chloro-biphenyl-2,5-diol; (4′Cl-2,3oQ), 3-(4-chloro-phenyl)-[1,2]benzoquinone; (4′Cl-3,4oQ), 4-(4-chloro-phenyl)-[1,2]benzoquinone.

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adducts through cysteine residues in treated cells (*19*). The specific cellular proteins that are modified by these compounds are not well characterized. However, the genotoxic effects of PCBs are consistent with the actions of topoisomerase II poisons, and a previous study found that these compounds inhibited enzyme-catalyzed DNA decatenation (*19*).

Topoisomerase II is an enzyme that removes knots and tangles from the genetic material by generating transient double-stranded breaks in the backbone of DNA (*20*−*27*). To maintain genomic integrity during this cleavage event, the enzyme forms covalent bonds between active site tyrosyl residues and the 5′-DNA termini created by scission of the double helix (*28*−*30*). These covalent topoisomerase II-cleaved DNA intermediates are known as *cleavage complexes*. Under normal conditions, they are present at low equilibrium levels and are tolerated by the cell (*20*, *21*, *23*, *24*, *27*). However, conditions that significantly increase the concentration of these complexes generate permanent DNA strand breaks that induce illegitimate recombination, chromosomal aberrations, and sister chromatid exchanges (*21*, *24*, *27*, *31*−*35*).

A variety of important anticancer drugs, such as etoposide, kill cells by increasing cellular levels of topoisomerase II-DNA cleavage complexes (*21*, *24*, *27*, *36*−*40*). Because these agents convert the type II enzyme to a potent cellular toxin, they are called *topoisomerase II poisons* (*21*, *24*, *27*, *37*, *39*, *40*). Most topoisomerase II poisons are believed to function in the active site of the enzyme via noncovalent interactions (*21*, *24*, *27*, *37*, *39*−*41*). However, recent studies indicate that sulfhydryl-reactive quinones also have the potential to increase levels of topoisomerase II-mediated DNA cleavage (*42*−*44*). It is believed that these compounds act by adducting amino acid residues that may lie outside the active site of the enzyme (*42*−*44*).

Humans encode two isoforms of topoisomerase II, α and β (*45*−*48*). Topoisomerase IIα levels increase dramatically during periods of cell growth, and this isoform appears to be a major target for anticancer drugs in rapidly proliferating cells (*22*, *26*, *49*−*51*). Because the genotoxic events triggered by PCBs resemble those of topoisomerase II poisons, the effects of quinone and hydroquinone PCB metabolites on human topoisomerase IIα were examined. Results indicate that quinone metabolites are potent topoisomerase II poisons in vitro and in cultured cells. These findings suggest that the type II enzyme may contribute to PCB genotoxicity. Finally, in vitro studies indicate that the ability of PCB metabolites to alter the activity of topoisomerase IIα is related to effects on the *N*-terminal clamp of the protein.

EXPERIMENTAL PROCEDURES

Enzymes and Materials. Human topoisomerase IIα was expressed in *Saccharomyces cerevisiae* and purified as described previously (*52*−*54*). Negatively supercoiled pBR322 DNA was prepared using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. ICRF-193 (Biomol) and etoposide (Sigma) were prepared as 20 mM stock solutions in 100% DMSO and stored at 4 °C. All other chemicals were of analytical reagent grade.

Synthesis of PCB Metabolites. 2-(2-Chloro-phenyl)-[1,4]-benzoquinone (2′Cl-2,5pQ), 2-(3-chloro-phenyl)-[1,4]benzoquinone (3′Cl-2,5pQ), 2-(4-chloro-phenyl)-[1,4]benzoquinone (4′Cl-2,5pQ), and 2-(3,5-dichloro-phenyl)-[1,4]benzoquinone (3′,5′Cl-2,5pQ) were synthesized by coupling the appropriate chloroaniline with 1,4-benzoquinone as described by Amaro et al. (*17*). 4′-Chloro-biphenyl-2,5-diol (4′Cl-2,5HQ) was generated by reducing 4′Cl-2,5pQ with sodium dithionite (*55*). 3-(4-Chloro-phenyl)-[1,2]benzoquinone (4′Cl-2,3oQ) and 4-(4-chloro-phenyl)-[1,2]benzoquinone (4′Cl-3,-4oQ) were generated by oxidizing the corresponding diol PCB derivatives with silver(I) oxide (*17*, 56). The [1,2]-benzoquinones were used within 48 h after their synthesis to minimize decomposition. In all cases, spectroscopic data were in agreement with proposed structures. Furthermore, the purity of all compounds was >98% as determined by gas chromatography ([1,4]benzoquinone) or gas chromatography−mass spectrometry (diols). The only exception were the two [1,2]benzoquinones, which were characterized with ^{1}H nuclear magnetic resonance spectroscopy and used without further purification. All PCB metabolites were prepared as 20 mM solutions in 100% DMSO and stored at −20 °C.

Plasmid DNA Cleavage. DNA cleavage reactions were carried out using the procedure described by Fortune and Osheroff (*57*). Assay mixtures contained 135 nM topoisomerase IIα and 10 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) that contained 0−200 μM of the PCB metabolites or etoposide. DNA cleavage was initiated by the addition of enzyme, and mixtures were incubated for 6 min at 37 °C to establish DNA cleavage-religation equilibria. Enzyme−DNA cleavage intermediates were trapped by adding 2 μL of 5% SDS and 1 μL of 375 mM EDTA, pH 8.0. Proteinase K was added (2 μL of 0.8 mg/mL), 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 15 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in a TAE buffer (40 mM Tris-acetate, pH 8.3, and 2 mM EDTA) that contained 0.5 μg/mL ethidium bromide. DNA cleavage was monitored by the conversion of negatively supercoiled plasmids to linear molecules. DNA bands were visualized by ultraviolet light and quantified using an Alpha Innotech digital imaging system.

To determine whether DNA cleavage by topoisomerase IIα was reversible, EDTA was added prior to treatment with SDS. To examine whether cleavage was protein-linked, proteinase K treatment was omitted. In reactions that tested whether PCB metabolite activity was affected by DTT, samples contained a final DTT concentration of 500 μM that was added either before or after cleavage complex formation and processed as above.

To analyze the effects of PCB metabolites on topoisomerase IIα in the absence of DNA, a metabolite was incubated with the enzyme for 0−3 min at 37 °C in 15 μL of cleavage buffer. Cleavage reactions were initiated by adding negatively supercoiled pBR322 DNA in 5 μL of cleavage buffer. The final concentrations of topoisomerase IIα, DNA, and PCB metabolite in the final reaction mixtures were 135 nM, 10 nM, and 100 μM, respectively. Topoisomerase IIα DNA cleavage−religation equilibria were

established and processed as described above.

Site-Specific DNA Cleavage Induced by PCB Metabolites. DNA sites cleaved by human topoisomerase IIα were determined by a modification (*44*) of the procedure described by O'Reilly and Kreuzer (*58*). A linear 4330 bp fragment (HindIII/EcoRI) of pBR322 plasmid DNA singly labeled with ^{32}P on the 5′-terminus of the HindIII site was used as the cleavage substrate. Reaction mixtures contained 0.35 nM DNA substrate and 60 nM topoisomerase IIα in 50 μL of cleavage buffer. Assays were carried out in the absence of compound, or in the presence of 12.5 μM etoposide or 25 μM PCB metabolite. Reactions were initiated by the addition of enzyme and 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. Topoisomerase IIα was digested with proteinase K (5 μL of 0.8 mg/mL) for 30 min at 45 °C. Reaction products were precipitated twice in 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% sequencing gel. The gel was then fixed in 10% methanol/10% acetic acid for 5 min, dried, and DNA cleavage products were analyzed on a Bio-Rad Molecular Imager FX.

DNA Religation. DNA religation mediated by topoisomerase IIα was monitored according to the procedure of Byl et al. (*59*). Topoisomerase IIα DNA cleavage−religation equilibria were established as described above in the absence of compound, or in the presence of 100 μM PCB metabolite or 100 μM etoposide. Religation was initiated by shifting reaction mixtures from 37 to 0 °C, and reactions were stopped at time points up to 40 s by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM NaEDTA, pH 8.0. Samples were processed and analyzed as described above for topoisomerase IIα plasmid DNA cleavage reactions.

DNA Binding. The binding of topoisomerase IIα to linear DNA substrates was assessed using a nitrocellulose filter-binding assay. Either the linearized pBR322 DNA substrate described above or a ^{32}P-labeled double-stranded 50-mer oligonucleotide that contained a single topoisomerase II cleavage site (*60*) were employed. The oligonucleotide was prepared on an Applied Biosystems DNA synthesizer. The sequences of the top and bottom strands were 5′-TTGGTATCTGCGCTCTGCTGAAGCC↓AGTTACCTTCGGAAAAAGAGTTGGT-3′ and 5′-ACCAACTCTTTTTCCGAAGGT↓AACTGGCTTCAGCAGAGCGCAGATACCAA-3′, respectively. The arrows indicate cleavage sites by topoisomerase IIα The enzyme was incubated for 0−5 min at 37 °C in 15 μL of DNA binding buffer (10 mM Tris-HCl, pH 7.9, 30 mM KCl, 0.1 mM NaEDTA, and 2.5% glycerol) that contained no compound, PCB metabolite, or etoposide. Binding equilibria were initiated by the addition of DNA in 5 μL of binding buffer and followed by incubation for 6 min at 37 °C. The final concentrations of topoisomerase IIα, DNA, and PCB metabolite (or etoposide) in the final reaction mixtures were 400 nM, 5 nM, and 100 μM, respectively. Nitrocellulose membranes (0.45 μm HA, Millipore) were prepared by incubation in DNA binding buffer for 10 min. Samples were applied to the membranes and filtered in vacuo. Membranes were washed three times with 1 mL of DNA binding buffer, dried, and submerged in 8 mL of scintillation fluid (Econo-Safe, Research Products International). Radioactivity remaining on the membranes was quantified using a Beckman LS 5000 TD Scintillation Counter.

Assays that monitored the binding of topoisomerase IIα to negatively supercoiled plasmid were carried out according to the procedure of Fortune et al. (*61*). Reaction mixtures contained 0−400 nM enzyme and 100 μM PCB metabolite (or etoposide) in 15 μL of binding buffer. Samples were incubated for 5 min at 37 °C. Binding reactions were initiated by adding DNA in 5 μL of binding buffer and incubated at 37 °C for an additional 6 min. The concentrations of topoisomerase IIα, DNA, and PCB metabolite in the final reaction mixtures were 0−400 nM, 5 nM, and 100 μM, respectively. Samples were loaded without further processing onto a 1% agarose gel and subjected to electrophoresis in 100 mM Tris-borate, pH 8.3, and 2 mM EDTA. Gels were stained for 30 min with 0.5 μg/mL ethidium bromide, and samples were analyzed as described above.

Protein Clamp Closing. Filter binding assays were used to analyze the salt-stable closed clamp of topoisomerase IIα (*62*). Briefly, 5 nM human topoisomerase IIα and 2 nM pBR322 were incubated for 5 min at 37 °C in a total of 90 μL of clamp-closing buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, and 8 mM $MgCl_2$). No compound, 100 μM 4′Cl-2,5pQ, 100 μM 4′Cl-2,5HQ, or a combination of 100 μM ICRF-193 + 2 mM ATP was added in a total of 10 μL of 10% DMSO. Mixtures were incubated for an additional 5 min at 37 °C.

In some experiments, 4′Cl-2,5pQ was incubated with topoisomerase IIα for 5 min in the absence of DNA. Following the addition of DNA, samples were incubated for an additional 5 min at 37 °C.

In all cases, binding mixtures were loaded onto glass fiber filters (Millipore) preincubated in clamp-closing buffer, and filtered in vacuo. Filters were washed three times with clamp-closing buffer (low salt wash), followed by three washes with clamp-closing buffer that contained 1 M NaCl (high salt wash), followed by three washes with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.5% SDS. DNA was precipitated with 2-propanol and loaded onto a 1% agarose gel in TAE buffer containing 0.5 μg/mL ethidium bromide. DNA was visualized as described above.

Protein Cross-linking. The ability of quinones to cross-link the protomer subunits of human topoisomerase IIα was assessed using SDS polyacrylamide gels. Cross-linking mixtures contained 135 nM topoisomerase IIα in a total of 60 μL of cleavage buffer. The enzyme was incubated at 37 °C for 0−2 min in the presence of 100 μM 4′Cl-2,5pQ or for 6 min in the absence of compound or in the presence of 100 μM 4′Cl-2,5pQ or 250 μM plumbagin. In dilution experiments, 80−800 nM topoisomerase IIα was incubated with 100 μM 4′Cl-2,5pQ for 1 min. In all cases, reactions were quenched by the addition of 2 μL of 5 mM DTT and were incubated at 37 °C for an additional 2 min. Samples were precipitated with TCA and resuspended in 10 μL of H_2O. Laemmli buffer (10 μL, Bio-Rad) was added, and samples were subjected to electrophoresis in a 7.5% denaturing acrylamide gel (Bio-Rad) at 200 v for 1 h. The protein was visualized by coumassie staining.

Partial Proteolytic Mapping. To identify peptides cross-linked by PCB metabolites, a modified Cleveland mapping protocol was utilized. Briefly, 3 μg of human topoisomerase

IIα was treated with 100 μM 4′Cl-2,5pQ for 0−2 min in 10 μL of cleavage buffer. Reactions were quenched with 1 μL of 5 mM DTT. Proteolysis was initiated by the addition of 0.4 μg of V8 protease (Pierce), and the samples were incubated at 37 °C for 30 min. The protease reactions were quenched with 3 μL of 10% SDS followed by the addition of 15 μL of Laemmli buffer. The samples were subject to electrophoresis in a 4−20% denaturing acrylamide gel (Bio-Rad) at 200 v for 1.5 h. Protein was visualized by silver staining (Bio-Rad).

DNA Cleavage Mediated by Topoisomerases in Cultured Human CEM Cells. Human CEM acute lymphoblastic leukemia cells (ATCC) were cultured under 5% CO_2 at 37 °C in an RPMI 1640 medium (Cellgro by Mediatech, Inc.) containing 10% heat-inactivated fetal calf serum (Hyclone) and 2 mM glutamine (Cellgro by Mediatech, Inc.). The in vivo complex of enzyme (ICE) bioassay (*63*, *64*) (as modified on the TopoGEN, Inc., website) was employed to determine the ability of PCB metabolites to induce topoisomerase IIα-mediated DNA breaks in CEM cells. Exponentially growing cultures were treated with 25 μM PCB metabolites for 6 h or with 10 μM etoposide or camptothecin for 2 h for comparison. The cells (∼5 × 10^6) were harvested by centrifugation and lysed by the immediate addition of 3 mL of 1% sarkosyl. Following gentle dounce homogenization, the cell lysates were layered onto a 2 mL cushion of CsCl (1.5 g/mL) and centrifuged in a Beckman NVT 90 rotor at 80 000 rpm (∼500 000*g*) for 5.5 h at 20 °C. The 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 cleavage complexes formed between topoisomerase IIα or topoisomerase I and chromosomal DNA were detected using a polyclonal antibody directed against human topoisomerase IIα (Kiamaya Biochemical Co.) at a 1:1000 dilution or a polyclonal antibody directed against human topoisomerase I (Biolegend) at a 1:2000 dilution.

RESULTS

PCB Metabolites Enhance DNA Cleavage Mediated by Human Topoisomerase IIα. The underlying basis for the chromosomal damage induced by PCBs is not understood. However, it is believed that these compounds act through a series of phenolic and quinone-based metabolites (*15*−*19*). Recent studies indicate that several quinones that promote chromosomal damage also act as topoisomerase II poisons (*42*−*44*). Therefore, the effects of a variety of PCB metabolites on DNA cleavage mediated by human topoisomerase IIα were examined.

The PCB metabolites used in the present study are shown in Figure 1. The compounds were either mono or dichlorinated and included a series of *p*-quinones and *o*-quinones as well as a *p*-hydroquinone.

All of the quinone-based PCB metabolites increased DNA cleavage mediated by topoisomerase IIα greater than 4-fold (Figure 2). As seen in the left panel, 4′Cl-2,5pQ was equipotent to etoposide and increased cleavage ∼8-fold. On the basis of titrations from 0 to 200 μM metabolites (not shown), levels of cleavage enhancement plateaued at ∼100 μM for all of the quinones examined. In contrast to 4′Cl-2,5pQ, which was the most active quinone metabolite, the

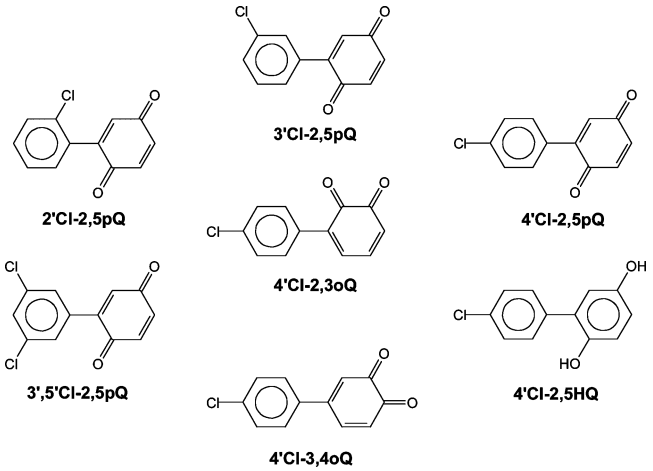

FIGURE 1: Stuctures of PCB metabolites.

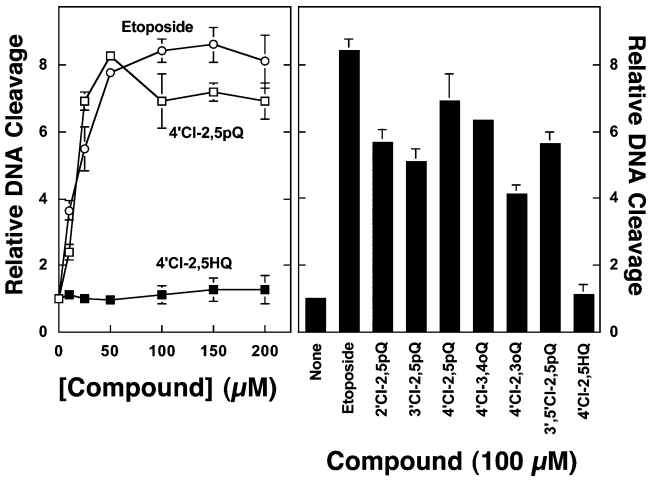

FIGURE 2: PCB metabolites stimulate DNA cleavage mediated by human topoisomerase IIα. Levels of DNA cleavage were quantified and expressed as a fold enhancement over reactions that were carried out in the absence of metabolites. Cleavage titrations are shown in the left panel. Reaction mixtures contained 0−200 μM 4′Cl-2,5pQ (□), or 4′Cl-2,5HQ (■). A parallel titration with etoposide (○) is shown as a control. Relative DNA cleavage enhancement induced by 100 μM PCB metabolites is shown in the right panel. Error bars represent the standard deviation of at least three independent experiments.

corresponding hydroquinone conger, 4′Cl-2,5HQ, displayed very little activity against topoisomerase IIα. No significant increase in DNA scission was observed up to 200 μM 4′Cl-2,5HQ (Figure 2, left panel). These results strongly suggest that PCB quinones are potent topoisomerase II poisons in vitro but that PCB hydroquinones are far less active against the enzyme.

Because 4′Cl-2,5pQ was the most active metabolite, it was used as the model quinone for control experiments designed to determine whether the enhanced DNA cleavage induced by PCB metabolites was mediated by topoisomerase IIα (Figure 3). First, no linear DNA was observed in reactions that contained 100 μM 4′Cl-2,5pQ 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 was added to reaction mixtures before cleavage complexes were trapped by the addition of SDS. This reversibility is inconsistent with

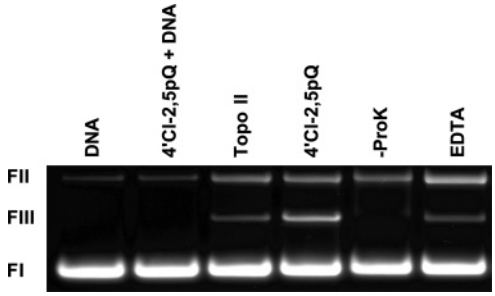

FIGURE 3: DNA cleavage induced by PCB metabolites is mediated by topoisomerase IIα. An ethidium bromide-stained agarose gel of DNA cleavage reactions is shown. DNA controls lacking enzyme were in the absence (DNA) or presence of 4′Cl-2,5pQ (4′Cl-2,-5pQ + DNA). DNA cleavage mediated by human topoisomerase IIα in the absence (Topo II) or presence (4′Cl-2,5pQ) of 100 μM 4′Cl-2,5pQ was examined. To determine whether the DNA cleavage observed in the presence of 4′Cl-2,5pQ was protein-linked, proteinase K treatment was omitted (-Pro K). Reversibility of reactions containing 4′Cl-2,5pQ was examined by adding EDTA prior to SDS treatment (EDTA). The electrophoretic mobilities of negatively supercoiled DNA (form I, FI), nicked circular plasmid (form II, FII), and linear molecules (form III, FIII) are indicated. Data are representative of two independent assays.

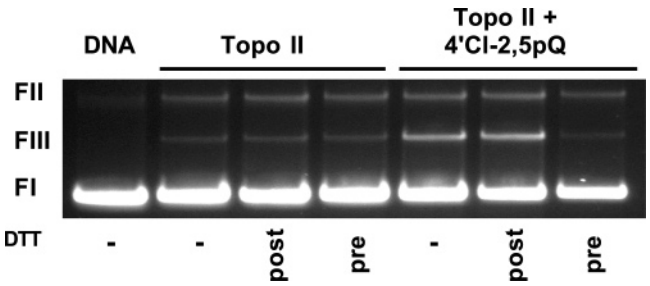

FIGURE 4: Effects of reducing agents on the ability of 4′Cl-2,5pQ to enhance DNA cleavage mediated by human topoisomerase IIα. A representative ethidium bromide-stained agarose gel is shown. The DNA substrate is shown as a control in lane 1. Reactions were carried out in the absence (lanes 2−4) or presence of 100 μM 4′Cl-2,5pQ (lanes 5−7). Reactions contained no reducing agent (-, lanes 1, 2 and 5), 500 μM DTT that was added to reaction mixtures after topoisomerase II−DNA cleavage complexes were established (post, lanes 3 and 6), or 500 μM DTT that was incubated with 4′Cl-2,-5pQ prior to the addition of the quinone to cleavage reactions (pre, lanes 4 and 7). The mobilities of supercoiled (FI), nicked circular (FII), and linear (FIII) DNA molecules are as those in Figure 3. Results are representative of four independent experiments.

a nonenzymatic reaction. Taken together, the above findings provide strong evidence that PCB quinones increase DNA cleavage through an enzyme-mediated reaction.

Previous studies suggest that quinones alter the activity of topoisomerase IIα by covalently modifying the enzyme (*19*, *42*, *43*). This is the primary reason quinones are believed to be more active toward the enzyme than hydroquinones (*19*, *65*). If protein adduction plays an important role in the actions of PCB metabolites, once a 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 the reducing agent DTT on the actions of 4′Cl-2,5pQ (Figure 4). As expected, no enhancement of enzyme-mediated DNA scission was observed when 100 μM quinone was incubated with 500 μM DTT prior to its addition to the topoisomerase IIα−DNA complex. Conversely, once a cleavage complex was established in the presence of 4′Cl-2,5pQ, DTT had no effect on the enhancement of DNA scission. In control experiments, DTT did not significantly alter levels of DNA cleavage mediated by topoisomerase IIα in the absence of the quinone (Figure 4) or in the presence of the anticancer drug etoposide (which does not form a covalent protein adduct; data not shown) (*19*, *42*, *43*). These findings indicate that PCB metabolites increase DNA strand breaks generated by topoisomerase IIα by forming covalent adducts with the enzyme.

Quinones can undergo enzyme-dependent redox cycling to generate free radicals that damage nucleic acids (*18*, *66*). In addition, some biologically relevant quinones can form adducts with DNA (*15*, *66*−*68*). Because many DNA lesions have been shown to poison human topoisomerase IIα (*54*, *69*−*73*), it is possible that the enhancement of DNA scission by the PCB metabolites is due to a modification of the plasmid substrate. To address this issue, 100 μM 4′Cl-2,-5pQ was incubated with negatively supercoiled pBR322 for 6 min but removed by gel filtration prior to the addition of the enzyme. No enhancement of DNA cleavage was observed under these conditions (data not shown). Thus, modification of DNA does not appear to contribute to the effects of PCB metabolites on topoisomerase IIα.

To determine the effects of PCB metabolites on the DNA cleavage site specificity of topoisomerase IIα, a singly end-labeled fragment of pBR322 was used as the substrate. This 4330 bp linear DNA allows cleavage to be monitored at specific sites. Four of the quinone metabolites were used for cleavage mapping, including both mono and dichlorinated *p*-quinones. All of them increased topoisomerase IIα-mediated DNA scission at a number of distinct sequences (Figure 5). As determined by visual inspection, the quinones displayed DNA cleavage site specificities that were identical to one another but differed significantly from that of etoposide. Furthermore, strong sites of cleavage enhancement appeared to correspond to sites cut by topoisomerase IIα in the absence of poisons. This finding suggests that PCB quinones may be acting distal to the active site of the enzyme, since drugs that act at the scissile bonds generally alter cleavage site utilization by topoisomerase II (*74*). Consistent with the global DNA cleavage data, 4′Cl-2,5HQ had little effect on enzyme-mediated DNA scission and yielded a cleavage pattern that was similar to that of the topoisomerase IIα control (Figure 5).

PCB Quinones Inhibit DNA Religation Mediated by Human Topoisomerase IIα. Topoisomerase II poisons increase levels of enzyme-mediated DNA breaks by two nonmutually exclusive mechanisms (*21*, *24*, *27*, *40*). Drugs such as etoposide act primarily by inhibiting the ability of topoisomerase IIα to religate DNA breaks (*75*, *76*). In contrast, drugs such as quinolones have little effect on strand rejoining and appear to act primarily by increasing the forward rate of DNA scission (*77*, *78*).

To determine the mechanism by which PCB metabolites increase levels of topoisomerase II-generated DNA strand breaks, their effects on rates of religation mediated by human topoisomerase IIα were assessed (Figure 6). All of the PCB quinones examined strongly inhibited the ability of the enzyme to reseal cleaved DNA molecules, suggesting that this inhibition is the primary mechanism by which these compounds increase levels of topoisomerase II−DNA cleavage complexes. Considerably less inhibition was seen for 4′Cl-2,5HQ.

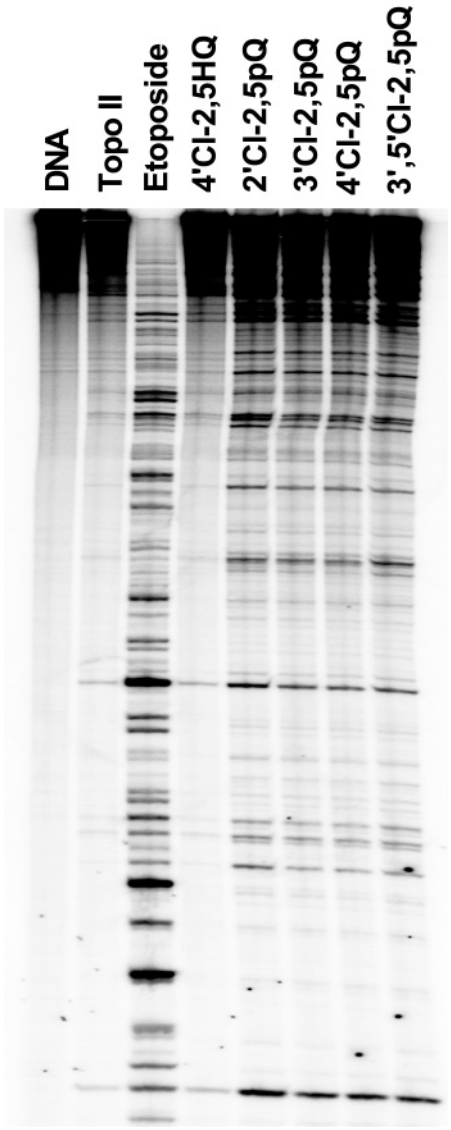

FIGURE 5: DNA cleavage site utilization by human topoisomerase IIα in the presence of PCB metabolites. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions contained no compound (Topo II), 12.5 μM etoposide, or 25 μM of the indicated PCB. A DNA control is shown in the far left lane. Data are representative of at least three independent experiments.

Exposure of Topoisomerase IIα to PCB Quinones in the Absence of DNA Inhibits Enzyme Activity. Although quinones act as topoisomerase II poisons when incubated with enzyme−DNA complexes, several studies indicate that they are potent inhibitors of enzyme activity when incubated with human topoisomerase IIα prior to the addition of DNA (*42*−*44*). It is not known whether these two properties of quinones share a common basis for action.

To determine whether PCB metabolites also display inhibitory properties, these compounds were incubated with topoisomerase IIα in the absence of DNA, and their effects on enzyme-mediated DNA cleavage were characterized. As seen in Figure 7, exposure of topoisomerase IIα to PCB quinones prior to the addition of plasmid rapidly decreased the ability of the enzyme to cleave DNA. Inhibition by all of the quinones (at 100 μM) was nearly complete within 30 s. In contrast, no inhibition was observed following a 3 min exposure to etoposide. While incubation with 4′Cl-2,5HQ decreased DNA cleavage, the rate of inhibition was substantially slower than that observed with the corresponding quinone. Taken together, these results indicate that PCB metabolites, like other quinone-based compounds (*19*, *42*, *43*) and ICRF-193 (which acts by trapping the *N*-terminal protein clamp of topoisomerase II) (*79*), are mixed function inhibitors of topoisomerase IIα. When incubated with the enzyme−DNA complex, they act as topoisomerase II poisons; when incubated with the enzyme in the absence of a nucleic acid substrate, they block topoisomerase II function.

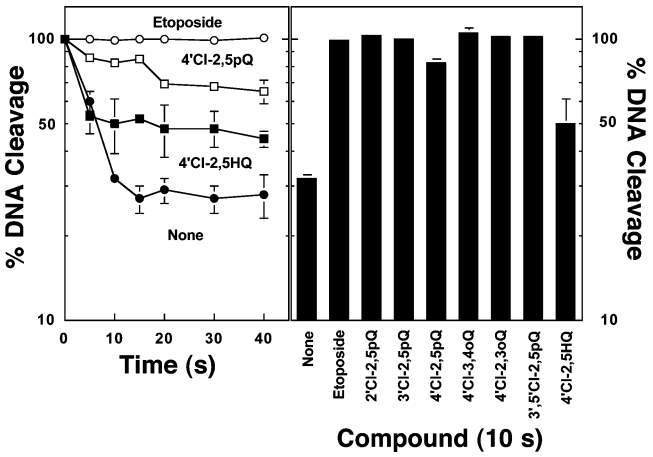

FIGURE 6: DNA religation mediated by human topoisomerase IIα is inhibited by PCB metabolites. Left panel: DNA religation was examined in the absence of compound (none, ●) or in the presence of 100 μM etoposide (○), 100 μM 4′Cl-2,5pQ (□), or 100 μM 4′Cl-2,5HQ (■). Right panel: Representative religation data for PCB metabolites at 10 s. In all cases, samples were incubated at 37 °C to establish DNA cleavage−religation equilibria and were then shifted to 0 °C to initiate religation. Levels of DNA cleavage observed at equilibrium were set to 100% at time zero. DNA religation was quantified by the loss of linear cleaved molecules. Error bars represent the standard deviation of at least three independent experiments.

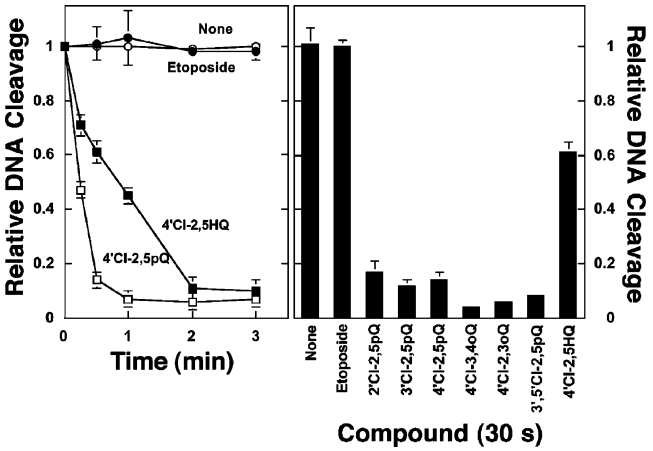

FIGURE 7: PCB metabolites rapidly inactivate human topoisomerase IIα in the absence of DNA. PCB metabolites were incubated with the enzyme prior to the addition of DNA, and the effects of these compounds on enzyme activity were assessed by DNA cleavage assays. Left panel: Time course for the incubation of topoisomerase IIα with no compound (●), 100 μM etoposide (○), 100 μM 4′Cl-2,5pQ (□), or 100 μM 4′Cl-2,5HQ (■). Right panel: Representative values for PCB metabolites at 30 s. Levels of DNA cleavage for reactions in which metabolites and DNA were added simultaneously (i.e., time zero) were set to 1.0. Error bars represent the standard deviation of at least three independent experiments.

Exposure of Topoisomerase IIα to PCB Quinones Prior to the Addition of DNA Inhibits the Binding of the Enzyme

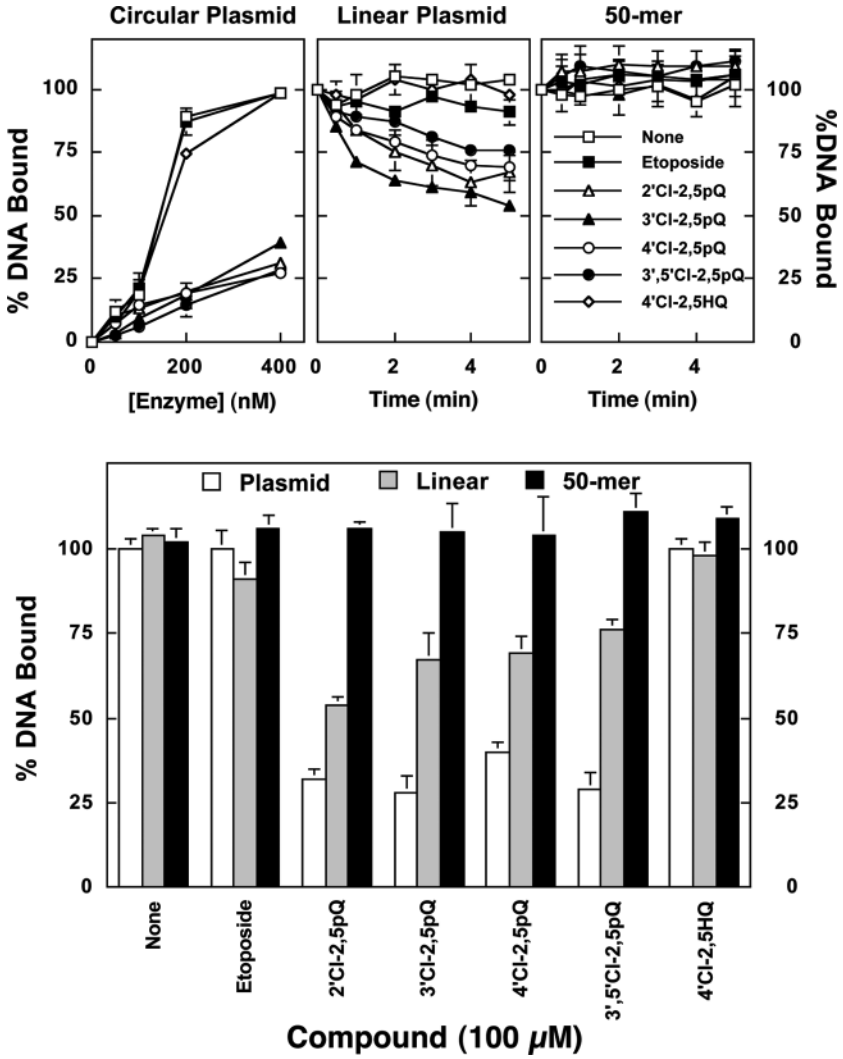

FIGURE 8: Substrate-dependent effects of PCB metabolites on topoisomerase IIα−DNA binding. Assays employed a negatively supercoiled circular plasmid (top left panel), a linear plasmid (top center panel), or a duplex 50-mer oligonucleotide (top right panel) as the substrate. For experiments that used the circular plasmid, 0−400 nM enzyme was incubated with no compound (none), 100 μM etoposide, or 100 μM PCB metabolite for 5 min prior to the addition of DNA. For assays that used linear substrates (linear plasmid or the 50-mer), 400 nM enzyme was incubated with no compound, 100 μM etoposide, or 100 μM PCB metabolite for 0−5 min prior to the addition of DNA. The bottom summary panel is a representative graph including all three substrates in assays carried out under identical reaction conditions (exposure of 400 nM enzyme to compounds for 5 min prior to the addition of DNA). Error bars represent the standard deviation of at least three independent experiments.

to Circular Plasmid Substrates. As a first step toward defining the mechanism by which incubation of PCB quinones with topoisomerase IIα in the absence of DNA impairs enzyme activity, the effects of these compounds on enzyme−DNA binding was assessed. Initial experiments utilized negatively supercoiled circular plasmids as substrates. Exposure of topoisomerase IIα to several *p*-quinones (at 100 μM) sharply decreased the formation of noncovalent enzyme−DNA complexes (Figure 8, top left panel and bottom summary panel). In contrast, equivalent concentrations of etoposide or 4′Cl-2,5HQ had no significant affect on binding.

Additional experiments examined the effects of PCB metabolites on the binding of topoisomerase IIα to linear plasmids. Similar patterns were observed with the linear substrate; however, the decrease in binding was less dramatic (Figure 8, top center panel and bottom summary panel). In general, the inhibition observed with linear DNA was ~1/2 that seen with circular substrates.

The linear plasmid substrates were >4300 bp in length. To further investigate the differences seen between linear and circular substrates, the effects of PCB metabolites on the ability of topoisomerase IIα to bind short DNA fragments was examined. The substrate used for these experiments was a duplex 50-mer that contained a DNA cleavage site for the human enzyme (*60*). As seen in Figure 8 (top right panel and bottom summary panel), exposure of topoisomerase IIα to PCB quinones prior to the addition of DNA had no substantial effect on the ability of the enzyme to bind the oligonucleotide.

In summary, PCB quinones sharply impair the binding of topoisomerase IIα to circular DNA substrates, display a smaller effect on the binding to linear molecules, and have no effect on the binding to oligonucleotides. These findings are not consistent with the idea that quinones adduct an amino acid residue in the active site of topoisomerase IIα. Rather, as discussed below, they suggest that the compounds may alter enzyme function by affecting the *N*-terminal gate of topoisomerase II.

4′Cl-2,5pQ Blocks the N-Terminal Protein Gate of Human Topoisomerase IIα. During the catalytic cycle of type II

topoisomerases, the T-helix (i.e., the DNA helix that is transported through the transient double-stranded break that the enzyme generates in a separate helix) enters the protein through a gate that is comprised of the *N*-terminal domains of the two protomer subunits of the enzyme (*21*, *23*, *24*, *27*, *80*). Upon ATP binding, topoisomerase II undergoes a conformational change that closes the *N*-terminal gate and induces passage of the T-helix through the DNA break (*21*, *23*, *24*, *27*, *80*−*82*). Formation of this *N*-terminal clamp does not allow the T-helix to exit the protein through the original gate (*21*, *23*, *24*, *27*, *80*−*84*). Thus, it promotes a unidirectional strand passage event (*82*). In addition, clamp closure does not allow a second T-helix to enter the enzyme through the *N*-terminal gate until the strand passage event is completed and the first T-helix has exited topoisomerase II through a *C*-terminal gate (*21*, *23*, *24*, *27*, *80*, *82*−*84*).

Closure of the *N*-terminal gate in the presence of DNA results in the formation of a protein clamp that topologically encircles the DNA, forming a noncovalent but salt−stable complex with circular nucleic acids (*81*, *83*). If the gate is closed prior to the addition of DNA, circular nucleic acids are prevented from entering the active site of topoisomerase II (*83*). Linear DNA molecules, however, are still able to access the active site by feeding directly through the central annulet of the enzyme (*81*, *83*). As discussed above, treatment of topoisomerase IIα with PCB quinones prior to the addition of DNA inhibits the binding of circular plasmid but has a distinctly lesser effect with the linear plasmid or oligonucleotide substrates. Because this finding is consistent with an effect on the *N*-terminal clamp, the ability of 4′Cl-2,5pQ and 4′Cl-2,5HQ to induce clamp closure was examined.

To assess clamp closing, topoisomerase IIα was incubated with negatively supercoiled plasmid, and the mixture was applied to a glass fiber filter. In the absence of a stable protein clamp, circular DNA (which does not bind to glass fibers) passes through the filter in the flow through, low salt (LS), or high salt (HS) washes. Alternatively, DNA trapped by stable protein clamps is eluted from the filter only after denaturation of the enzyme with SDS (Figure 9, inset).

Previously, stable clamps have been observed for wild-type human topoisomerase IIα only in the presence of a nonhydrolyzable ATP analogue or in the presence of ICRF-193 and ATP (*81*, *83*, *84*). Similar results were seen in the present study (Figure 9, left panel). Less than 3% of the circular plasmid substrate formed a salt-stable complex with the enzyme in the absence of an ATP analogue, whereas ∼10% did so in the presence of 100 mM ICRF-193 and ATP. Remarkably, when 100 μM 4′Cl-2,5pQ was incubated with a mixture of topoisomerase IIα and DNA, even in absence of ATP, ∼20% of the initial plasmid substrate formed a salt-stable noncovalent complex with the enzyme (left panel). In a set of parallel experiments, ∼8% of the plasmid formed a salt−stable complex following incubation with 100 μM 4′Cl-2,5HQ. Finally, incubation of topoisomerase IIα with 100 μM 4′Cl-2,5pQ prior to the addition of DNA reduced the formation of the salt-stable complex by nearly 10-fold (right panel).

Taken together, the above results strongly suggest that the exposure of topoisomerase IIα to PCB quinones blocks the *N*-terminal protein gate even in the absence of a high-energy cofactor.

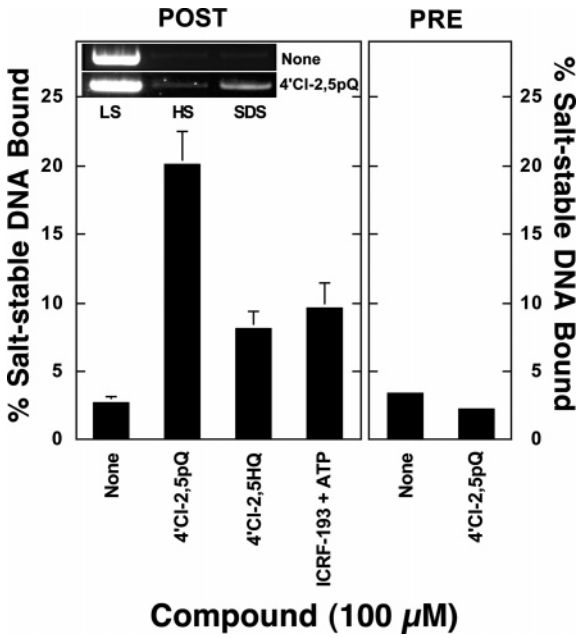

FIGURE 9: 4′Cl-2,5pQ blocks the *N*-terminal gate of human topoisomerase IIα. Filter binding assays were used to analyze the salt-stable closed-clamp of topoisomerase II. Reactions contained no compound (None), 100 μM 4′Cl-2,5pQ or 100 mM 4′Cl-2,-5HQ in the absence of ATP, or 100 μM ICRF-193 + 2 mM ATP. DNA eluted from filters after sequential low salt (LS), high salt (HS), or SDS (SDS) washes was subjected to electrophoresis in an agarose gel. Salt−stable noncovalent enzyme−DNA complexes were quantified by the amount of plasmid that did not elute until the SDS wash relative to that of the total plasmid eluted in all three washes. Left panel: Compounds were incubated with topoisomerase IIα after the addition of plasmid (POST). Right panel: 4′Cl-2,5pQ was incubated with enzyme prior to the addition of plasmid (PRE). The error bars represent the standard deviation of four independent experiments. A representative ethidium bromide-stained agarose gel is shown in the inset in the left panel.

4′Cl-2,5pQ Cross-Links the Subunits of Topoisomerase IIα. Data shown in Figure 4 indicate that PCB quinones alter the activity of topoisomerase IIα by covalently adducting the enzyme. Adduction results in the formation of a covalent bond between the sulfhydryl (or other amino acid group such as an amine) and one of the hydrogenated carbons on the quinone ring (*85*, *86*).

Quinone compounds are capable of cross-linking proteins in the cell (*85*, *87*, *88*). Previous studies demonstrate that benzoquinone (and derivatives) as well as PCB quinones are capable of adducting to more than one sulfhydryl group (*19*, *85*). To this point, a single molecule of 2′Cl-2,5pQ has been shown to adduct to three separate glutathione molecules, one at each of the compounds' electrophilic sites (*19*) (see Figure 11E). Thus, the PCB quinones shown in Figure 1 are able to act as protein cross-linking agents.

Therefore, three possible mechanisms are consistent with the actions of PCB quinones (Figure 10). First, covalent modification of topoisomerase IIα by quinones may induce the *N*-terminal gate to close in a manner that is similar to that observed in the presence of ATP (model A). While this model is formally possible, it should be noted that clamp closure in the absence of a nucleotide cofactor has not been reported (*81*, *83*, *84*). Second, quinones may adduct residues above the position where the T-segment normally rests prior to strand passage and sterically block the entrance or exit of the helix (model B). However, since there are no reported

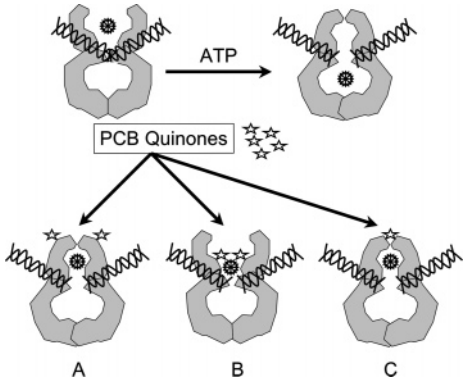

FIGURE 10: Models for how PCB Quinones block the *N*-terminal gate of human topoisomerase IIα. (A) PCB quinones induce natural clamp closure of the enzyme as seen with ATP (above). (B) PCB quinones adduct to positions above the T-segment that sterically block the entrance or exit of the helix. (C) PCB quinones cross-link the two protomers of human topoisomerase IIα.

structures of topoisomerase IIα with an intact but open *N*-terminal gate, it is not known whether PCB metabolites or other quinones would have enough bulk to act in this fashion. Third, PCB quinones may cross-link the two protomers of topoisomerase IIα, thereby blocking the *N*-terminal gate of the protein (model C).

To help distinguish between these three models, the ability of 4′Cl-2,5pQ to cross-link the two subunits of human topoisomerase IIα was assessed (Figure 11A and B). Following incubation with the PCB quinone, a distinct new protein band appeared with an apparent molecular mass of ~330 kDa. This mass is approximately twice that of the protomer molecular mass of topoisomerase IIα (170 kDa), suggesting that two subunits of the protein were cross-linked in the presence of 4′Cl-2,5pQ. Partial proteolytic mapping experiments demonstrated that specific topoisomerase IIα peptides were cross-linked by the quinone (Figure 11C). These results favor model C.

In addition to the dimer band, an apparent high molecular weight protein species was observed at the origin at incubation times of 1 min or longer (Figure 11A). After a 6 min exposure to 100 μM 4′Cl-2,5pQ, most of the enzyme remained at the origin (Figure 11E). It is notable that this protein species was soluble and did not precipitate upon centrifugation. Consequently, the protein at the origin does not appear to be an insoluble aggregate and is believed to represent a high molecular species that is too large to effectively enter the gel. Thus, it appears that the quinone is able to establish cross-links between different topoisomerase IIα homodimers (i.e., intermolecular cross-links).

If model C is correct, however, 4′Cl-2,5pQ must be able to cross-link the two promoter subunits of an individual enzyme homodimer (i.e., intramolecular cross-link). Therefore, dilution experiments (from 800 down to 80 nM topoisomerase IIα) were carried out to determine whether the 330 kDa band resulted from an inter or intramolecular cross-linking event. If the 330 kDa band arose from the cross-linking of two separate topoisomerase IIα molecules, it should decrease proportionally as the concentration of the protein decreases. Conversely, if the band arose from the cross-linking two protomers of an individual topoisomerase IIα homodimer, its intensity should be independent of protein concentration. As seen in Figure 11D, the 330 kDa band did not decrease at lower protein concentrations. This finding indicates that the dimer band most likely is formed by an intramolecular cross-linking event. On the basis of these data, we propose that PCB quinones block the *N*-terminal gate of topoisomerase IIα, at least in part, by cross-linking the two protomer subunits of the enzyme.

As a control, the effects of plumbagin on topoisomerase IIα were examined (Figure 11E). Although similar in size to the PCB metabolites, this compound has a quinone ring that contains only a single reactive site (marked by the asterisk in the figure). In comparison, 4′Cl-2,5pQ has three potential sites of adduction (also marked by asterisks). Even though plumbagin covalently adducts to cysteine residues on human topoisomerase IIα (determined by mass spectrometry), it displayed very little ability to enhance enzyme-mediated DNA cleavage (*42*) or to inhibit enzyme activity in the absence of nucleic acids (data not shown). Following a 6 min incubation with 250 μM plumbagin, no protein dimers or high molecular weight species were observed. Hence, as expected for a quinone that has only a single site of adduction, plumbagin displays no ability to induce protein−protein cross-links. These findings are not consistent with models A or B, which require only a single site of adduction per quinone molecule. Rather, they support the importance of protomer−protomer cross-links, as postulated in model C.

PCB Metabolites Increase Levels of DNA Cleavage Mediated by Topoisomerase IIα in Cultured Human Cells. Because PCB metabolites act as topoisomerase II poisons in vitro, the ICE bioassay was employed to determine whether these compounds increase DNA cleavage mediated by topoisomerase IIα in human cells. In this assay, cultured CEM leukemia cells were lysed with an ionic detergent, and proteins that were covalently attached to genomic DNA were separated from free proteins by sedimentation through a CsCl cushion. The pelleted DNA from cultures treated with no compound or 25 μM PCB metabolites for 6 h was blotted and probed with a polyclonal antibody directed against human topoisomerase IIα. Results for cells treated with 10 μM etoposide for 2 h are shown for comparison. In all cases, less than 10% cell death was observed over the course of the experiment. As seen in Figure 12, levels of topoisomerase IIα that were covalently attached to DNA increased 3- to 4-fold following treatment of cells with PCB metabolites (as compared to ~7-fold with etoposide).

The covalent topoisomerase II−DNA complexes monitored in the ICE bioassay are believed to represent cleavage complexes. However, since some quinones can induce protein−DNA cross-links, it is possible that a portion of the observed complexes may actually reflect cross-links rather than cleavage. We do not believe that this is the case for the following reasons. First, no protein−DNA cross-linking was observed in experiments with purified topoisomerase IIα, even under conditions that employed enzyme/base pair ratios (~1:325) that were >20 times higher than those estimated (~1:7500) for CEM cells (*89*) and quinone concentrations (100 μM) that were 4 times higher than those used in the cellular studies (Figure 3). Second, treatment of CEM cells with 25 μM 4′Cl-2,5pQ did not increase levels of covalent topoisomerase I−DNA complexes (Figure 12). It is notable that 4′Cl-2,5pQ does not stimulate topoisomerase I-mediated DNA cleavage in vitro (data not shown). Because the type

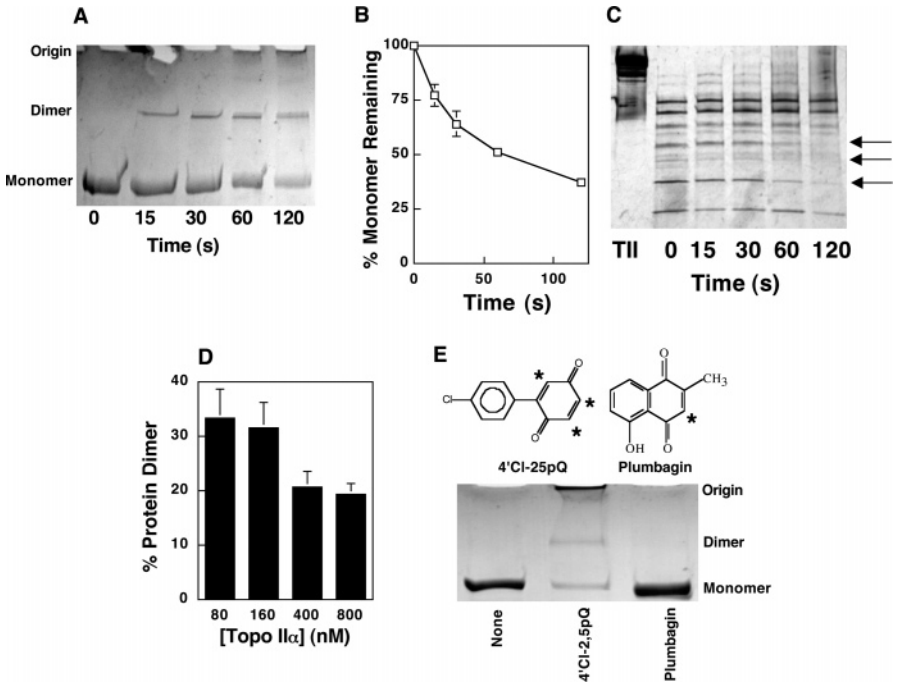

FIGURE 11: 4′Cl-2,5pQ cross-links the protomer subunits of human topoisomerase IIα. Panel A: Coumassie-stained denaturing polyacrylamide gel in which 135 nM topoisomerase IIα was treated with 100 μM 4′Cl-2,5pQ for 0−2 min. The mobility of a topoisomerase IIα subunit monomer and dimer, as well as the origin of the gel, is indicated as well as the origin of the gel. The gel is representative of two independent experiments. Panel B: The loss of the monomeric topoisomerase IIα subunit over time as a result of cross-linking by 4′Cl-2,5pQ. Data were derived from the gel shown in Panel A and a parallel experiment. Error bars represent the standard error of the mean for these two experiments. Panel C: Display of a partial proteolytic map of topoisomerase IIα following exposure to 100 μM 4′Cl-2,5pQ over time. A silver-stained polyacrylamide gel that is representative of four independent experiments is shown. Arrows indicate specific polypeptide bands that diminished following exposure to the PCB quinone. Panel D: The formation of SDS-stable dimers when 80−800 nM topoisomerase IIα was treated with 100 μM 4′Cl-2,5pQ for 1 min. Error bars represent the standard deviation of four independent experiments. Panel E: Coumassie stained denaturing polyacrylamide gel in which 135 nM topoisomerase IIα was treated with no compound (None), 100 μM 4′Cl-2,5pQ, or 250 μM plumbagin for 6 min. The structures of both compounds are shown with sites of potential protein adduction marked by asterisks. The gel is representative of two independent experiments.

I enzyme is intimately associated with chromosomal DNA, this latter finding argues against 4′Cl-2,5pQ acting as a general protein−DNA cross-linking reagent. On the basis of these two control experiments, we conclude that PCB metabolites are topoisomerase II poisons in cultured human cells.

Although 4′Cl-2,5HQ displayed minimal cleavage enhancing activity in vitro, it induced topoisomerase II-mediated DNA scission in CEM cells. However, since these leukemic cells display peroxidase activity (*90*), it is likely that the hydroquinone was activated to 4′Cl-2,5pQ within the cell.

DISCUSSION

Exposure to PCBs induces a variety of chromosomal aberrations in humans and treated human cells (*1*, *3*), but the underlying basis for this genomic damage has yet to be defined. Multiple pathways are likely to be involved, such as oxidative damage due to redox cycling, glutathione depletion, or the adduction of protein thiols (*15*−*19*).

Because the genotoxic effects of PCBs are consistent with the actions of topoisomerase II poisons and quinones have been shown to enhance DNA scission mediated by the type II enzyme (*42*−*44*), we examined the effects of several PCB metabolites on the activity of human topoisomerase IIα. Results indicate that a variety of PCB quinones, including mono and dichlorinated compounds and both *p*- and *o*-quinones, are potent topoisomerase IIα poisons in vitro and in cultured human cells. Furthermore, as demonstrated previously with other bioreactive quinones (*42*−*44*), incubation of topoisomerase IIα with PCB metabolites in the absence of DNA leads to a rapid loss of enzyme activity.

Although quinones appear to act by covalently adducting topoisomerase IIα (*42*−*44*), the mechanism by which these compounds alter enzyme activity is not understood. Moreover, it is not known whether the same series of events is responsible for both the enhancement of DNA cleavage and the inhibition of enzyme activity. Results with PCB metabolites suggest that these two opposing actions of quinones may be linked, at least in part, to an effect on the *N*-terminal gate of topoisomerase IIα. On the basis of the differential ability of the quinone-treated enzyme to bind circular and linear forms of DNA and the generation of salt-stable noncovalent complexes between topoisomerase IIα and circular plasmids in the presence of 4′Cl-2,5pQ, it appears that PCB metabolites block the *N*-terminal gate of the protein.

The effects of 4′Cl-2,5pQ on topoisomerase IIα are in many respects similar to those described for ICRF-193 and related bisdioxopiperazines. These compounds alter enzyme function by stabilizing the closed-clamp form of topoisomerase II (*84*, *91*, *92*). If the clamp is closed in the absence of a nucleic acid substrate, DNA cleavage is inhibited because DNA is unable to enter the active site of the enzyme (*84*). Conversely, if the clamp closed after DNA binding, levels of cleavage rise modestly (2- to 3-fold) (*93*). This latter effect presumably is due to the increased concentration of noncovalent enzyme−DNA complexes. In this regard, cleav-

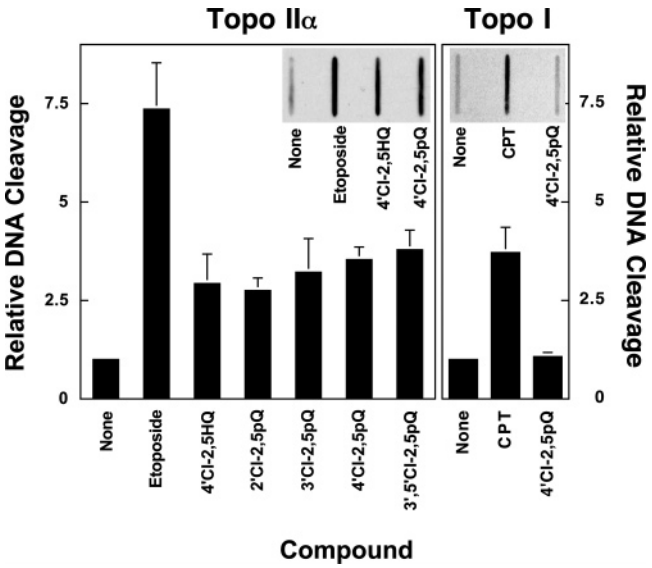

FIGURE 12: PCB metabolites increase topoisomerase IIα-mediated DNA cleavage in human CEM cells. The ICE bioassay was used to monitor the level of cleavage complexes in cells treated with PCB metabolites. DNA (10 μg) from cultures treated with no compound (None), 25 μM of the indicated PCB metabolite for 6 h, or 10 μM etoposide or 10 μM camptothecin (CPT) for 2 h was blotted onto a nitrocellulose membrane. Blots were probed with a polyclonal antibody directed against human topoisomerase IIα (left) or human topoisomerase I (right). Relative DNA cleavage was calculated relative to levels of cleavage complexes observed in untreated cells. Error bars represent the standard deviation of three independent experiments. Representative immunoblots are shown in the insets.

age enhancement by 4′Cl-2,5pQ (~8-fold) was considerably higher than that induced by ICRF-193. However, PCB quinone also was more efficient at trapping a salt−stable complex between topoisomerase IIα and supercoiled plasmid.

The above similarities notwithstanding, the mechanism by which 4′Cl-2,5pQ traps the salt-stable complex is very different than that seen with ICRF-103. While bisdioxopiperazines act in a strictly noncovalent manner (*84*), the quinones appear to require covalent adduction to the protein (*42*−*44*). Furthermore, bisdioxopiperazines stabilize the closed *N*-terminal protein clamp by inhibiting ATP hydrolysis (*79*). In contrast, PCB quinones induce a salt-stable enzyme−DNA complex even in the absence of a nucleotide triphosphate cofactor and have only a modest effect on ATP hydrolysis (data not shown). On the basis of protein cross-linking experiments, we propose that PCB quinones block the *N*-terminal gate of topoisomerase IIα by cross-linking the two protomer subunits of the enzyme (Figure 10, model C). If this hypothesis is correct, then reactive amino acids (most likely cysteines) on the two protomers must be in close enough proximity in the absence of ATP (<8 Å) to bond to the same quinone molecule. Since the *N*-terminal gate of topoisomerase IIα has to be at least 20 Å across to accommodate the T-segment, this proposal implies that the *N*-terminal domain of topoisomerase IIα must be very flexible and dynamic in the absence of ATP.

Two complicating points should be noted. First, while exposure to 4′Cl-2,5pQ resulted in the preferential loss of individual peptides, no specific higher molecular mass peptide species were identified (Figure 11C). Rather, a high molecular mass smear was observed. This finding suggests that the quinone cross-linking of the topoisomerase IIα protomers may involve multiple sites of adduction rather than unique amino acid residues. Second, under conditions that paralleled those employed for DNA cleavage experiments, high molecular mass topoisomerase IIα multimers were observed following exposure to PCB quinones (Figure 11A and E). This indicates that intermolecular protein−protein cross-linking also is taking place. It is not known how (or if) these intermolecular events contribute to either the enhancement of enzyme-mediated DNA cleavage or the inhibition of enzyme activity by quinones.

Finally, although the evidence indicates that blocking the *N*-terminal protein gate plays an important role in the actions of quinones against topoisomerase IIα, additional possibilities cannot be excluded. To this point, the quinone-treated enzyme binds oligonucleotide substrates, but the adducted protein is not capable of cleaving the bound 50-mers (data not shown). Furthermore, levels of clamp closing and protein cross-linking are not as great as levels of enzyme inhibition under similar conditions. Thus, in the absence of structural data, alternative mechanisms must still be considered.

In conclusion, PCB metabolites are reactive chemicals that are health hazards to humans and probable carcinogens. The toxic and mutagenic effects of these compounds are complex and most likely driven by a variety of pathways. Although the ultimate relationship of our findings to the health effects of PCBs is not known, the present work suggests that topoisomerase IIα may play a role in mediating at least some of the chromosomal damage induced by these environmental contaminants.

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