N-Acetyl-*p*-benzoquinone Imine, the Toxic Metabolite of Acetaminophen, Is a Topoisomerase II Poison†

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ABSTRACT: Although acetaminophen is the most widely used analgesic in the world, it is also a leading cause of toxic drug overdoses. Beyond normal therapeutic doses, the drug is hepatotoxic and genotoxic. All of the harmful effects of acetaminophen have been attributed to the production of its toxic metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI). Since many of the cytotoxic/genotoxic events triggered by NAPQI are consistent with the actions of topoisomerase II-targeted drugs, the effects of this metabolite on human topoisomerase IIα were examined. NAPQI was a strong topoisomerase II poison and increased levels of enzyme-mediated DNA cleavage >5-fold at 100 μM. The compound induced scission at a number of DNA sites that were similar to those observed in the presence of the topoisomerase II-targeted anticancer drug etoposide; however, the relative site utilization differed. NAPQI strongly impaired the ability of topoisomerase IIα to reseal cleaved DNA molecules, suggesting that inhibition of DNA religation is the primary mechanism underlying cleavage enhancement. In addition to its effects in purified systems, NAPQI appeared to increase levels of DNA scission mediated by human topoisomerase IIα in cultured CEM leukemia cells. In contrast, acetaminophen did not significantly affect the DNA cleavage activity of the human enzyme in vitro or in cultured CEM cells. Furthermore, the analgesic did not interfere with the actions of etoposide against the type II enzyme. These results suggest that at least some of the cytotoxic/genotoxic effects caused by acetaminophen overdose may be mediated by the actions of NAPQI as a topoisomerase II poison.

Acetaminophen is the most widely used analgesic in the United States and the world and is contained in more than one hundred prescription and nonprescription products (*1*, *2*). Unfortunately, the drug is also the second leading cause of toxic drug overdoses in the United States and accounts for ~50% of hospital admissions for poisoning in the United Kingdom (*3*, *4*). Over 56000 cases of acetaminophen poisoning were reported in the United States in the year 2000, resulting in 100 deaths (*3*, *4*).

Acetaminophen is metabolized in the liver (*5*). Normally, the drug is cleared from the body by sulfation or glucuronidation followed by renal excretion (*1*, *6*−*9*). However, if these two processes become overwhelmed or if the cytochrome P450 system has been induced by prior insult to the liver (such as alcohol ingestion), acetaminophen is converted to *N*-acetyl-*p*-benzoquinone imine (NAPQI)[1] by CYP2E1 and to a lesser extent by CYP1A2 and CYP3A4 (*1*, *6*−*12*). NAPQI is cleared primarily by conjugation to glutathione (*1*, *6*−*9*).

All of the harmful effects of acetaminophen have been attributed to the formation of NAPQI (*1*, *6*−*9*, *13*). This reactive metabolite is toxic to cells and induces apoptosis and necrosis in cultured cells and in vivo (*13*, *14*). In addition, the compound is genotoxic in humans and genotoxic and carcinogenic in animals (*13*, *15*). It generates DNA strand breaks, gaps, and other chromosomal aberrations and induces sister chromatid exchange (*13*, *15*). The mechanistic basis for the actions of NAPQI is not fully understood. Since the compound can alkylate and oxidize proteins, it was initially believed that NAPQI toxicity was due to a direct inactivation of cellular enzymes (*6*, *13*, *16*). More recent studies, however, suggest that toxicity is facilitated by the depletion of cellular glutathione (*6*, *13*, *17*). This depletion opens the cell to a variety of reactive chemicals (including oxygen radicals), which inactivate enzymes and damage the genetic material (*13*, *17*). Finally, since depletion of glutathione allows the accumulation of NAPQI in cells, it is once again believed that (at least) some of the cytotoxic/genotoxic effects of NAPQI may be caused by the direct modification and/or oxidation of cellular proteins by the compound (*13*).

The specific cellular proteins that are modified by NAPQI are not well characterized. However, the genotoxic effects

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[1] Abbreviations: NAPQI, *N*-acetyl-*p*-benzoquinone imine; ICE bioassay, *i*n vivo *c*omplex of *e*nzyme bioassay.

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of the coupound are consistent with the actions of topoisomerase II-targeted drugs. Topoisomerase II is an enzyme that removes knots and tangles from the genome and is necessary for proper chromosome structure and segregation (*18*−*25*). Although lower eukaryotes, such as yeast and *Drosophila*, contain only a single form of topoisomerase II, vertebrates contain two isoforms of the enzyme, topoisomerase IIα and II*β* (*26*−*33*). 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 (*21*, *34*−*38*).

Topoisomerase II acts by passing an intact double helix through a transient double-stranded break that it creates in a separate segment of DNA (*18*−*20*, *22*−*24*). To maintain genomic integrity during the DNA strand passage event, the enzyme forms covalent bonds between active site tyrosyl residues and the 5′-DNA termini created by cleavage of the double helix (*39*−*41*). These covalent topoisomerase II-cleaved DNA intermediates (known as *cleavage complexes*) normally are present in low steady-state levels and are tolerated by the cell (*18*−*20*, *22*−*24*). However, conditions that significantly increase the concentration or longevity of cleavage complexes induce many of the genotoxic and cytotoxic events described above, including the generation of DNA strand breaks, chromosomal aberrations, sister chromatid exchange, and the induction of apoptosis (*20*, *23*, *24*, *42*−*46*).

A variety of widely used anticancer drugs, such as etoposide, kill cells by increasing cellular levels of topoisomerase II−DNA cleavage complexes (*20*, *23*, *24*, *47*−*51*). These drugs are referred to as *topoisomerase II poisons* (to distinguish them from catalytic inhibitors of the enzyme) because they convert an essential enzyme into a potent cellular toxin (*20*, *23*, *24*, *47*, *49*, *51*, *52*). Most topoisomerase II poisons are believed to function in the active site of the enzyme (*20*, *23*, *24*, *47*, *49*, *51*). However, recent evidence suggests that some sulfhydryl-reactive chemicals, such as quinones, also have the potential to increase levels of DNA cleavage mediated by human topoisomerase IIα (*53*, *54*).

Since the genotoxic events triggered by NAPQI resemble those of topoisomerase II poisons, the effects of the compound on human topoisomerase IIα were examined. Results indicate that NAPQI is a topoisomerase II poison that increases levels of DNA cleavage mediated by the human enzyme at physiologically relevant concentrations in vitro and in cultured cells. Acetaminophen, the parent compound, has little effect on the activity of topoisomerase IIα and does not antagonize the actions of etoposide against the enzyme.

EXPERIMENTAL PROCEDURES

Enzymes and Materials. Human topoisomerase IIα was expressed in *Saccharomyces cerevisiae* (*55*) and purified as described previously (*56*, *57*). Negatively supercoiled pBR322 DNA was prepared using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. NAPQI and acetaminophen were purchased from Sigma, prepared as 20 mM stock solutions in 100% DMSO and water, respectively, and used fresh for all experiments. Etoposide (Sigma) was prepared as a 20 mM stock solution in 100% DMSO and stored at 4 °C. All other chemicals were of analytical reagent grade.

DNA Cleavage. DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (*58*). 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 NAPQI, acetaminophen, or etoposide. Assays that examined the ability of acetaminophen to compete with etoposide contained 0−500 μM acetaminophen in a constant background of 25 μM 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 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 plasmid DNA to linear molecules. DNA bands were visualized by ultraviolet light and quantified using an Alpha Innotech digital imaging system.

In reactions that determined whether DNA cleavage by human topoisomerase IIα was reversible, EDTA was added prior to treatment with SDS. To determine whether cleavage was protein-linked, proteinase K treatment was omitted.

To analyze the effects of NAPQI on human topoisomerase IIα in the absence of DNA, the drug (or equivalent levels of DMSO solvent) 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 NAPQI 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 NAPQI. DNA sites cleaved by human topoisomerase IIα were determined as described by O'Reilly and Kreuzer (*59*). A linear 4330 bp fragment (*Hin*dIII/*Eco*RI) of pBR322 plasmid DNA singly labeled with ^{32}P on the 5′-terminus of the *Hin*dIII site was used as the cleavage substrate. Reaction mixtures contained 0.35 nM DNA substrate and 60 nM human topoisomerase IIα in 50 μL of cleavage buffer. Assays were carried out in the absence of drug or in the presence of 25 μM etoposide or 100 μM NAPQI. Reactions were started by the addition of the 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 and dried, and DNA cleavage products were analyzed on a Bio-Rad Molecular Imager FX.

DNA Religation. The DNA religation reaction of human topoisomerase IIα was monitored according to the procedure of Byl et al. (*60*). Topoisomerase IIα DNA cleavage−religation equilibria were established as described above in the presence of no drug or 25 μM NAPQI. 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 followed by 1 μL of 375 mM NaEDTA, pH 8.0. Samples were processed and analyzed as described above for topoisomerase IIα cleavage reactions.

DNA Relaxation. DNA relaxation reactions were carried out as described by Fortune and Osheroff (*58*). 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 that contained 0−25 μM NAPQI. DNA relaxation was initiated by the addition of enzyme, and mixtures were incubated for 30 s at 37 °C. Reactions were stopped by adding 1 μL of 375 mM EDTA, pH 8.0, followed by 2 μL of 0.5% SDS and 77 mM EDTA. Proteinase K was added (2 μL of 0.8 mg/mL), and reactions were incubated for 30 min at 45 °C to digest the type II enzyme. Samples were subjected to electrophoresis in 1% agarose gels containing 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.

Topoisomerase II-Mediated DNA Cleavage in CEM Cells. Human CEM acute lymphoblastic 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 (*61*, *62*) (as modified on the TopoGEN, Inc., website) was employed to determine the ability of NAPQI to induce topoisomerase IIα-mediated DNA breaks in treated CEM cells. Exponentially growing cultures were treated with 50 or 100 μM NAPQI for 4 h or with 10 μM etoposide for 1 h for comparison. In assays that examined the ability of acetaminophen to compete with etoposide, cells were treated with 150 μM acetaminophen for 4 h in the absence or presence of 10 μM etoposide (added for the last hour of treatment). Cells ($\sim 5 \times 10^6$) were harvested by centrifugation and lysed by the immediate addition of 3 mL of 1% sarkosyl. Following gentle dounce homogenization, cell lysates were layered onto a 2 mL cushion of CsCl (1.5 g/mL) and centrifuged in a Beckman NVT 90 rotor at 80000 rpm ($\sim$500000*g*) for 5.5 h at 20 °C. DNA pellets were isolated, resuspended in 25 mM sodium phosphate (pH 6.9), 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 1:1000 dilution.

RESULTS

NAPQI Increases DNA Cleavage Mediated by Human Topoisomerase IIα. The reactive metabolite of acetaminophen, NAPQI, is genotoxic (*1*, *6*−*9*, *13*). It induces DNA strand breaks, chromosomal aberrations, and sister chromatid exchanges in a variety of mammalian cells (*13*, *15*). The mechanisms underlying the genotoxic effects of NAPQI are not well characterized. However, many of the cellular responses to the compound are characteristic of topoisomerase II poisons (*20*, *23*, *24*, *42*−*46*). Therefore, the effects of NAPQI on DNA cleavage mediated by human topoisomerase IIα were examined.

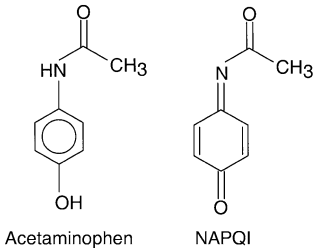

FIGURE 1: Stuctures of acetaminophen and NAPQI.

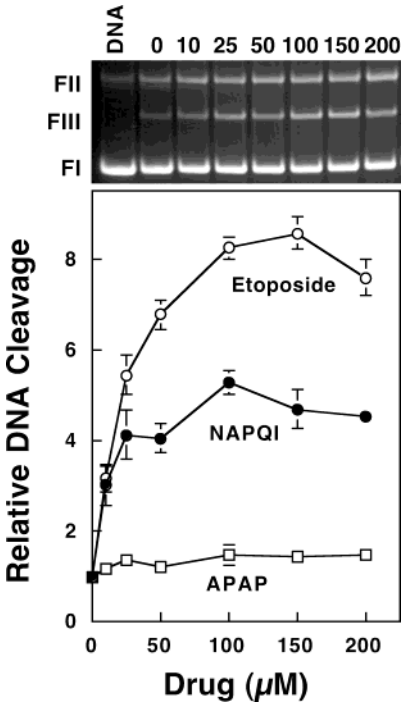

FIGURE 2: NAPQI stimulates DNA cleavage mediated by human topoisomerase IIα. An ethidium bromide-stained agarose gel of DNA cleavage reactions carried out in the presence of 0−200 μM NAPQI is shown at the top. A lane containing DNA in the absence of enzyme (DNA) is shown as a control. The mobilities 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 drug. Assays mixtures contained NAPQI (closed circles), etoposide (open circles), or acetaminophen (APAP, open squares). Error bars represent the standard deviation of three independent experiments.

The plasma concentration of acetaminophen in humans following a standard drug dose ranges from 50 to 100 μM (*1*, *6*−*9*, *13*, *63*). The average plasma concentration in patients who suffer accidental acetaminophen overdose is $\sim$250 μM drug, while that in patients who intentionally overdose is $\sim$950 μM drug (*3*). With these values in mind, the effects of 0−200 μM acetaminophen and NAPQI on DNA cleavage mediated by human topoisomerase IIα were determined (Figure 2).

NAPQI increased levels of topoisomerase II-mediated double-stranded DNA breaks in a concentration-dependent manner. The highest relative amount of DNA scission was

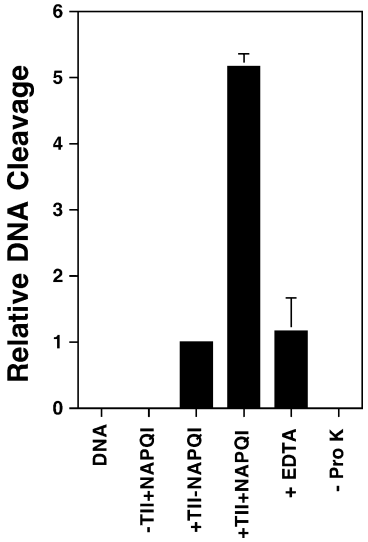

FIGURE 3: DNA cleavage in the presence of NAPQI is mediated by topoisomerase IIα. Levels of DNA cleavage were quantified and expressed as a fold enhancement over reactions that were carried out in the absence of drug. Data for DNA alone (DNA) or with NAPQI (−TII + NAPQI) in the absence of enzyme are shown. DNA cleavage mediated by human topoisomerase IIα in the absence (+TII − NAPQI) or presence (+TII + NAPQI) of 100 μM NAPQI was examined. Reversibility of reactions containing NAPQI was examined by adding EDTA prior to SDS treatment (+EDTA). To determine whether the DNA cleavage observed in the presence of NAPQI was protein-linked, proteinase K treatment was omitted (−Pro K). Error bars represent the standard deviation of three independent experiments.

observed at 100 μM NAPQI and was >5-fold higher than the no drug control. This cleavage enhancement is as compared to the widely used anticancer drug, etoposide, which stimulated enzyme-mediated DNA cleavage ~8-fold at a similar concentration. In marked contrast, acetaminophen had a marginal effect on the DNA cleavage activity of human topoisomerase IIα. These results indicate that metabolism of acetaminophen to NAPQI converts the parent compound to a strong topoisomerase II poison.

Several controls were carried out to ensure that the DNA cleavage seen in Figure 2 was mediated by topoisomerase IIα (Figure 3). First, no linear DNA was generated by NAPQI in the absence of enzyme. Second, scission was reversed when EDTA was added to the reaction prior to denaturation of topoisomerase IIα with SDS. This reversibility is consistent with an enzyme-mediated reaction. Third, the electrophoretic mobility of the cleaved DNA (i.e., linear band) was dramatically retarded in the absence of proteinase K treatment, indicating that all of the cleaved plasmid molecules were covalently attached to topoisomerase IIα. These findings provide strong evidence that the double-stranded DNA breaks observed in the presence of NAPQI are generated by human topoisomerase IIα.

To further examine the effects of NAPQI on DNA scission mediated by the human type II enzyme, singly end-labeled linear pBR322 plasmid was used as a substrate. This linear DNA allows cleavage to be monitored at the site-specific level. As seen in Figure 4, NAPQI increased DNA scission at every site cleaved by the enzyme in the absence of drug. It also induced cleavage at a number of additional DNA sites. Levels of cleavage induced by 100 μM NAPQI were similar to those observed in reactions that contained 25 μM

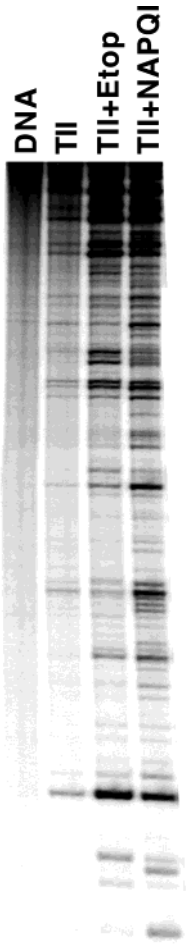

FIGURE 4: DNA cleavage site utilization by human topoisomerase IIα in the presence of NAPQI. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions contained no drug (TII), 25 μM etoposide (TII + Etop), or 100 μM NAPQI (TII + NAPQI). A DNA control is shown in the far left lane (DNA). Data are representative of three independent assays.

etoposide. This result is consistent with assays that employed negatively supercoiled plasmid substrate (see Figure 2). While the DNA sites cleaved by topoisomerase IIα in the presence of the two drugs were similar, site utilization differed. A number of sequences appeared to be preferentially cleaved when NAPQI was present in reactions.

Recent reports indicate that agents that are reactive toward protein sulfhydryl groups increase levels of DNA breaks generated by human topoisomerase IIα when they are incubated with the enzyme in the presence of DNA (*53*, *54*). A common characteristic of these "sulfhydryl-reactive topoisomerase II poisons" is that they rapidly inactivate topoisomerase IIα when they are incubated with the enzyme in the absence of its DNA substrate (*53*). Since NAPQI is reactive toward protein sulfhydryl groups and modifies a number of proteins (*13*, *16*), similar incubation studies were performed (Figure 5). NAPQI rapidly inactivated human topoisomerase IIα in the absence of DNA. Within 1 min, the enzyme lost ~90% of its DNA cleavage activity. These results suggest that the effects of NAPQI on the human enzyme may be due (at least in part) to a covalent modification of the protein.

NAPQI Inhibits DNA Religation Mediated by Human Topoisomerase IIα. Topoisomerase II poisons increase levels

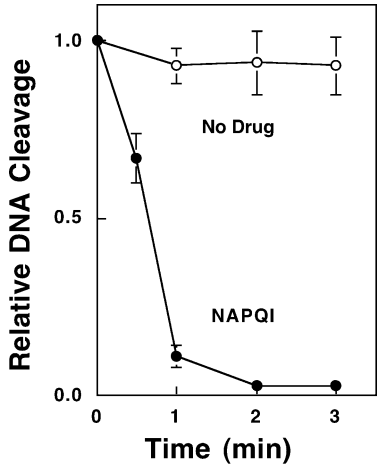

FIGURE 5: NAPQI rapidly inactivates human topoisomerase IIα in the absence of DNA. Reaction mixtures were incubated in the absence (open circles) or presence of 100 μM NAPQI (closed circles) for 0−3 min prior to the addition of DNA. Levels of DNA cleavage in reaction mixtures that were not preincubated (i.e., time zero) were set to 100%. Error bars represent the standard deviation of three independent experiments.

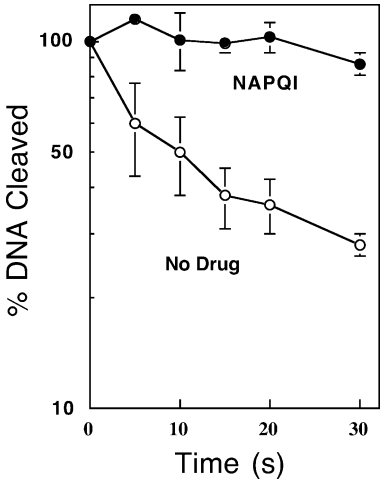

FIGURE 6: DNA religation mediated by human topoisomerase IIα is inhibited by NAPQI. DNA religation mediated by topoisomerase IIα was examined in the absence of drug (open circles) or in the presence of 25 μM NAPQI (closed circles). Samples were incubated at 37 °C to establish DNA cleavage/religation equilibria and were then shifted to 0 °C to initiate religation. The amount of DNA cleavage observed at equilibrium for each drug was set to 100% at time zero. DNA ligation was quantified by the loss of linear cleaved molecules. Points represent the average of three independent experiments.

of enzyme-mediated DNA breaks by two nonmutually exclusive mechanisms. Whereas etoposide appears to act primarily by inhibiting the ability of topoisomerase II to religate DNA breaks, other poisons such as quinolones appear to act primarily by enhancing the forward rate of DNA scission (*20*, *23*, *24*, *64*−*66*).

To determine the mechanistic basis for the actions of NAPQI against human topoisomerase IIα, the effects of the compound on enzyme-mediated DNA religation were examined (Figure 6). The rate of religation in the presence of NAPQI was severalfold slower than observed in the absence of drug and approached levels of inhibition seen with etoposide (not shown). While the enzyme religated <15% of the cleaved DNA in 30 s in reactions that contained 25 μM NAPQI, the $t_{1/2}$ value for religation in the absence of drug was ∼10 s. On the basis of these values, it is proposed that NAPQI increases levels of topoisomerase II-mediated DNA breaks primarily by inhibiting the ability of the enzyme to religate DNA.

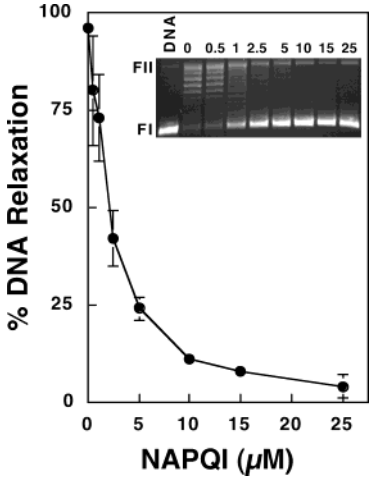

FIGURE 7: NAPQI inhibits the overall catalytic activity of topoisomerase IIα. DNA relaxation reactions catalyzed by topoisomerase IIα were carried out for 30 s in the presence of 0−25 μM NAPQI. DNA relaxation was quantified by the disappearance of negatively supercoiled DNA substrate. Error bars represent 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 0−25 μM NAPQI. A lane containing DNA in the absence of enzyme (DNA) is shown as a control. The mobilities of negatively supercoiled DNA (form I, FI) and nicked circular plasmid (form II, FII) are indicated.

NAPQI Inhibits DNA Strand Passage Catalyzed by Human Topoisomerase IIα. A number of well-characterized topoisomerase II poisons inhibit the overall catalytic activity of the enzyme (*67*−*71*). However, in many cases, the ability to increase DNA cleavage and inhibit overall catalytic activity requires significantly different drug concentrations. This finding implies that different drug binding sites on topoisomerase II may be responsible for the two events. Therefore, to determine the relationship between cleavage enhancement and catalytic inhibition for NAPQI, the effects of the drug on topoisomerase II-catalyzed DNA relaxation were examined.

NAPQI was a strong inhibitor of DNA relaxation catalyzed by human topoisomerase IIα. When catalytic levels of the enzyme (∼5 nM) were employed in assays, 50% inhibition was observed at nearly stoichiometric levels of NAPQI (∼5 nM) (not shown). To compare results of catalytic and DNA cleavage assays directly, relaxation was carried out using the same concentration of topoisomerase IIα that was utilized for DNA scission (∼135 nM). Short time courses (30 s) and low ATP concentrations (250 μM) were employed in these latter experiments so that inhibition could be monitored accurately. As seen in Figure 7, 50% inhibition of DNA relaxation at high topoisomerase IIα concentrations was observed at ∼1.5 μM NAPQI. This drug concentration is as compared to the ∼15 μM NAPQI that was required to enhance DNA cleavage to 50% of its maximal value. These results suggest that the inhibition of overall catalytic activity and the stimulation of DNA cleavage by NAPQI may be due to separate events.

NAPQI Is a Topoisomerase II Poison in Cultured Human Cells. The ICE bioassay was employed to determine whether

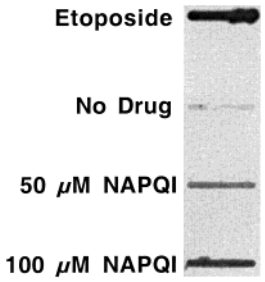

FIGURE 8: NAPQI enhances human topoisomerase IIα-mediated DNA cleavage in treated human CEM cells. The ICE bioassay was used to monitor the level of cleavage complexes in cells treated with NAPQI. DNA (10 μg) from cultures treated with no drug, 50 or 100 μM NAPQI, or 10 μM etoposide was blotted onto a nitrocellulose membrane. Blots were probed with a polyclonal antibody directed against human topoisomerase IIα. Results are representative of two independent experiments.

NAPQI affects levels of DNA cleavage mediated by topoisomerase IIα in cultured human CEM cells. In this assay, 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 cultures treated with no drug, 50 or 100 μM NAPQI, or 10 μM etoposide (for comparison) was blotted and probed with a polyclonal antibody specific for human topoisomerase IIα. As seen in Figure 8, levels of topoisomerase IIα that were covalently attached to DNA following treatment with NAPQI increased severalfold over the drug-free control.

In order for the ICE bioassay to accurately reflect physiological levels of topoisomerase II−DNA cleavage complexes, the assay is carried out under conditions that do not result in high levels of cell death. At the 4 h exposure times employed for the assays shown in Figure 8, 83% and 72% cell viability was observed at 50 and 100 μM NAPQI, respectively. When the exposure time was increased to 48 h, cell viability dropped to 4% and 1%, respectively. Therefore, NAPQI is cytotoxic to CEM cells under conditions that induce topoisomerase II-mediated DNA scission.

It should be noted that cleavage-independent topoisomerase IIα−DNA cross-linking was observed in vitro in DNA cleavage assays that contained NAPQI. For example, 54% of the uncleaved plasmid DNA was cross-linked to the enzyme in the presence of 50 μM NAPQI. Therefore, it is possible that a portion of the covalent topoisomerase IIα−DNA complex observed in NAPQI-treated CEM cells actually reflects 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 (*72*). When in vitro experiments were carried out using topoisomerase IIα:DNA base pair ratios that approximated the cellular condition, ∼1% of the uncleaved plasmid DNA was cross-linked to the enzyme in the presence of 50 μM NAPQI. Therefore, we believe that NAPQI acts as a topoisomerase II poison in cultured human cells.

Acetaminophen Does Not Compete with Etoposide for Human Topoisomerase IIα. Acetaminophen does not significantly increase enzyme-mediated DNA cleavage in vitro (see Figure 2). However, it still is possible that the drug interacts with topoisomerase IIα at the same site as anticancer agents such as etoposide but does not induce DNA cleavage. Such is the case for the antibacterial quinolone ciprofloxacin. This drug targets prokaryotic type II topoisomerases and has little effect on DNA scission mediated by the eukaryotic enzyme (*68*, *73*, *74*). However, ciprofloxacin competes with etoposide and several other anticancer drugs, blocking their ability to increase DNA cleavage by topoisomerase II (*74*).

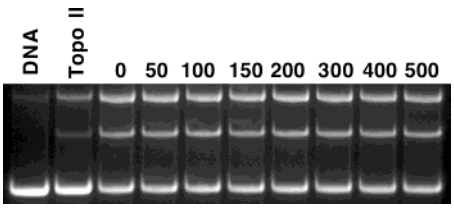

FIGURE 9: Acetaminophen does not interfere with the ability of etoposide to increase DNA cleavage mediated by human topoisomerase IIα in vitro. An ethidium bromide-stained agarose gel of DNA cleavage reactions carried out in the presence of 25 μM etoposide and 0−500 μM acetaminophen is shown. Reactions containing DNA in the absence (DNA) or presence of topoisomerase IIα (Topo II) but in the absence of drugs are shown as controls.

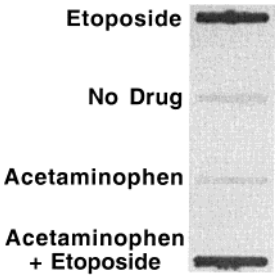

FIGURE 10: Acetaminophen does not alter DNA cleavage mediated by human topoisomerase IIα in cultured CEM cells. The ICE bioassay was used to monitor the level of cleavage complexes in cells. DNA (10 μg) from cultures treated with no drug, 150 μM acetaminophen, 10 μM etoposide, or 150 μM acetaminophen plus 10 μM etoposide was blotted onto a nitrocellulose membrane. Blots were probed with a polyclonal antibody directed against human topoisomerase IIα. Results are typical of two independent experiments.

Considering the wide use of products that contain acetaminophen, it is important to know whether this drug alters the efficacy of topoisomerase II-targeted chemotherapeutic agents. Therefore, two experiments were carried out to determine the effects of acetaminophen on the actions of etoposide against human topoisomerase IIα. First, an in vitro competition assay was performed. In this assay, etoposide (25 μM) was mixed with acetaminophen (0−500 μM) and then added to a topoisomerase IIα−DNA complex. Even at a concentration of 500 μM acetaminophen (which is 3−5 times higher than the upper range of the normal nontoxic dose found in blood), no decrease in etoposide-induced DNA cleavage was observed (Figure 9). Therefore, if acetaminophen interacts with topoisomerase IIα, it probably does not interact with the binding site utilized by anticancer drugs such as etoposide.

Second, ICE bioassays were performed to examine the effects of acetaminophen on covalent topoisomerase IIα−DNA complexes in human CEM cells. As seen in Figure 10, no increase in complex formation was observed in the presence of 150 μM acetaminophen (the upper limit for nontoxic drug doses). Furthermore, preincubation of cells with 150 μM acetaminophen had no effect on levels of topoisomerase IIα−DNA complexes that were induced by

10 μM etoposide. Thus, it appears that normal therapeutic doses of acetaminophen do not significantly interfere with the actions of etoposide on topoisomerase IIα in treated cells.

DISCUSSION

Although acetaminophen is the most widely used analgesic in the world, it causes hepatotoxicity if ingested in doses that are beyond its normal therapeutic window (*1*−*4*, *6*−*9*, *13*). Acetaminophen-induced liver damage also can occur if the organ has been compromised by previous insults (*7*−*9*, *11*, *12*). All of the harmful effects of acetaminophen have been attributed to its reactive metabolite, NAPQI (*7*−*9*, *63*). This compound induces DNA strand breaks and other genotoxic events in a variety of treated cells and also promotes apoptosis and necrosis (*13*−*15*). Results of the present study indicate that NAPQI is a potent poison of human topoisomerase IIα and induces enzyme-mediated double-stranded DNA breaks approximately half as well as etoposide, a widely prescribed anticancer drug.

Relationships between the enhanced topoisomerase IIα-mediated DNA cleavage and the cytotoxic/genotoxic effects of NAPQI are not known. However, exposure of cells to topoisomerase II poisons causes DNA strand breaks and many of the other cytotoxic/genotoxic events seen following NAPQI treatment (*20*, *23*, *24*, *42*−*46*). While NAPQI reacts with a number of proteins in vivo and induces oxidative damage in cells (*13*), the present findings are consistent with a role for topoisomerase IIα in mediating the toxicity of this acetaminophen metabolite.

It is presumed that NAPQI increases levels of cellular DNA cleavage by human topoisomerase IIα by a mechanism that involves the direct interaction of the compound with the enzyme−DNA complex. However, NAPQI treatment also generates DNA adducts in cells by inducing oxidative stress (*13*). Many of these adducts enhance topoisomerase II-mediated DNA cleavage or are converted to abasic sites, which are strong topoisomerase II poisons (*57*, *75*−*81*). Therefore, it is possible that some of the increase in enzyme-mediated DNA scission that is observed following cellular exposure to NAPQI results from an interaction of topoisomerase IIα with drug-induced DNA damage.

As determined by experiments carried out in vitro or in cultured human cells, acetaminophen does not significantly raise levels of DNA cleavage generated by topoisomerase IIα. Furthermore, the analgesic does not antagonize the poisoning effects of etoposide. Therefore, normal doses of acetaminophen should neither cause topoisomerase II-mediated DNA damage nor decrease the efficacy of topoisomerase II-based cancer chemotherapy.

Structural changes between acetaminophen and NAPQI (i.e., the loss of two hydrogen molecules; see Figure 1) are relatively minor. The primary difference between the two compounds is related to their chemical reactivity. While hydroquinones (i.e., acetaminophen) are relatively inert toward proteins, quinones (i.e., NAPQI) are very reactive toward protein sulfhydryl groups and other residues (*13*). Recent work suggests that thiolation agents enhance topoisomerase II-mediated DNA scission (*53*, *54*). Thus, it is possible that covalent modification of topoisomerase IIα by NAPQI plays a role in stabilizing DNA cleavage complexes.

In summary, exposure to NAPQI increases levels of DNA cleavage mediated by topoisomerase IIα in vitro and in cultured cells. These findings suggest that at least some of the cytotoxic/genotoxic effects caused by acetaminophen overdose may be mediated by the actions of NAPQI as a topoisomerase II poison.

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