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Topoisomerase II inhibition by the bioactivated benzene metabolite hydroquinone involves multiple mechanisms

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ABSTRACT

While benzene is widely recognized as a human and animal carcinogen, the key mechanisms underlying its carcinogenic effects remain unknown. Inhibition of topoisomerase II (topoII) by benzene and its metabolites represents a potential mechanism by which benzene could induce its chromosome-altering and leukemogenic effects. Previous work from our laboratory and others has demonstrated that bioactive benzene metabolites are capable of inhibiting topoII in isolated enzyme and cell culture systems. Similarly, a decrease in topoII activity has been seen in the bone marrow of mice administered benzene *in vivo*. The objective of these studies was to further investigate the mechanisms by which the bioactivated benzene metabolite, hydroquinone (BAHQ), inhibits topoII *in vitro*, and to identify the point(s) in the enzyme's catalytic cycle where inhibition occurs. Our experiments indicate that BAHQ inhibits topoII at the DNA binding stage as well as in the closed clamp stage in the catalytic cycle, thereby interfering with either the binding to, or the release of, DNA from the enzyme. While increases in the cleavable complex were also seen with BAHQ treatment, our results suggest that this is related to a shift in equilibrium due to an accumulation of the topoII enzyme at the closed clamp stage rather than a major inhibitory effect on the religation step. An increase in cleavable complex formation as well as the inhibition of enzymatic activity at the closed clamp and other stages of the catalytic cycle in bone marrow cells would likely result in DNA breakage, the formation of chromosomal aberrations, and could potentially result in leukemia-associated chromosomal translocations, similar to those seen in leukemias induced by the bisdioxopiperazine type of catalytic topoII inhibitors.

1. Introduction

Occupational exposure to benzene (BZ) has long been recognized as resulting in hematotoxicity with the major effects being pancytopenia, aplastic anemia, and leukemia. Despite efforts to elucidate the mechanism(s) of BZ-induced hematotoxicity, several key steps in the mechanisms have yet to be determined. Exposure to BZ results in the production of numerical and structural chromosomal aberrations in the peripheral blood of humans and it has been hypothesized that similar chromosomal alterations occurring in bone marrow stem cells play a critical role in the development of leukemia [1]. Notably, BZ and its metabolites are only weakly mutagenic in standard gene mutation assays [2,3] and bind poorly to DNA [4–6], therefore suggesting that an alternative mechanism of genotoxicity and carcinogenesis is involved. Hepatic metabolism of BZ has been shown to be a prerequisite for BZ-induced hematotoxicity [7]. Metabolism of BZ in the liver by CYP2E1 results in the production of several oxidative metabolites including the phenolic metabolites phenol (PH), hydroquinone (HQ), 1,2,4-benzenetriol (BT) and catechol (CT) [8]. It has been proposed that these metabolites are further oxidized in the bone marrow by myeloperoxidase (MPO) to electrophilic metabolites such as 1,4-benzoquinone (BQ), 1,2-BQ, and the putative metabolite 4,4′-diphenoquinone, [9,10]. It is currently believed that the formation of these quinone metabolites contributes to the clastogenic and hematotoxic effects of BZ [11].

For a number of years we have focused on the inhibition of topoisomerase II (topoII) as a potential mechanism of BZ-induced hematotoxicity [12–14]. TopoII is a nuclear ATP-dependent enzyme that plays an important role in several DNA manipulating processes including relieving the torsional stress that occurs in DNA during replication and transcription, the decatenation of sister chromatids, and the condensation of chromatin to metaphase chromosomes. The role of topoII throughout the cell cycle renders it a prime target for numerous chemotherapeutic agents [15–17]. TopoII inhibitors

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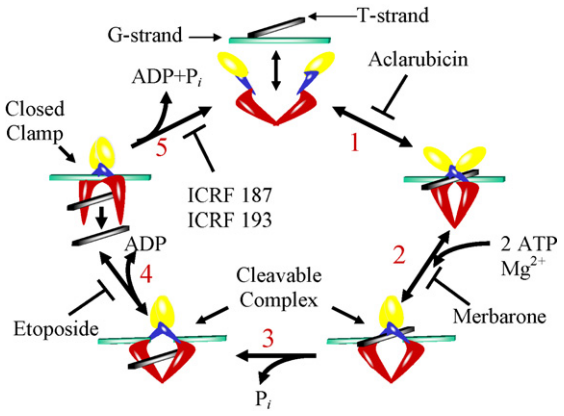

Fig. 1. The catalytic cycle of topoisomerase II. A description of the catalytic cycle of topoisomerase II is illustrated as well as the sites of inhibition of various topoII inhibitors. (1) The binding of topoII to two segments of double stranded DNA, the gate segment (G-segment) and the transport segment (T-segment). These two segments may be from the same or two different double helices. The binding of topoII to DNA promotes the closure of the N-terminal clamp. This step can be inhibited by aclarubicin. (2) The binding of 2 ATP molecules promotes the dimerization of the ATPase domains and the production of a transient double strand break in the G-segment of DNA resulting in a stabilized DNA enzyme complex known as the cleavable complex. Merbarone has been reported to inhibit this step of the cycle. (3) The hydrolysis of one ATP and release of the hydrolyzed phosphate induces the passage of the T-segment of DNA through the G-segment. (4) The release of the ADP promotes the religation of the G-segment and release of the T-segment through the C-terminal gate of the enzyme resulting in the formation of the topoII enzyme in the closed clamp configuration. This step can be inhibited by etoposide and other topoII poisons. (5) The hydrolysis and release of ADP + P_i causes the disassociation of the ATPase domains and the opening of the N-terminal gate to allow the release of the G-segment and the return of topoII to its native state. This last step can be inhibited by the bisdioxopiperazine class of drugs such as ICRF 187 and 193, and as described later, BAHQ.

are grouped into two general classes, topoII poisons and topoII catalytic inhibitors. TopoII poisons are those agents such as etoposide and teniposide that stabilize a specific stage in the enzyme's catalytic cycle referred to as the cleavable complex [16,18], while catalytic inhibitors such as aclarubicin, merbarone, and the bisdioxopiperazines ICRF 187 and ICRF 193 inhibit the enzyme at other stages in the catalytic cycle [19–22]. During the catalytic cycle, the enzyme binds two DNA double helixes and passes one through a transient double strand break in the other. The stage in which the double strand break exists and the enzyme is covalently bound to both ends of the cleaved DNA is referred to as the cleavable complex [23]. An illustration of the topoII catalytic cycle with a more detailed description is presented in Fig. 1.

The hypothesis that BZ-induced hematotoxicity is the result of the inhibition of topoII activity stems from the observation that there are several shared features between BZ and topoII inhibitors, most importantly is the induction of acute myelogenous leukemia in humans [6,24–28]. Other similarities include myelotoxicity, the induction of numerical and structural chromosomal abnormalities that include sister chromatid exchanges, micronuclei, reciprocal translocations, chromosomal breakage and loss [29–33], an induction of a G_2/M block [12,34–36], and a parent compound or metabolite that has a phenolic or quinoid structure [3,10,15,25,35,37]. Based on this original hypothesis, our lab has tested the effect of several common and putative BZ metabolites on purified topoII. These metabolites fell into two categories, those that directly inhibited the enzyme, such as BQ and t,t-muconaldehyde, and those that required the addition of a bioactivation system that included horseradish peroxidase (HRP), a model enzyme for MPO, and hydrogen peroxide. The metabolites that required bioactivation included HQ, PH, CT, 4,4′-biphenol, 2,2′-biphenol, and BT [12,13]. The rationale for using a bioactivation system lies in the observation that the bone marrow contains high levels of MPO [38] that can potentially oxidize these metabolites to their respective quinones or other reactive intermediates such as semiquinone or phenoxy radicals. It should be noted that the concentrations of the bioactivated and reactive metabolites present within the bone marrow have been estimated for only a few benzene metabolites such as 1,2-BQ and BQ, and the concentrations of these have been reported to vary considerably between species [39,40]. More recently the inhibition of topoII activity was observed in the nuclear extracts of cultured HL60 cells treated with benzene metabolites and of the bone marrow of mice treated with benzene itself [13,14,41]. Together these results indicate that inhibition of topoII may contribute to BZ-induced hematotoxicity.

In an effort to classify the BZ metabolites, including HQ, bioactivated HQ (BAHQ), and BQ, as topoII poisons or catalytic inhibitors, Baker et al. [42] tested the ability of various metabolites to stabilize the cleavable complex. The authors reported that the BZ metabolites did not significantly increase the amount of cleavable complexes over that of the control, and in fact reduced the amount of cleavable complexes stabilized by the addition of etoposide. The authors concluded that because these metabolites did not stabilize the cleavable complex, it was unlikely that BZ-induced acute myelogenous leukemia resulted from chromosomal aberrations caused by the inhibition of topoII. This rationale stemmed from a belief at that time that topoII inhibiting agents that do not stabilize the cleavable complex could not be clastogenic and therefore would not induce the chromosomal aberrations necessary for acute myelogenous leukemia. However, more recently it has been shown that several topoII catalytic inhibitors including ICRF 187 and merbarone are highly clastogenic [43–46], thereby indicating that the inhibition of topoII by BZ metabolites remains a potential mechanism through which BZ causes leukemia.

More recently Lindsey et al. [47] also addressed the ability of BQ to stabilize the cleavable complex. Contrary to the findings of Baker et al., this group found that BQ was as potent as etoposide at stabilizing the cleavable complex. However in contrast to etoposide, the authors suggested that BQ increased the level of cleavable complexes via an increased rate of DNA cleavage as opposed to an inhibition of the DNA ligation step of the catalytic cycle. Since that time other studies by that group [48] suggest that BQ stabilizes the cleavable complex in part by inhibiting the ligation step as well as other stages of the catalytic cycle.

A re-evaluation of the ability of BQ to stabilize the cleavable complex by Lindsey et al. [47] was prompted by their interpretation of the data presented in several articles [49–53] suggesting that individuals who are heterozygous or homozygous for the *C609T* polymorphism of the *NQO1* gene display an increasingly higher risk for leukemias with 11q23 chromosomal translocations. In addition, some sulfhydryl-reactive chemicals, such as quinones, have been shown to increase levels of DNA cleavage mediated by human topoII [54–57]. Aberrations at chromosomal band 11q23 are a hallmark of exposure to topoII poisons such as etoposide. However, the peripheral blood of BZ exposed individuals also contain chromosomal aberrations such as reciprocal translocations between chromosomes 8 and 21 that have been detected in patients treated with the bisdioxopiperazine class of drugs that include bimolane, ICRF187, and ICRF193 [58,59]. These agents are catalytic inhibitors of topoII that inhibit the enzyme by preventing the release of DNA and the recycling of the enzyme [60]. Inhibition at this stage results in the formation of a salt-stable closed clamp complex in which the enzyme is trapped around DNA [19]. The observation that exposure to BZ may result in the production of chromosomal aberrations similar to those caused by agents that inhibit topoII at different points in the catalytic cycle suggests that the BZ metabolites may themselves inhibit topoII at various points throughout the catalytic cycle.

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It should be noted that in evaluating the potency of the benzene metabolites some caution is warranted as many of the earlier experiments mentioned above contained high levels of dithiothreitol (up to 0.5 mM; DTT) in the reactions. The quinone metabolites have been shown to undergo a Michael addition with thiol-containing agents such as DTT and GSH that results in the conjugation and/or reduction of the metabolite to an inactive form [61]. It has also been demonstrated that the addition of reduced glutathione or DTT to the topoII reaction prior to the addition of enzyme abrogates the inhibitory effect of the metabolites on topoII activity [12,47,62]. The fact that inhibition was seen at concentrations as low as 1 μM of the BZ metabolites in reactions containing 500 μM DTT suggest that upon activation these metabolites may be much more potent inhibitors than previously thought.

The aim of this research is to clarify the contradictory results of the above mentioned studies regarding the ability of these metabolites to stabilize the cleavable complex and to identify the point(s) in the enzyme's catalytic cycle at which these metabolites inhibit topoII activity. In addition, we also re-evaluate the inhibition of topoII activity by the BZ metabolites BQ, HQ, and BAHQ in the absence of reducing agents.

2. Materials and methods

2.1. Materials

Hydroquinone and 1,4-benzoquinone (≥98% purity) were purchased from Aldrich Chemical Co. Q-sepharose fast flow, protease inhibitors, HRP (type VI), and etoposide were purchased from Sigma Chemical Co. P-11 phosphocellulose and GF/C glass fiber filters were purchased from Whatman, and ICRF 187 was obtained from the National Cancer Institute. The *Crithidia fasciculata* that was used to produce kDNA was provided by Dr. Dmitri Maslov (University of California, Riverside). The topoII expression vector YEpWOB6 and the yeast strain JEL1Δtop1 were the kind gifts of Dr. John Nitiss (St. Jude Children's Research Hospital). All test agents were suspended in DMSO at 100 mM and stored at −20 °C and all topoII reactions contained 1% DMSO.

2.2. Induction and purification of topoII

The htopIIα cDNA was contained in the expression vector YEpWOB6 and expressed in the yeast strain JEL1Δtop1. Purification of the enzyme was carried out by the protocol described by Lindlsey [63].

2.3. Purification of kDNA

The purification of kDNA was performed as described by Shapiro et al. [64].

2.4. HQ bioactivation

HQ was bioactivated to BQ in 10 μl (topoII activity and cleavage assays) or 80 μl (topoII closed clamp assays) reactions containing 0.05 U/ml HRP and 200 μM H_2O_2 and 2× the final HQ concentration in the topoII assays. The reaction was incubated for 30 min at room temperature and placed on ice until the addition of the respective topoII assay components.

2.5. TopoII activity

The activity of topoII was assessed via the decatenation of catenated kDNA. Subsequent to the activation of HQ to BQ, 8 μl of a 2.5× topoII activity mix containing 250 ng kDNA was added to the 10 μl bioactivation reactions. The activity assay was initiated by the addition of enzyme (2 μl at 30 ng/μl). The final concentrations in the 20 μl reactions were 50 mM Tris–Cl pH 7.3, 5 mM $MgCl_2$, 1 mM ATP, 100 mM KCl, 0.1 mM EDTA, 2.5% glycerol, 0.025 U/ml HRP, 100 μM H_2O_2, 12.5 ng/μl kDNA and 9 nM topoII dimer and the indicated concentration of test chemical. The reactions were incubated at 37 °C for 30 min and stopped by the addition of 3 μl stop buffer (2.5% SDS, 0.05% bromophenol blue, and 50% glycerol) followed by incubation at 70 °C for 2 min. The DNA was then separated on a 1% agarose gel in TAE containing 0.5 μg/ml ethidium bromide at 100 V for one hour. Gels were photographed and DNA bands were quantified using the public domain NIH Image Program 1.61 Gel Plotting Macro developed at the U.S. National Institutes of Health and available on line at http://rsb.info.nih.gov/nih-image/.

2.6. Cleavable complex formation

The method utilized to test the ability of BAHQ to stabilize the cleavable complex was based on those previously described [54] with minor modifications to accommodate the bioactivation reaction. Subsequent to the activation of HQ to BQ, 8 μl of a 2.5× topoII cleavage mix containing 175 ng pUC18 DNA were added to the 10 μl bioactivation reactions. The reaction was initiated by the addition of topoII (2 μl at 240 ng/μl) and incubating at 37 °C for 6 min. The final concentrations in the 20 μl reactions were 50 mM Tris–Cl pH 7.3, 5 mM $MgCl_2$, 1 mM ATP, 135 mM KCl, 0.1 mM EDTA, 2.5% glycerol, 0.025 U/ml HRP, 100 μM H_2O_2, 5 nM pUC18 DNA, 70 nM topoII dimer, and the indicated concentration of BAHQ. The reaction was stopped and the cleavable complexes were trapped by rapidly denaturing the enzyme with 2 μl of 5% SDS followed by the addition of 1 μl of 375 mM EDTA pH 8.0. The enzyme was digested by the addition of 2 μl of proteinase K (0.8 mg/ml) and incubation at 45 °C for 30 min. The samples were then mixed with 3 μl of loading buffer (0.05% bromophenol blue and 70% glycerol), heated at 70 °C for 2 min, and subjected to electrophoresis in a 1.5% agarose gel in TAE containing 0.5 μg/ml ethidium bromide. DNA cleavage was monitored by the conversion of supercoiled plasmid to linear molecules. Gels were photographed and DNA bands were quantified as described above. Reversibility of the cleavable complexes was determined by adding high salt (2 μl of 5 M NaCl) or EDTA (1 μl of 375 mM) prior to the addition of SDS.

2.7. TopoII/DNA binding

The ability of topoII to bind DNA was determined based on the protocol described by [65] with slight modifications. The reaction concentrations were as described above for the cleavage assay with the exception that the enzyme and DNA concentrations were doubled. Immediately following the 6-min incubation, 3 μl of loading buffer (±3.5% SDS) at 37 °C were added to the samples. The samples were immediately separated on a 0.8% agarose gel in TAE at 100 V for 1.5 h.

2.8. Preparation of the GF/C filter apparatus

GF/C filters were cut with a cork borer and placed at the bottom of a 2 ml conical centrifuge tube in the bottom of which a hole had previously been punched using an 18-gauge needle. To catch the flow through, the 2 ml tube was placed in the cap of a 15 ml centrifuge tube possessing a hole large enough to accommodate the 2 ml tube. The filters were equilibrated with 200 μl of a filter equilibration buffer, consisting of buffer A (50 mM Tris–Cl pH 7.3, 5 mM $MgCl_2$, 0.1 mM EDTA, and 100 mM KCl) and 100 μg/ml herring sperm DNA sheared to <600 bp. The equilibration buffer was removed by centrifugation at 500 × *g* in a Beckman TJ6 swing bucket rotor at room temperature for 3 min. The filter was washed once with buffer A and the 2 ml tube containing the filter was placed

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in the cap of a clean 15 ml tube (tube A) just prior to the application of the topoII closed clamp reaction to the filter. All washes were performed by centrifugation at 500 × *g* at room temperature for 3 min.

2.9. Closed clamp formation

The methods utilized to test the ability of BAHQ to stabilize the closed clamp were based on those described previously [19] with minor modifications. Following the activation of HQ to BQ, 78 μl of 2× topoII activity mix containing 400 ng pUC18 were added to the 80 μl bioactivation reactions. The topoII reaction was initiated by the addition of topoII (2 μl at 240 ng/μl) and incubating at 37 °C for 10 min. The final concentrations in the 160 μl reactions were 50 mM Tris–Cl pH 7.3, 5 mM $MgCl_2$, 1 mM ATP, 100 mM KCl, 0.1 mM EDTA, 2.5% glycerol, 0.025 U/ml HRP, 100 μM H_2O_2, 1.5 nM pUC18 and 9 nM topoII dimer. After a 10-min incubation the reactants were immediately loaded onto the equilibrated GF/C filters. The reactants were eluted from the filter by centrifugation and then washed once with 500 μl buffer A containing 500 mM NaCl. The washes were saved and the filter apparatus was placed in the cap of a clean 15 ml tube (tube B). The DNA trapped in the closed clamp was removed from the enzyme by adding 500 μl of buffer A containing 1% SDS to the filter and incubating at 37 °C for 5 min to denature the enzyme. The enzyme and DNA were removed from the filters by centrifugation as described above. The DNA in the washes of tubes A and B was then precipitated with isopropanol, pelleted by centrifugation, and washed once with 70% ethanol. The precipitated DNA was resuspended in 10 μl TE followed by the addition of 2 μl loading buffer. The DNA was separated by electrophoresis on a 1% agarose gel in TAE for one hour at 100 V.

3. Results

3.1. Inhibition of topoII

The inhibition of topoII by BAHQ and BQ has previously been demonstrated with inhibition of topoII activity occurring in the low micromolar range. However, the previous work regarding the inhibition of topoII was performed using the standard topoII activity assay containing the reducing agents DTT or β-mercaptoethanol at concentrations as high as 500 μM. Because these reducing agents readily conjugate or reduce quinones, rendering them non-reactive, the presence of these agents in the assay may have decreased the effectiveness of BAHQ and BQ against topoII. We therefore tested the potency of these metabolites after eliminating all reducing agents from the last step of the enzyme purification process as well as all of the assays studying the effect of these metabolites on topoII.

Following the elimination of reducing agents from the assay significant decreases in topoII activity were seen at concentrations as low as 25 nM BAHQ and BQ (Fig. 2). This is less than a three-fold molar excess over the topoII concentration, demonstrating the high potency of these metabolites. Surprisingly we also detected a partial inhibition of topoII activity by HQ at sub-micromolar concentrations (Fig. 2). We believe this is the result of autooxidation of HQ to BQ that was prevented in previous studies by the presence of the reducing agents. While the autooxidation may be the result of oxygen present in the buffers, sparging the buffers with helium had no significant effect (not shown). However, there was a direct relationship between HQ potency and pH. At pH 8.0 HQ was as potent an inhibitor of topoII as was BQ and BAHQ, whereas at pH 7.3 HQ was significantly less effective at inhibiting topoII activity (Fig. 3). Although we believe the effect of pH is largely due to the autooxidation of HQ to BQ, it is also possible that the elevated pH may also

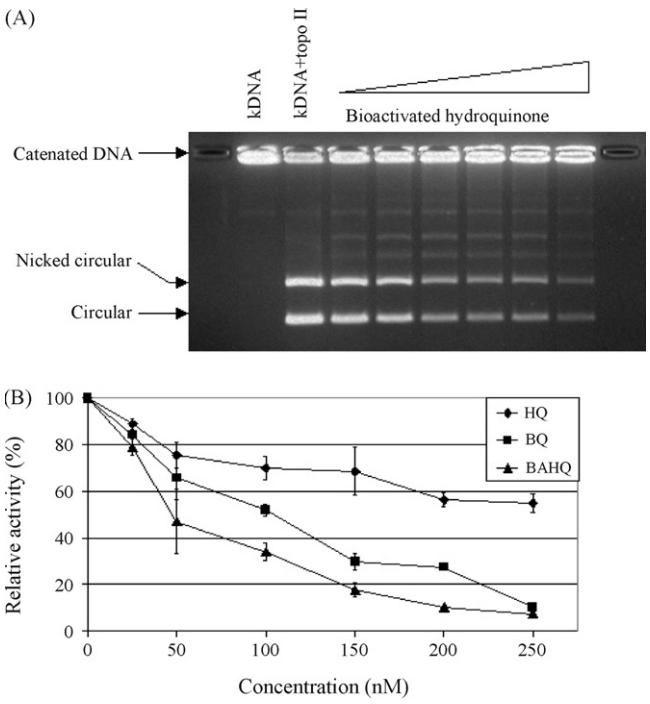

Fig. 2. The inhibition of topoIIα by BAHQ, HQ and BQ in the absence of reducing agents. TopoIIα activity was assessed via the decatenation of catenated DNA. (A) Agarose gel illustrating the inhibition of topoIIα by BAHQ, representative of four experiments. Decatenation of the kDNA by topoIIα allows the migration of the mini-circles through the gel. Compare the kDNA and kDNA + topoII lanes. (B) Graphical representation of the inhibition of topoIIα by BAHQ, HQ, and BQ. Activity of the treated samples is compared to the DMSO control. Error bars represent the standard error of the mean of four experiments.

alter the reactivity of a critical cysteine to which BQ may bind. This possibility may also explain why BQ and BAHQ were significantly less potent at pH 7.3 than at pH 8.0 (Fig. 3).

In an effort to determine if the inhibition of topoII activity was due to an interaction of BAHQ to DNA or topoII, we devised an assay in which we were able to treat either the enzyme or DNA individually with BAHQ without treating the other. Either the enzyme or DNA was incubated with the complete reaction mix containing the indicated concentration of BAHQ for 5 min at 37 °C. After the 5-min incubation, DTT was added to a final concentration of 1 mM to convert BAHQ to a non-reactive form followed by the addition of the missing reaction component, either the enzyme or DNA. The results clearly demonstrate that treating the DNA with BAHQ has no effect

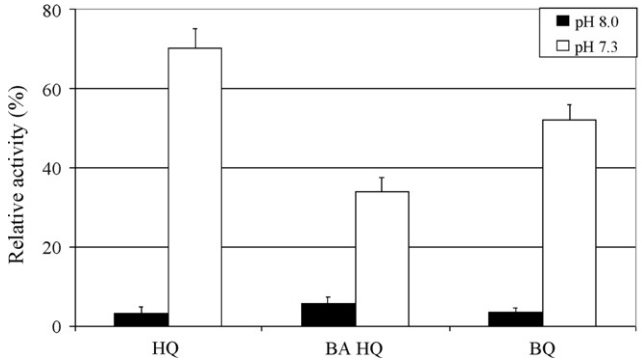

Fig. 3. The influence of pH on the inhibition of topoIIα by BAHQ, HQ, and BQ. TopoIIα was treated with 100 nM BAHQ, HQ, and BQ at pH 7.3 and 8.0. The inhibition of topoIIα at each pH is compared to that of the control without treatment at the respective pH values. Error bars represent the standard error of the mean of three or more experiments.

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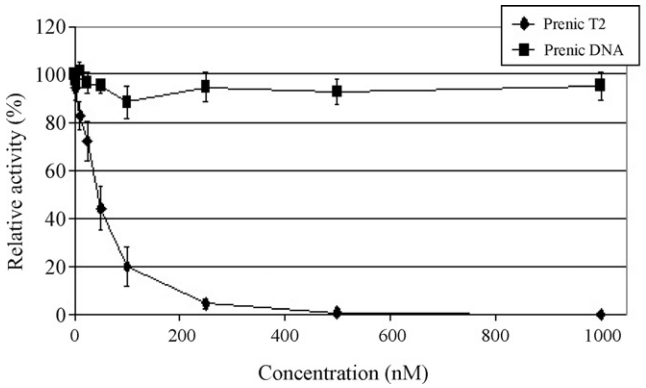

Fig. 4. BAHQ inhibits topoII activity via a direct interaction with the enzyme and not DNA. Either topoIIα or DNA were incubated in the complete reaction mix containing the indicated concentrations of BAHQ for 5 min at 37 °C prior to the addition of 1 mM DTT. DTT converts BAHQ to a non-reactive form. After a 5-min incubation at room temperature, the missing reaction component, either topoIIα or DNA, was added to the reaction mix and incubated at 37 °C for 30 min. The DNA was then separated as described in Section 2. Error bars represent the standard error of the mean of three experiments.

on topoII activity and that the enzyme is clearly the target of this metabolite (Fig. 4).

3.2. Stabilization of the cleavable complex

TopoII inhibitors can inhibit the enzyme at various points in the enzyme's catalytic cycle (Fig. 1). The ability of BAHQ or BQ to stabilize the enzyme in the cleavable complex has been evaluated by others with contradictory results. We tested the ability of BAHQ to stabilize the cleavable complex using a standard cleavage assay that uses a circular plasmid as the enzyme substrate. The addition of SDS to the reaction after a short incubation rapidly denatures the enzyme. After denaturation and digestion of the enzyme with proteinase K, the circular plasmid that is covalently bound to the enzyme via the cleavable complex will be linear and will migrate between the supercoiled and nicked circular plasmids in an agarose gel (Fig. 5A). Consistent with the findings of Lindsey et al. [47] with BQ, we found that without preincubation BAHQ is a potent topoII poison that produced levels of cleavable complexes similar to that of etoposide (Fig. 5B). The level of cleavable complexes peaked at between 25 and 50 μM with about an eightfold increase over that of control. In contrast to etoposide, however, BAHQ significantly inhibited topoII activity at concentrations below those that produced the highest level of cleavable complexes, suggesting that BAHQ and etoposide may induce increases in cleavable complexes via alternative mechanisms.

Several controls were performed to confirm that the linear DNA band used to quantitate the cleavable complexes was the result of topoII mediated cleavable complexes as opposed to BAHQ alone and also to test the reversibility of the cleavable complexes (Fig. 5C). As expected no cleavable complexes were detected either when topoII or proteinase K were omitted from the assay. The reversibility of the cleavable complexes was determined by the addition of salt (2 μl of 500 mM NaCl) or EDTA (1 μl of 175 mM) prior to the addition of SDS and proteinase K. As shown in Fig. 5C, the cleavable complexes were completely reversed demonstrating that they are not due to a total inhibition of the ligation step of the catalytic cycle as has been demonstrated with etoposide [47] and suggested for BQ by others [48].

We then proceeded to test the ability of BAHQ and etoposide to stabilize the cleavable complex with and without preincubation of the enzyme with the respective inhibitors. The results demonstrate that preincubation had no effect on the ability of etoposide to stabilize the cleavable complex. In contrast, preincubating the enzyme with BAHQ completely abolished cleavable complex formation (Fig. 5D). These results confirm that BAHQ inhibits topoII by multiple mechanisms and that it stabilizes the cleavable complex by a mechanism that differs from that of etoposide.

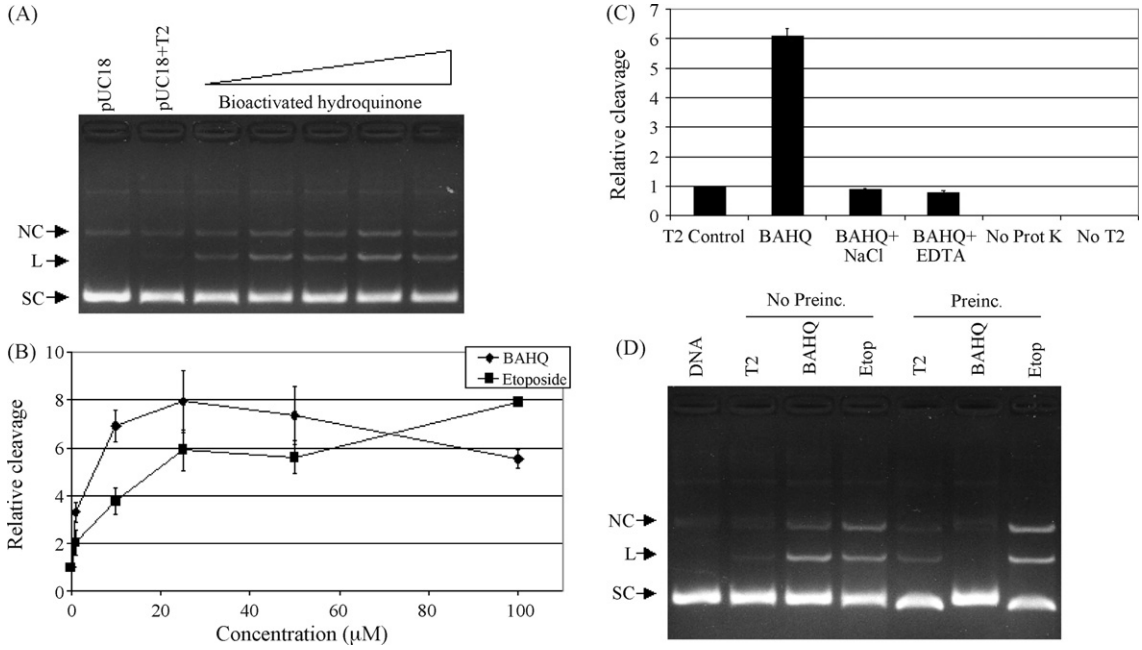

Fig. 5. BAHQ stimulates the formation of the cleavable complex. Stabilization of the cleavable complex was observed by the conversion of circular pUC18 (SC) to linear DNA (L). (A) Representative gel of three experiments demonstrating the effect of BAHQ on the formation of the cleavable complex. (B) Graphical representation of the stabilization of the cleavable complex by BAHQ and etoposide. Error bars represent the standard error of the mean of three experiments. (C) The reversibility the cleavable complexes stimulated by BAHQ was tested by adding NaCl (2 μl of 5 M) or EDTA (1 μl of 375 mM) to the reaction just prior to the addition of SDS. The control lacking enzyme (No T2) was performed to confirm that the linear molecules were not produced through an interaction between BAHQ and DNA alone. (D) The effect of preincubating the enzyme with BAHQ or etoposide on the formation of the cleavable complex was evaluated by preincubating the enzyme with the respective chemicals for 5 min at 37 °C prior to the addition DNA.

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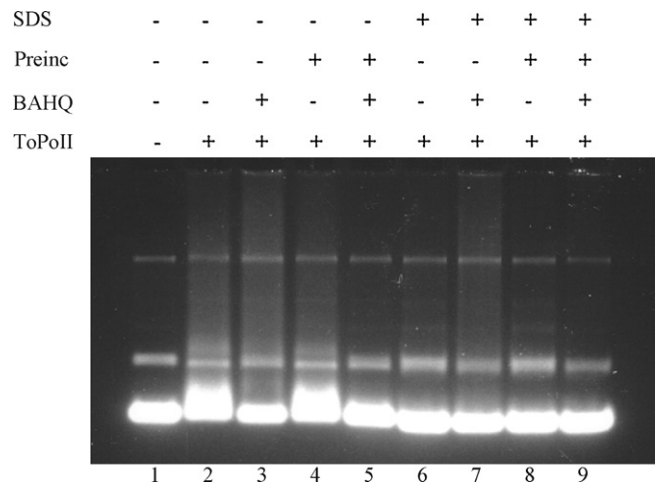

Fig. 6. The preincubation of topoIIα with BAHQ inhibits the binding of the enzyme to DNA. TopoIIα was treated with BAHQ ± preincubation at 37 °C for 5 min prior to the addition of DNA. Samples were then incubated for another 6 min at 37 °C followed by the immediate addition of 3 μl loading buffer ±3.5% SDS and subjected to electrophoresis as described in materials and methods.

3.3. Binding of topoII to DNA

To further investigate the mechanism(s) through which BAHQ inhibits topoII activity, we evaluated the effect of BAHQ on the binding of topoII to DNA with and without preincubation. The binding of topoII to DNA alters the migration of the plasmid through the agarose gel and results in the production of a DNA smear (compare lanes one and two, Fig. 6). As seen in the image, the addition of BAHQ without preincubation (lane three) intensifies the shift in migration whereas the preincubation with BAHQ (lane five) results in the plasmid migrating just as in the no enzyme control. This confirms that the preincubation of topoII with BAHQ inhibits the binding of topoII to DNA as reported previously [42]. In addition, we also tested the effect of adding SDS to the samples prior to electrophoresis on the amount of topoII that remains bound to DNA. Although the pattern of migration in the BAHQ treated sample with SDS is similar to that in the BAHQ treated sample without SDS, the amount of DNA that migrates as a smear is somewhat diminished (compare lanes 3 and 7) suggesting that not all of the bound enzyme is trapped in the cleavable complex.

3.4. Cleavable complex formation with extended incubation times

The above results suggested multiple mechanisms of inhibition. The stabilization of the cleavable complex via an increased rate of DNA cleavage alone does not adequately explain the inhibition of topoII activity. Preincubating the enzyme with BAHQ however, inhibited the binding of topoII to DNA. These results suggest that if the cleavable complex assays are allowed to proceed for an extended time, the level of cleavable complexes should decrease. However, Fig. 7 demonstrates that extended incubation times have little effect on the amount of cleavable complexes stabilized by BAHQ.

3.5. Stabilization of the closed clamp

We next proceeded to test the ability of BAHQ to stabilize the enzyme in the closed clamp. This assay utilizes glass fiber filters that bind topoII but not DNA. After loading the reaction components on the filter, the filter was washed with a high salt buffer to remove free DNA and DNA that was not specifically bound to the enzyme and filter. The enzyme was then denatured with SDS to release any

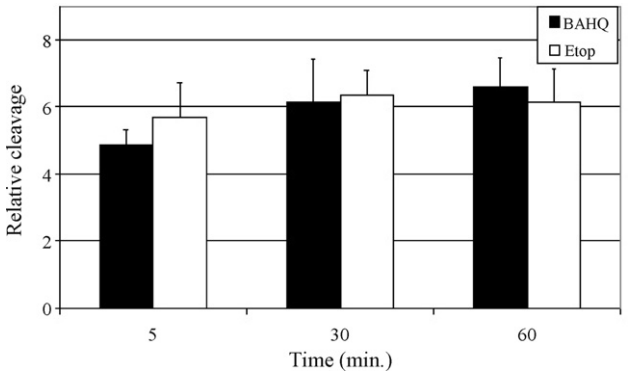

Fig. 7. The level of BAHQ stimulated cleavable complexes does not change with increasing reaction times. Cleavable complex reactions were carried out as described in Section 2 with the extended incubation times.

DNA trapped in the closed clamp. After washing with salt and SDS, the DNA in the washes was then precipitated and separated on an agarose gel. DNA found in the SDS wash represents DNA that was present in the closed clamp. As can be seen in Fig. 8A, there was no detectable DNA in the SDS washes of the DNA and topoII controls, but DNA was present in the SDS washes of the BAHQ and ICRF 187 (positive control) treated samples confirming the presence of enzymes trapped in the closed clamp. It was possible that the presence of the closed clamp was due to reversibility of the cleavable complex by high salt. Therefore the assay was also performed with a low salt wash. This also detected the presence of the closed clamp (Fig. 8B). This indicates that a fraction of the enzyme inhibited by BAHQ is present in the closed clamp form.

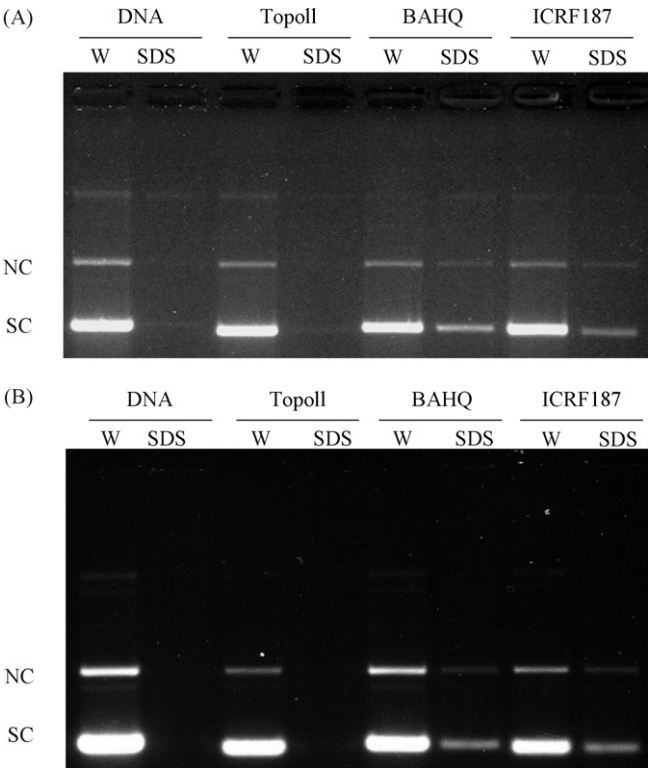

Fig. 8. BAHQ stimulates the stabilization of the closed clamp. Samples were treated as described in Materials and Methods. Samples labeled topoIIα alone and ICRF187 represent the negative and positive controls respectively. Samples were washed with high salt (A) or low salt (B) prior to eluting the DNA from topoIIα with SDS. The DNA sample did not contain topoIIα. *Abbreviations:* NC–nicked circular, SC–supercoiled, W–salt wash, SDS–SDS wash.

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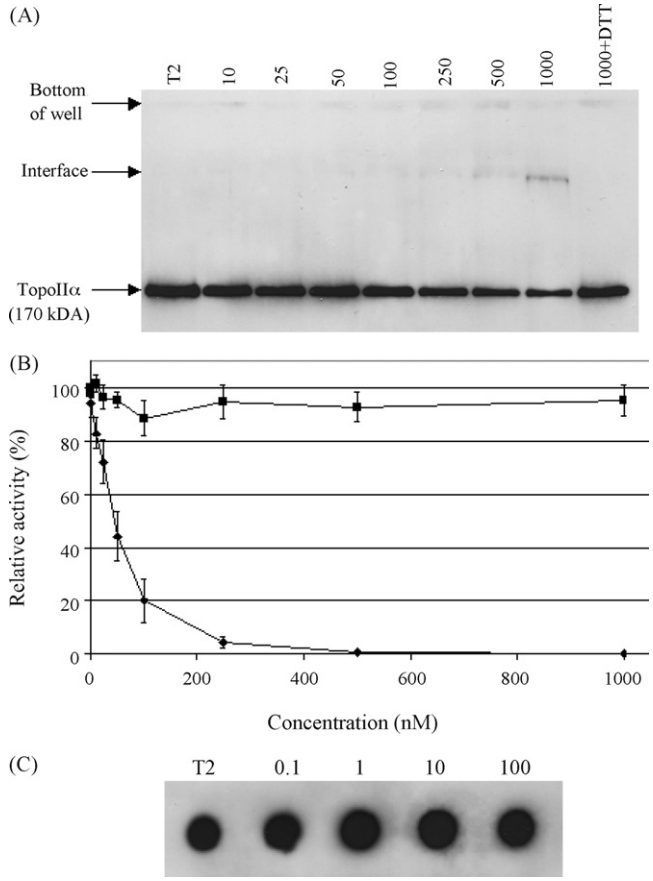

Fig. 9. BAHQ inhibits topoII's ability to migrate through a polyacrylamide gel but does not interfere with the antibody's recognition of the epitope. (A) TopoIIα was treated with BAHQ (10–1000 nM) for 5 min prior to the addition of 1 mM DTT to prevent cross-linking after denaturation of the enzyme. Samples were then separated via SDS–PAGE and immunoblotted using standard protocols. DTT was added to the BAHQ prior to the addition of topoIIα in the 1000 + DTT sample. (B) The inhibition of topoIIα activity under the same conditions as the gel shift assay was evaluated. TopoIIα was incubated with BAHQ at the indicated concentrations for 5 min at 37 °C prior to the addition DNA and completion of the activity assay as described in Section 2. 1 mM DTT to was added to the reaction prior to topoIIα (■) or after the 5-min incubation of topoIIα with BAHQ (♦). (C) The ability of BAHQ to inhibit the antibody from recognizing the enzyme's epitope was examined by treating topoII (T2) with up to 100 μM BAHQ and loading onto a nitrocellulose membrane with a Dot Blot manifold (Bio-Rad). The enzyme was visualized by ECL using standard protocols.

3.6. *Cross-linking of topoII*

It has recently been shown that the bisdioxopiperizines stabilize the closed clamp by forming a non-covalent cross-link between the two ATPase domains of the topoII dimer which inhibits the release of DNA. Because the ATPase domain contains several cysteine residues to which BAHQ can bind, it is possible that BAHQ may also stabilize the closed clamp via the same mechanism. We tested this possibility by treating the enzyme with the indicated concentration of BAHQ followed by the addition of DTT (1 mM final concentration) to convert BAHQ to a non-reactive form prior to the addition of SDS sample buffer. This was done to prevent further cross-linking after denaturation of the enzyme. The samples were then subjected to SDS–PAGE and Western blotting. In order to make a direct comparison between the activity assays and the cross-linking assays, we utilized the same concentrations of enzyme and BAHQ as well as the same incubation periods. As Fig. 9 illustrates, the concentrations of BAHQ that resulted in complete inhibition of topoII activity also resulted in decreased amounts of topoII that migrated as the 170 kDa topoII monomer. Those samples that did not migrate at the monomer molecular weight traveled as a smear that migrated only a short distance past the interface of the stacking and separating gels (faintly visible in the scanned image). Of note, the observed pattern of migration is very similar to the results of Bender et al. [66] that observed the cross-linking of the two monomers by selected PCB quinone metabolites. The observation that the monomers remained cross-linked in our experiment after denaturation indicates that BAHQ is covalently bound to the enzyme.

It has been suggested that the lack of detectable topoII migrating at the monomer molecular weight from samples of nuclear extracts of cells treated with HQ may be due to a modification of the enzyme's epitope that prevents the recognition of the enzyme by the antibody. To test this possibility, we treated topoII with BAHQ at concentrations that far surpassed those that resulted in a decrease in the amount of enzyme that migrates at 170 kDa, and performed dot blots on these samples. As can be seen, BAHQ does not inhibit the recognition of topoII by the antibody (Fig. 9C). These results indicate that the amount of enzyme detected in the smear of the SDS–PAGE represents essentially all of the topoII in the sample.

4. Discussion

Previous experiments investigating the inhibition of topoII by benzene metabolites contained high levels of reducing agents (β-mercaptoethanol or DTT) in the reaction buffers making it difficult to establish the potency of these metabolites. In the studies presented here we have eliminated all reducing agents from the topoII reactions. As demonstrated by our results, it is clear that under these conditions BAHQ and BQ are extremely potent inhibitors of topoII. Inhibition occurred at lower concentrations than has been reported for any other agent to our knowledge. We were also somewhat surprised to detect inhibition of topoII activity by the generally non-reactive HQ. Previously the inhibition of topoII activity by this metabolite in the absence of bioactivation was not seen until the concentration of HQ surpassed that of the reducing agent concentration in the reaction mix. Given the potency of BQ and BAHQ, we believe that the inhibition of topoII by HQ itself at submicromolar concentrations is due to a pH dependent autoxidation of HQ to BQ. This is supported by the observation that the potency of HQ was directly proportional to pH, which is known to affect the autoxidation of HQ. However, we also found that the potency of BQ and BAHQ was also affected to some degree by pH. It is therefore possible that the effect of pH on the potency of these agents may be due to a chemical reduction or an altered reactivity of a critical cysteine to which these metabolites may bind. Therefore the presence of reducing agents in previous studies may have reduced the potency of these agents by both reducing the metabolites to non-reactive forms as well as maintaining a pH-sensitive critical cysteine in a reduced state.

TopoII inhibitors have been demonstrated to inhibit the enzyme either by binding to DNA or the enzyme itself. Examples include the intercalation of aclarubicin into DNA that inhibits the binding of topoII to DNA and the non-covalent binding of ICRF187 to the enzyme promoting the bridging of the two ATPase domains and stabilizing the closed clamp. Because BZ metabolites have been reported to bind to both DNA and protein [67–73], we felt it necessary to examine if the inhibition of topoII by BAHQ was due to the targeting of DNA and/or the enzyme. Using the method described above we were able to treat either the enzyme or DNA independently within the same reaction. The results of these experiments demonstrate that the inhibition of topoII by BAHQ is due to a direct interaction with the enzyme as opposed to binding to DNA. This is in agreement with recent findings that BQ binds covalently to C170, C392, C405, and C455 on the topo II enzyme [48,74].

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The ability of BAHQ and BQ to stabilize the cleavable complex has been tested previously with various results. It is possible that slight differences in the protocols used by the investigators could explain the various outcomes. We therefore tested the ability of BAHQ to stabilize the cleavable complex with and without preincubating the enzyme with the metabolite. The results clearly indicate that BAHQ is a potent topoII poison when the enzyme is added to both DNA and BAHQ simultaneously but inhibits the formation of the cleavable complex when the enzyme is preincubated with the metabolite. In addition, the reversibility of the cleavable complexes stabilized by BAHQ suggest that this metabolite does not stabilize the cleavable complex by inhibiting the ligation step of the reaction such has previously been shown with etoposide, and therefore the stabilization of the cleavable complex does not appear to be the sole mechanism through which this metabolite inhibits topoII activity.

Based on the results of Baker et al. [42] we examined the effect of preincubating topoII with BAHQ on the binding of the enzyme to DNA. The results demonstrate that the binding of topoII to DNA is inhibited if BAHQ has the opportunity to interact with the enzyme prior to the binding of the enzyme to its DNA substrate. This is consistent with previous findings [48] that BQ inhibits binding to DNA under the same conditions. In addition, the results of the experiments utilizing SDS in the BAHQ samples without preincubation suggest that a fraction of the enzyme that is locked on DNA is not covalently bound and therefore is not trapped in the cleavable complex (Fig. 6). These results also suggest another mechanism of topoII inhibition.

If the increase in cleavable complexes is in fact due to an increase in the rate of DNA cleavage as opposed to inhibiting ligation as proposed by others [47,48], the enzyme should proceed through the catalytic cycle and eventually release DNA. Therefore no inhibition of topoII activity should occur. Because the enzyme has already been targeted by BAHQ, it should not be able to bind DNA once again and enter the catalytic cycle and proceed to the cleavable complex. If this proposed mechanism is correct, an extended incubation time should have allowed the enzyme to proceed through the catalytic cycle and release the DNA, therefore resulting in decreasing levels of cleavable complexes at extended time points. The proposed mechanism was not supported by our results (Fig. 7). There was little difference between the level of cleavable complexes observed at early and extended incubation times of up to 1 h. These results suggest that the enzyme must also be inhibited at a later point prior to the release of DNA.

The step between the cleavable complex and the release of DNA is the closed clamp. We therefore tested the ability of BAHQ to stabilize the enzyme at this point in the catalytic cycle. Typically this assay is only performed if the compound of interest has been demonstrated not to stabilize the cleavable complex and therefore the presence of high salt would have little if any effect on the results of the experiment. Based on the above observations, we felt that BAHQ may have the capacity to stabilize topoII in both the cleavable complex and the closed clamp. Because it is not known if high salt reverses the BAHQ stabilized cleavable complex to the closed clamp, we performed the experiment with and without the high salt buffer. In both cases the results demonstrated that BAHQ does in fact stabilize the enzyme in the closed clamp form. To exclude the possibility that the cleavable complexes stabilized by BAHQ are reversed to the closed clamp at some point in the processing of the samples, we maintained constant salt and temperature conditions throughout the processing of the samples.

Our data indicate that BAHQ can inhibit topoII by multiple mechanisms, by inhibiting the binding of topoII to DNA, and through stabilization of the closed clamp. We propose that the observed increase in cleavable complexes is the result of an equilibrium established between the closed clamp and the cleavable complex. This mechanism may result in a perception that BAHQ retards the DNA ligation step as reported by others [48]. Given that less than 1% of the enzyme is trapped in the cleavable complex at any given time [75], it would seem reasonable that a block in the closed clamp would shift the equilibrium and result in an increase in cleavable complexes. There is precedent for this hypothesis in that ICRF193 does in fact cause a modest increase in cleavable complexes in addition to stabilizing the closed clamp. One might argue that if both agents inhibit topoII by stabilizing the closed clamp they should produce comparable levels of cleavable complexes. We feel that it is possible that the two agents can stabilize the closed clamp via alternative mechanisms and therefore one conformational state may be favored over another in the presence of different inhibitors. This would occur in spite of the fact that the two inhibitors may both inhibit the same step of the catalytic cycle, the release of DNA in this case.

The cross-linking of topoII may also account for the multiple mechanisms of inhibition. Due to the multiple cysteine residues in the N-terminal region of the enzyme, it is possible that BAHQ may be cross-linking the two monomers at either the ATPase domains or the N-terminal clamp as has been demonstrated in the case of PCB quinone metabolites [66]. If the enzyme is cross-linked at either of these sites prior to the binding of topoII to DNA, it would result in an inhibition of topoII binding to its DNA substrate. If, on the other hand, the cross-linking occurs after DNA binding and the initiation of the catalytic cycle, the enzyme could proceed through the catalytic cycle to the closed clamp but would not be able to release DNA. An equilibrium could then be established between the cleavable complex and the closed clamp.

The stabilization of the cleavable complex observed in these and other experiments provides a potential mechanism through which exposure to benzene could produce the chromosomal aberrations seen in the bone marrow of benzene-exposed leukemia patients. However, it should be noted that translocations involving 11q23 that are characteristic of leukemias induced by topoII poisons have been infrequently seen in benzene- or solvent-exposed leukemia patients and have not been reported in the peripheral blood lymphocytes of benzene-exposed workers [76]. This suggests that the induced leukemias could arise via another mechanism. In addition, acute myelogenous leukemias that result from the exposure to topoII poisons tend to be of the myelomonocytic (M4) or monocytic (M5) FAB (French-American-British) subtypes which are also less frequently seen in benzene-exposed patients [59,62]. On the other hand, reciprocal translocations between chromosomes 8 and 21 have been reported to occur at increased frequencies in the peripheral blood of benzene-exposed workers [31].

Many of the acute leukemias induced by the bisdioxopiperazine class of topoII inhibitors (e.g. bimolane and ICRF 154) are characterized by reciprocal translocations between specific regions on chromosomes 8 and 21, and chromosomes 15 and 17 [59,62]. In addition, the acute myelogenous leukemias resulting from exposure to the bisdioxopiperazine class of inhibitors tend to be of the acute myeloblastic leukemia with maturation (M2) and acute promyelocytic leukemia (M3) FAB subtypes. These subtypes of AML (M2 and less frequently M3) have been reported to occur in leukemia patients previously exposed to benzene [62,77]. Similar results have recently been reported in the multivariate analyses of a hospital-based case-control study of Chinese leukemia patients [78], in which a significant association was seen between benzene exposures and the development of AML with the 8;21 translocation (M2 subtype), and a near significant association was observed between benzene exposure and acute promyelocytic leukemia (M3), which characteristically exhibits the 15;17 translocation. The similarities between the subtypes of leukemias and types of chromosomal aberrations produced by both the bisdioxopiperizines and benzene, as well as the mechanistic studies described above, provide indirect but complementary evidence that inhibition of

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topoII by stabilizing the closed clamp as opposed to enhancing formation of the cleavable complex may be responsible for the chromosomal aberrations observed in benzene-exposed patients and workers.

In summary, we have demonstrated that BAHQ can inhibit topoII through multiple mechanisms, each of which can potentially lead to DNA double strand breakage, and subsequently form the chromosomal alterations characteristic of BZ exposure. Our experiments indicate that BAHQ inhibits topoII at the DNA binding stage (step #1 in Fig. 1) as well as in the closed clamp stage (step #5 in Fig. 1) in the catalytic cycle, thereby interfering with either the binding to, or the release of, DNA from the enzyme, and inhibiting subsequent enzymatic activity. While increases in the cleavable complex are seen with BAHQ treatment, our results suggest that this is related to a shift in equilibrium due to an accumulation of the topoII enzyme at the closed clamp stage rather than a major inhibitory effect on the religation step. However, the observed increase in cleavable complex formation as well as the inhibition of enzymatic activity at the closed clamp and other stages of the catalytic cycle would likely result in DNA breakage, the formation of chromosomal aberrations, and could potentially result in leukemia-associated chromosomal translocations. Furthermore, inhibition of topoII activity and the related inability of the enzyme to properly function in chromosome condensation and decatenation could also result in the G_2/M cell cycle block and contribute to the bone marrow toxicity that has been observed with benzene exposure.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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