

Inhibition of Human Topoisomerase II *in Vitro* by Bioactive Benzene Metabolites

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Benzene is a clastogenic and carcinogenic agent that induces acute myelogenous leukemia in humans and multiple types of tumors in animals. Previous research has indicated that benzene must first be metabolized to one or more bioactive species to exert its myelotoxic and genotoxic effects. To better understand the possible role of individual benzene metabolites in the leukemogenic process, as well as to further investigate inhibition of topoisomerase II by benzene metabolites, a series of known and putative benzene metabolites, phenol, 4,4'-biphenol, 2,2'-biphenol, hydroquinone, catechol, 1,2,4-benzenetriol, 1,4-benzoquinone, and *trans-trans*-muconaldehyde were tested for inhibitory effects *in vitro* on the human topoisomerase II enzyme. With minor modifications of the standard assay conditions, 1,4-benzoquinone and *trans-trans*-muconaldehyde were shown to be directly inhibitory, whereas all of the phenolic metabolites were shown to inhibit enzymatic activity following bioactivation using a peroxidase activation system. The majority of compounds tested inhibited topoisomerase II at concentrations at or below 10 μ M. These results confirm and expand upon previous findings from our laboratory and indicate that many of the metabolites of benzene could potentially interfere with topoisomerase II. Since other inhibitors of topoisomerase II have been shown to induce leukemia in humans, inhibition of this enzyme by benzene metabolites may also play a role in the carcinogenic effects of benzene. — Environ Health Perspect 104(Suppl 6):1319–1323 (1996)

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Introduction

Benzene is a known leukemia-inducing agent in humans and a multisite carcinogen in rodents (1). However, the mechanisms by which benzene exerts its leukemogenic effects remain unknown. A number of studies have shown that benzene-exposed workers exhibit increased frequencies of structural and numerical chromosome aberrations in their peripheral blood and bone marrow cells (2,3). In addition, animal studies have shown that after benzene exposure, increases in micronuclei, chromosome loss, and breakage are seen in the hematopoietic cells of mice and rats

(1,4,5). These chromosomal effects, combined with observations that benzene is only weakly mutagenic in standard gene mutation assays (6,7) and binds poorly to DNA (8,9) suggest that indirect genetic mechanisms that result in translocations, deletions, aberrant recombination, or aneuploidy may be important in benzene's genotoxic effects (10,11). It is also likely that the chromosome damage seen in the blood cells of occupationally exposed populations reflects alterations occurring in the hematopoietic stem cells, which contribute to leukemogenesis. In addition to benzene,

a number of different types of leukemia-inducing agents have been identified (12). Topoisomerase-interactive agents make up one recently identified class of leukemogens that exhibit similarities to benzene in their cellular, genotoxic, and leukemogenic effects (13).

Topoisomerase II enzymes relieve torsional strain on DNA that occurs during replication and transcription by creating transient breaks in both strands of double-stranded DNA and allowing passage of a second DNA strand (14). These enzymes are also important structural components of interphase nuclei and are believed to function during recombination and chromosome condensation (14,15). Over the past several years, a number of potent anti-cancer drugs have been identified that target topoisomerase II (16). These drugs can be divided up into three distinct groups. First are the intercalating compounds such as the drug classes of anthracyclines, ansamycin, and ellipticines that stimulate the formation of the cleavable complex (17). Second are the epipodophyllotoxins, which form the cleavable complex, but do not intercalate into DNA (17). A third class of compounds has been identified in the last few years that includes compounds such as the dioxopiperazine and coumarin derivatives merbarone and novobiocin that inhibit topoisomerase II activity but do not intercalate or induce formation of the cleavable complex (15,17–19). These topoisomerase II-interactive agents (referred to here as "topoisomerase inhibitors") act through a variety of different mechanisms including the prevention of topoisomerase II binding to DNA, inhibition of strand passage, stabilization of the enzyme–DNA complex, or interference with the religation of the DNA strands (14). Topoisomerase II inhibitors tend to be strong clastogens in mammalian cells (14,20). In addition, these agents have been shown to induce sister chromatid exchanges and illegitimate recombination as well as inhibit chromosome separation and DNA and RNA synthesis (14,20). These inhibitors have also been demonstrated to delay cells in the G₂ to M phases of the cell cycle, altering differentiation and perturbing the topoisomerase enzyme, thus rendering it error prone (14,16,21–23).

Previous work in our laboratory has shown that some of the known and putative metabolites of benzene inhibit topoisomerase II *in vitro* at relatively high concentrations (13). Using a model peroxidase

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activation system similar to the system believed to exist in the bone marrow (24,25), inhibition was also seen at much lower concentrations with phenol, 2,2'-biphenol and 4,4'-biphenol. Based on these observations, we proposed that inhibition of topoisomerase II may contribute to the clastogenic and carcinogenic effects of benzene (13). These initial studies were conducted using the standard topoisomerase assay protocol recommended by the supplier of the enzyme. In addition, high peroxidase concentrations were employed for bioactivation with incubations being performed for 1 hr to ensure that metabolism was complete and to minimize effects due to short-lived radical species. In these earlier studies, reduced glutathione (GSH) was shown to protect the topoisomerase enzyme from inhibition by activated 4,4'-biphenol in spite of the presence of dithiothreitol in the assay buffer. This suggested that the assay conditions might significantly influence the outcome of the topoisomerase assay. Furthermore, subsequent spectrophotometric studies indicated that activation occurred very rapidly, which may have affected the assay results by allowing degradation of the reactive intermediates during the remaining 1-hr incubation. In this study, the peroxidase concentrations and incubation times were shortened to reduce these possibilities. In addition, metabolites that we had not previously tested with metabolic activation were tested for inhibitory effects after incubation with a peroxidase activation system. The results of these studies demonstrate that a variety of reactive metabolites of benzene are capable of inhibiting topoisomerase II *in vitro* when tested under appropriate assay conditions or with peroxidase activation.

Materials and Methods

Phenol, hydroquinone, catechol, 1,4-benzoquinone, 1,2,4-benzenetriol, 2,2'-biphenol, and 4,4'-biphenol (all \geq 98% purity) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Glutathione (GSH reduced form, 98–100%), hydrogen peroxide (H_2O_2 ; 30%), horseradish peroxidase (HRP), type VI (250 U/mg) were obtained from Sigma Chemical Co. (St. Louis, MO). Distilled deionized water was obtained from MallinKrodt Chemical, Inc. (Paris, KY) *trans-trans*-Muconaldehyde was a generous gift of Dr. G. Witz (Rutgers University, Piscataway, NJ). Human topoisomerase II, catenated kinetoplast DNA (kDNA), and *m*-amsacrine were obtained from TopoGEN, Inc. (Columbus, OH).

2,2'-Biphenol, 4,4'-biphenol, and *m*-amsacrine were dissolved in 100% dimethylsulfoxide (DMSO) at 100 mM concentrations, with subsequent dilutions in 1% DMSO. *trans-trans*-Muconaldehyde was dissolved and diluted in 100% ethanol. Phenol, hydroquinone, catechol, 1,4-benzoquinone, and 1,2,4-benzenetriol were prepared in distilled, deionized H_2O . Chemical concentrations are reported as final nominal concentrations in the total assay volume. The initial concentration to which the enzyme is exposed will vary depending on the order that the reaction components were added.

An HRP solution [1 U/ μl] was prepared in 0.1 M Tris-Cl, pH 8. All dilutions of peroxidase and H_2O_2 solutions were made in distilled, deionized water. The final enzyme and H_2O_2 concentrations for all the incubations except those containing 2,2'-biphenol were 0.07 U/ml HRP and 55 μM H_2O_2 . 2,2'-Biphenol was bioactivated with 0.1 U/ml HRP and 55 μM H_2O_2 concentrations to facilitate more complete metabolism. Peroxidase bioactivation reactions were run for 5 min at room temperature, with the exception that the 2,2'-biphenol incubations were performed for 30 min. Each reaction tube was then placed on ice, at which time the assay buffer, kDNA, and topoisomerase II enzyme were added sequentially for the topoisomerase assay.

The testing of benzene metabolites was done using a commercially available topoisomerase II inhibition assay (TopoGEN). The kit included purified human topoisomerase II (2 U/ μl), kDNA (0.1 $\mu\text{g}/\mu\text{l}$), decatenated kinetoplast DNA, and 10 \times assay buffer [0.5 M Tris-Cl, pH 8.0, 1.2 M KCl, 100 mM MgCl_2 , 5 mM ATP, 5 mM dithiothreitol, and 300 $\mu\text{g}/\text{ml}$ bovine serum albumin (BSA)]. Assays were performed in the presence and absence of test chemicals or solvents (1 μl added to assay) by mixing 4 U enzyme with 0.2 μg kDNA and 3 μl 10 \times assay buffer. The reaction was brought to a final volume of 30 μl with deionized, distilled H_2O (0.2 μm filtered). For the standard assay method, the order of addition to the assay was H_2O , 10 \times assay buffer, followed by either the test compound, the solvent, or the metabolic reaction mixture, kDNA and topoisomerase II. For the direct method, the metabolite or the reaction mix was added directly to the topoisomerase II, followed by H_2O , 10 \times assay buffer, and kDNA. The incubations were run for 1 hr at 37°C, and the reaction was terminated by adding

6 μl of a stop solution consisting of 5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol in H_2O . The DNA products and decatenated marker kDNA were separated by electrophoresis using a 1% agarose gel and 1 \times (Tris-acetate-EDTA [TAE] [0.04 M Tris-acetate, 0.001 M EDTA]) buffer containing 0.03 $\mu\text{g}/\text{ml}$ ethidium bromide. The DNA-containing bands were visualized using an ultraviolet light box. All experiments were repeated a minimum of three times.

Results

Topoisomerase II activity was determined by assaying the decatenation of kDNA. After electrophoresis, the appearance of either open circular or linearized kDNA indicated an active and functional enzyme. If inhibition occurred, the kDNA remained in the catenated form and did not migrate from the well (13,26). All of the benzene metabolites that were tested inhibited the ability of topoisomerase II to decatenate kDNA. The inhibitors fell into two distinct classes, each of which are described below. The first group consisted of metabolites that were inhibitory when added directly to the enzyme. The second and larger group of metabolites required bioactivation by peroxidase enzymes to inhibit topoisomerase or for inhibition to be seen at low micromolar concentrations. For this second group of inhibitors, the order in which the reagents were added to the topoisomerase II assay did not substantially alter the inhibitory effects.

1,4-Benzoquinone and *trans-trans*-muconaldehyde inhibited topoisomerase II when added directly to the enzyme before addition of the assay buffer and kDNA. This modified reaction assembly procedure was necessary to prevent 1,4-benzoquinone and *trans-trans*-muconaldehyde from reacting with components of the assay buffer. When these two compounds were mixed with the buffer before enzyme addition, the inhibitory effects were greatly reduced. Spectrophotometric studies (data not shown) indicated that dithiothreitol reacts directly with these benzene metabolites to form UV-absorbing products. When the reaction was assembled using the standard procedure, sulfhydryl groups of dithiothreitol almost certainly reacted with these two electrophilic chemicals, preventing them from interacting with topoisomerase II and inhibiting its enzymatic activity. Table 1 shows the metabolites and concentrations tested and indicates the concentrations at which inhibitory effects were

Table 1. Direct inhibitors of topoisomerase II.

Chemical	Concentration, μM	Inhibition ^a
1,4-Benzoquinone	500	++
	100	++
	10	+
	1	-
<i>t-t</i> -Muconaldehyde	100	++
	10	+/-
<i>m</i> -Amsacrine (positive control)	3000	++
Ethanol control	3.3% (v/v)	-
Water control	3.3% (v/v)	-

The benzene metabolites 1,4-benzoquinone and *trans-trans*-muconaldehyde were added directly to the topoisomerase II enzyme with the 10 \times assay buffer, kDNA, and water being added shortly thereafter. The reaction was initiated by placing the reaction tube into a 37°C water bath for 1 hr. ^aInhibition reported as: ++ = total inhibition; + = partial inhibition; - = no inhibition of enzyme activity; +/- = inconsistent inhibitory results.

seen. 1,4-Benzoquinone inhibited topoisomerase II at concentrations $\geq 10 \mu\text{M}$. *trans-trans*-Muconaldehyde exhibited consistent inhibitory effects at concentrations $\geq 100 \mu\text{M}$. Inhibition of topoisomerase II was also seen at lower concentrations, but the effects were somewhat inconsistent.

Phenol, 4,4'-biphenol, 2,2'-biphenol, hydroquinone, catechol, and 1,2,4-benzenetriol all required bioactivation by peroxidase in the presence of hydrogen peroxide to inhibit topoisomerase II at low micromolar concentrations. Table 2 shows the concentrations tested and the concentrations at which inhibitory effects were seen. Direct addition of the metabolic products to the topoisomerase did not substantially alter the inhibitory concentrations. 2,2'-Biphenol was the only compound from this group to give somewhat variable results. Activated 2,2'-biphenol consistently inhibited topoisomerase II at concentrations $\geq 100 \mu\text{M}$. However, inconsistent results were seen at the lower 10 μM concentration.

Discussion

The results of these *in vitro* assays demonstrate that most of the benzene metabolites are capable of inhibiting topoisomerase II *in vitro* at relatively low concentrations and indicate that a variety of benzene metabolites may be involved in the inhibition of this enzyme *in vivo*. For the reactive benzene metabolites 1,4-benzoquinone and *trans-trans*-muconaldehyde, inhibition occurred when the assay was modified so that the metabolites were added directly to the topoisomerase II enzyme. For the phenolic metabolites, incubation in the

Table 2. Metabolites of benzene requiring peroxidase activation in order to inhibit topoisomerase II.

Chemical	Concentration, μM	Inhibition ^a
Phenol	100	++
	10	++
	1	-
4,4'-Biphenol	100	++
	10	++
	1	-
2,2'-Biphenol	100	++
	10	+/-
Hydroquinone	100	++
	10	++
	1	+
Catechol	100	++
	10	++
	1	-
1,2,4-Benzenetriol	100	++
	10	++
	1	-
<i>m</i> -Amsacrine (positive control)	3000	++
Controls ^b		-

All the compounds in this table, with the exception of 2,2'-biphenol, were incubated with 0.07 U/ml horseradish peroxidase and 55 μM hydrogen peroxide for 5 min, followed by the addition of the 10 \times assay buffer, kDNA, and topoisomerase II enzyme. 2,2'-Biphenol was incubated with 0.1 U/ml horseradish peroxidase and 55 μM hydrogen peroxide; all other steps were the same. ^aInhibition reported as: ++, total inhibition; +, partial inhibition; -, no inhibition of enzyme activity; +/-, inconsistent inhibitory results. ^bControls consisted of horseradish peroxidase (0.07-0.1 U/ml), H₂O₂ (55 μM), and dimethylsulfoxide (0.03-3.3%) or water.

presence of a peroxidase/hydrogen peroxide activation system was necessary for inhibition of topoisomerase II to be seen at low micromolar concentrations. The rationale for the use of a peroxidase activation system is that the bone marrow contains high levels of myeloperoxidase as well as other peroxidase enzymes (27,28) and increases in oxygen radicals have been shown to occur in this organ after benzene administration (10,29). In addition, relatively high levels of phenolic metabolites have been recovered in the bone marrow after benzene exposure (30).

Recently, a number of benzene metabolites were screened by our laboratory for their inhibitory effects on human topoisomerase I and II *in vitro* (13). In these initial experiments, assay conditions recommended by the supplier of the enzyme were used as well as relatively long (1-hr) bioactivation incubations with high levels of peroxidase enzymes. In the studies reported here, shorter bioactivation periods

(5 min) with reduced enzyme concentrations were used. The results of the two series of experiments are quite consistent but illustrate how modifications in the assay conditions can significantly alter the test results. For example, by adding 1,4-benzoquinone directly to the enzyme, inhibition was seen at a 10 μM (final) concentration, whereas inhibition was only seen at 500 μM when this reactive metabolite was added to the dithiothreitol- and BSA-containing buffer. In addition, by altering the bioactivation procedure, inhibition of topoisomerase II was seen at lower concentrations for phenol, 2,2'-biphenol and 4,4'-biphenol.

While the majority of the compounds assayed in these studies were inhibitory at 10 μM , two of the compounds, *trans-trans*-muconaldehyde and 2,2'-biphenol, required higher concentrations (100 μM) to consistently inhibit the topoisomerase enzyme. However, these compounds were sometimes observed to inhibit the enzyme at lower concentrations. We believe that these somewhat inconsistent results were due to instability of *trans-trans*-muconaldehyde in aqueous solutions and the incomplete metabolism and formation of polymers in incubations containing 2,2'-biphenol. Attempts to use higher concentrations of peroxidase and H₂O₂ to complete the metabolism of 2,2'-biphenol were problematic in that occasionally inhibition of topoisomerase II was observed in the absence of added inhibitors, making interpretation of the experiments difficult. However, at the 0.07 to 0.1 U/ml HRP and 55 μM hydrogen peroxide concentrations used for bioactivation in the experiments described here, this problem was not seen. As a result of the reactive nature and instability of certain metabolites, the likely presence of nucleophilic constituents in the topoisomerase-containing solution as well as the influence of the assay conditions, we believe that the absolute inhibitory concentrations observed in the topoisomerase II assay should be interpreted with caution.

The inhibitory effects seen with 1,4-benzoquinone, *trans-trans*-muconaldehyde, and the bioactivated phenolic metabolites as well as observed protection of the enzyme from inhibition by GSH (13) suggest that there is a sulfhydryl or other nucleophilic residue on the topoisomerase enzyme that is critical for enzymatic activity. These results also indicate that the standard assay conditions used to screen pharmaceutical and toxicological agents for topoisomerase-inhibitory activity may not detect certain classes of inhibitory compounds

such as those that react with dithiothreitol or other components of the topoisomerase assay. Despite the limitations inherent in the *in vitro* assay, it is still clear that most of the benzene metabolites (known and putative) tested inhibited topoisomerase II *in vitro* in the low micromolar concentration range when assayed under appropriate test conditions or with metabolic activation.

Beyond these early *in vitro* studies, there is also mounting evidence in the literature suggesting that topoisomerase II inhibition may contribute to benzene-induced leukemogenesis. While the evidence is inconclusive at this point, there are a number of similarities in characteristics exhibited by the chemotherapeutic topoisomerase II inhibitors and benzene (or its metabolites) (Table 3). In addition, topoisomerase II inhibitors induce high frequencies of chromosome alterations which are due, at least in part, to action on specific DNA recognition sites (31,32). Disrupting topoisomerase II function frequently results in chromosome translocations and deletions, including alterations affecting chromosome regions 11q23 and 21q22, which have become hallmarks of therapy-related acute myelogenous leukemia induced by topoisomerase inhibitors (12,33,34). Translocations have been reported in the blood cells of workers exposed to benzene and in patients who have developed leukemia after exposure to benzene or benzene-containing solvents such as petroleum (35–40). In a number of these cases, the translocations and deletions that have been reported are identical to those that are characteristic of topoisomerase-interactive agents (Table 4). It should be noted, however, that chromosome

Table 3. Similar characteristics of benzene and selected topoisomerase II inhibitors.

Characteristic	References
Parent or metabolite has a phenolic or quinonoid structure	(6,14,16,17,24,44)
Increased toxicity to actively dividing cells	(14,45,46)
Alter differentiation of immature myeloid cells	(21,46,47)
Block dividing cells at G ₂ /M stage	(13,14,23,45)
Yield high frequencies of structural chromosomal alterations	(2,4,12,14,48)
Induce acute myelogenous leukemia	(2,12,33,34,44,46)

Table 4. Chromosome alterations characteristic of topoisomerase II inhibitors reported in leukemia patients with previous exposure to benzene-containing solvents.

Sex	Age	Chromosome alteration	Source of benzene exposure	Reference
Male	37	t(8;21)(q22;q22)	Petroleum products	(37)
Male	36	t(8;21)(q22;q22)	Petroleum products	(37)
Male	19	del(11)(q23;q25)	Petroleum products	(37)
Female	55	t(4;11)(q21;q23)	Benzene-containing solvents	(38)
Male	64	t(3;21)(q26.2;q22.1)	Benzene-containing solvents	(39)

alterations similar to those seen after treatment with alkylating chemotherapeutic agents such as loss of all or part of the long arms of chromosomes 5 and 7 (12) have been reported in benzene-exposed workers as well as in leukemia patients previously exposed to benzene (35,40,41). In addition, trisomy or tetrasomy of a C-group chromosome, occasionally identified as chromosome 8 or 9, has been associated with benzene exposure (11,40,42,43). This suggests that multiple types and mechanisms of genotoxicity may be occurring. Our current working hypothesis is that both the alkylating-type of chromosomal alterations (aneuploidy and deletions) as well as topoisomerase-type alterations (translocations and deletions) are occurring in benzene-exposed individuals. The combination of these types of chromosomal alterations

confers increased risks for leukemia similar to that reported for cancer patients who have been treated with both alkylating agents and topoisomerase inhibitors (34).

In summary, our *in vitro* topoisomerase II inhibition results, combined with the characteristics shared by both the bioactive benzene metabolites and topoisomerase II inhibitors in addition to the karyotypic alterations seen in benzene-exposed individuals, provide support for the hypothesis that interference with the normal function of topoisomerase II is involved in benzene-induced leukemia. Additional studies are currently being performed to identify the species involved in topoisomerase inhibition *in vitro* and to determine whether inhibition of topoisomerase enzymes contribute to the myelotoxic and carcinogenic effects of benzene *in vivo*.

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