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Mutagenic and cytotoxic activity of benzo[*a*]pyrene 4,5-, 7,8-, and 9,10-oxides and the six corresponding phenols

(*Salmonella typhimurium*/reversion of histidine auxotrophs/Chinese hamster cells/8-azaguanine resistance)

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ABSTRACT **The benzo[*a*]pyrene 4,5-, 7,8-, and 9,10-oxides and the six corresponding phenols (4-, 5-, 7-, 8-, 9-, and 10-hydroxybenzo[*a*]pyrene) have been tested for mutagenic and cytotoxic activity in bacteria and in a mammalian cell culture system. Benzo[*a*]pyrene 4,5-oxide (K-region) was highly mutagenic in two histidine-dependent strains (TA1537 and TA1538) of *Salmonella typhimurium* which detect frameshift mutagens. In contrast, benzo[*a*]pyrene 7,8- and 9,10-oxides were less than 1% as mutagenic as the 4,5-oxide. Benzo[*a*]pyrene 7,8- and 9,10-oxides were unstable in aqueous media, whereas the 4,5-oxide was stable for several hours. This difference in stability could not account for the different mutagenic activities of the three arene oxides. The benzo[*a*]pyrene oxides were inactive in a strain (TA1535) that is reverted by base pair mutagens such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or in a strain (TA1536) that detects frameshift mutagens similar to the acridine half-mustard ICR-191. Benzo[*a*]pyrene and the six phenols were all stable in aqueous media, but they had little or no mutagenic activity in any of the four *Salmonella* strains. Conversion of 8-azaguanine-sensitive Chinese hamster V79 cells to 8-azaguanine-resistant variants was increased by benzo[*a*]pyrene 4,5-oxide, whereas the 9,10-oxide was considerably less active. Benzo[*a*]pyrene and the other derivatives had little or no effect. Benzo[*a*]pyrene 4,5-oxide was more cytotoxic to the Chinese hamster V79 cells than the 7,8- and 9,10-oxides, while 8-hydroxybenzo[*a*]pyrene was the most cytotoxic of the six phenols.**

Recent studies have indicated that much of the biologic activity of polycyclic aromatic hydrocarbons and the covalent binding of these compounds to macromolecules can be attributed to metabolic products. The end products of aromatic hydrocarbon metabolism, which include phenols, quinones, *trans*-dihydrodiols, and thiol conjugates, result from reactive arene oxide intermediates (1, 2). The ability of certain K-region arene oxides to bind covalently to DNA (3–5), to induce malignant transformations in cell culture (6–10), and to elicit mutations in both bacteria (11) and in cultured mammalian cells (12), suggests that arene oxides may be proximate or ultimate carcinogens of polycyclic hydrocarbons. Unfortunately, studies with arene oxides have generally been limited to the more stable K-region arene oxides. Since metabolic oxidation of polycyclic hydrocarbons occurs at multiple sites in these molecules, it is important to systematically study the biologic activity of all of the potential metabolites.

In this communication, we report the mutagenic properties of the chemically synthesized 4,5-, 7,8-, and 9,10-oxides of benzo[*a*]pyrene (BP) and the six corresponding phenols (4-, 5-, 7-, 8-, 9-, and 10-hydroxybenzo[*a*]pyrene) in histidine-dependent auxotrophs of *Salmonella typhimurium* and in V79 Chinese hamster cells. Neither the bacteria (13) nor the V79 cells (14) contain enzymes that metabolize polycyclic hydrocarbons. We have chosen initially to study the mutagenic properties of BP derivatives because the mutation test systems are generally well characterized, relatively rapid, and provide an index of an interaction of the BP derivatives with genetic material under conditions that result in an altered phenotype.

MATERIALS AND METHODS

Synthesis and Handling of BP Derivatives. The unequivocal synthesis of the BP 4,5-, 7,8-, and 9,10-oxides (15, 16) and the six corresponding phenols[§] have been reported, as have structural proofs and criteria of purity. All compounds used in this study were greater than 98% pure. Arene oxides were dissolved in acetone/NH_4OH (1000:1), while BP and phenols were dissolved in acetone. Derivatives were stored at −90° in amberized glass tubes, and their stability was confirmed prior to each experiment by chromatography on silica gel thin layer sheets (Eastman 13181) using a benzene/chloroform/ethylacetate (1:1:1) solvent system. Decomposition of the arene oxides on silica gel was prevented by adding triethylamine to the solvent system (5% v/v) and prespotting the origin of the plate with 10 μl of triethylamine immediately before application of the arene oxides.

Bacterial Mutagenesis Assay. For studies on the mutagenic effects of BP derivatives in bacteria, we have used four histidine-dependent auxotrophs of *Salmonella typhimurium* developed by Ames *et al.* (13, 17). As a result of a different DNA base sequence at the site of histidine mutation, each of the four *Salmonella* tester strains has a different sensitivity profile to a series of mutagens. Strain TA1535 detects base pair mutagens, while strains TA1536, TA1537, and TA1538 detect frameshift mutagens. Two additional mutations, one eliminating excision repair of DNA and the other producing an outer cell membrane deficient in lipopolysaccharide, increase the sensitivity of all four strains to mutagen-induced reversion. The mutagenic activity of a

Abbreviations: BP, benzo[*a*]pyrene; 4-HOBP, 4-hydroxybenzo[*a*]pyrene; 5-, 7-, 8-, 9-, and 10-HOBP, other BP phenols; 8-AG, 8-azaguanine.

§ H. Yagi, G. M. Holder, P. Dansette, O. Hernandez, H. J. C. Yeh, R. A. LeMahieu, and D. M. Jerina, "Synthesis and Spectral Properties of the Isomeric Hydroxybenzo[*a*]pyrenes," submitted for publication.

Table 1. Reversion of histidine auxotrophs of *Salmonella typhimurium* by BP oxides and phenols

Compound	μg added	His+ revertants per plate* 1535	1536	1537	1538
—	—	27 ± 3	0	7 ± 2	15 ± 4
BP	1.0	28 ± 3	0	8 ± 2	11 ± 5
	5.0	31 ± 6	0	8 ± 2	11 ± 3
BP 4,5-oxide	0.25	29 ± 3	0	63 ± 7	606 ± 56
	1.0	39 ± 4	0	229 ± 23	3800†
	5.0	40 ± 5	0	1235 ± 200	—
BP 7,8-oxide	0.25	31 ± 5	0	8 ± 2	28 ± 3
	1.0	30 ± 6	0	8 ± 4	43 ± 4
	5.0	28 ± 6	0	7 ± 1	73 ± 8
BP 9,10-oxide	0.25	33 ± 6	0	7 ± 2	22 ± 4
	1.0	30 ± 5	0	11 ± 1	31 ± 3
	5.0	28 ± 5	0	11 ± 3	61 ± 8
4-HOBP	1.0	32 ± 6	0	8 ± 4	9 ± 2
	10.0	36 ± 7	0	13 ± 3	16 ± 2
5-HOBP	1.0	40 ± 9	0	9 ± 2	13 ± 4
	10.0	32 ± 6	0	17 ± 3	12 ± 2
7-HOBP	1.0	38 ± 11	0	11 ± 3	28 ± 4
	10.0	42 ± 3	0	6 ± 1	41 ± 5
8-HOBP	1.0	31 ± 8	0	8 ± 2	13 ± 3
	10.0	34 ± 6	0	7 ± 3	10 ± 2
9-HOBP	1.0	32 ± 5	0	10 ± 3	16 ± 3
	10.0	41 ± 6	0	9 ± 10	16 ± 3
10-HOBP	1.0	30 ± 4	0	11 ± 4	9 ± 3
	10.0	41 ± 5	0	13 ± 5	10 ± 4

Mutagenesis testing was performed using a pour plate procedure as described in *Materials and Methods*. BP and the BP oxides were dissolved in acetone/NH_4OH (1000:1), and the phenols were dissolved in acetone. Neither solvent had any affect on the spontaneous mutation frequency and were nontoxic at the volume used.

* Numbers represent the mean and standard deviation of five replicate plates.

† Because of the large number of revertants, only one plate was counted.

compound is quantified by counting the number of bacterial colonies that grow on a histidine-free medium.

The four *Salmonella typhimurium* tester strains were obtained from Dr. B. Ames, University of California at Berkeley. Growth and maintenance of the bacteria and the pour plate procedure for mutagen testing were essentially as has been described in detail elsewhere (17).

BP or its derivatives were added in 100 μl of acetone/NH_4OH (1000:1) or acetone to 2.0 ml of top agar which contained 0.6% NaCl and 0.6% agar (Difco) and was 0.05 mM in histidine and biotin. Approximately 2×10^8 bacteria in 100 μl of nutrient broth (Difco) were added just prior to the addition of the polycyclic hydrocarbons.

Cytotoxicity and Mutagenicity Assay with Cultured Cells. For studies with eukaryotic mammalian cells, we used an established line of V79 Chinese hamster cells developed by Chu and Malling (18, 19). Resistance to the lethal effects of the purine analog 8-azaguanine (8-AG) was used as the mutagenic marker. For the purposes of this work, we, like others (12), define a mutation as a stable and heritable change in phenotype.

The Chinese hamster cell line, V79-6, was generously provided by Dr. E. H. Y. Chu, University of Michigan, Ann Arbor. The cells were grown in Eagle's minimum essential medium (Gibco) which contained a 1.5-fold higher than normal concentration of glutamine and nonessential amino acids and 10% dialyzed and heat-inactivated fetal calf serum (Reheis). Medium for stock cultures contained no antibiotics but contained 10 μM hypoxanthine, 10 μM thymidine, 10 μM glycine, and 3.3 μM aminopterin to select against spontaneously formed 8-AG-resistant variants (19). Four days prior to an experiment, the medium was removed and replaced with medium free of aminopterin. Medium used for experiments contained none of the compounds in the hypoxanthine/thymidine/glycine/aminopterin medium, but contained 100 units of penicillin, and 100 μg of streptomycin per ml of medium.

The cytotoxicity of BP and its derivatives was determined by comparing the plating efficiency of treated cultures to cultures receiving solvent alone. The procedure used was essentially as described by Huberman *et al.* (12), except that 100 cells were added to 60-mm culture dishes (Falcon). Seven days after treatment, the cells were fixed and stained with Giemsa, and colonies of more than 50 cells were counted. Induction of 8-AG-resistant variants of Chinese hamster cells was done according to the procedure developed by Chu and Malling (18) as modified by Huberman *et al.* (12). Cells (1×10^4) were added to 60 × 15 mm culture dishes in 5 ml of medium 18 hr prior to treatment. Test compounds were added to the culture medium in 20 μl of acetone/NH_4OH (1000:1) or acetone. Addition of the hydrocarbons and all subsequent treatment of the cells was done in subdued light. After 1 hr at 37°, the medium and hydrocarbon were removed, the cells were washed once with 5 ml of phosphate-buffered saline, pH 7.2, and then cultured in 5 ml of fresh medium. After 2 days, drug-resistant variants were selected by adding 8-AG (10 μg/ml of medium) to the cultures. Preliminary experiments indicated that when dialyzed fetal calf serum was used in the medium, a 10 μg/ml concentration of 8-AG was four to five times the minimum dose necessary to kill all 8-AG-sensitive cells. Culture medium was replaced on day 7 with 5 ml of fresh medium containing the same concentration of 8-AG. This procedure, using one medium change after selection, was used to minimize the chance that secondary colonies would arise from the reattachment and growth of cells dislodged from a primary colony during manipulation (19). Drug-resistant colonies were fixed with methanol, stained with Giemsa, and counted on day 14.

RESULTS

Salmonella test system

The number of reversions to histidine independence that was induced in four strains of *Salmonella typhimurium* by BP, three BP oxides, and by six phenols of BP is shown in Table 1. BP 4,5-oxide (K-region) was highly mutagenic in strains TA1537 and TA1538. As little as 0.25 μg of the K-region arene oxide induced a greater than 40-fold increase in the mutation rate in strain TA1538. In contrast to the results with BP 4,5-oxide, BP 7,8- and 9,10-oxides were only weakly mutagenic, even at high concentrations. None of the arene oxides was capable of inducing histidine independence in strains TA1535 and TA1536. The data in Table 1 also show the lack of appreciable activity of BP or any of the six phenols that could form during spontaneous isomerization of BP 4,5-, 7,8-, and 9,10-oxides in an aqueous environment. These data indicate that the phenols or their potential breakdown products are without significant mutagenic activity under the conditions of these experiments and that the activity of BP 4,5-oxide is not due to 4- or 5-hydroxybenz[*a*]pyrene (HOBP).

Table 2. Reversion of strain TA1538 of *Salmonella typhimurium* after repeated additions of BP 7,8-oxide and BP 9,10-oxide

Additions	Total amount added (μg)	His^+ revertants per plate
Acetone/NH_4OH	—	14 ± 6
BP 4,5-oxide	0.625	534 ± 31
BP 7,8-oxide	15	55 ± 6
	75	99 ± 10
BP 9,10-oxide	15	61 ± 6
	75	164 ± 17

The incubation mixture consisted of 5.0 ml of nutrient broth with an initial cell density of 1×10^8 bacteria per ml. BP 4,5-oxide was added once at 0 time in 20 μl of acetone/NH_4OH (1000:1). At 20-min intervals thereafter, 20 μl of acetone/NH_4OH was added. BP 7,8- and 9,10-oxides were added in a volume of 20 μl at 0 time and at 20-min intervals thereafter for 100 min (six separate additions). For the solvent control, acetone/NH_4OH (1000:1) was added in the same volume and at the same time intervals. Cells were incubated for 2 hr at 37° with shaking (200 excursions per minute, 1-inch stroke). At 0 time or at the end of the 2 hr, 0.1 ml of the incubation mixture was added to 2.0 ml of top agar and plated as described in *Materials and Methods*. The number of revertants observed in the 0 time samples were never significantly above the background. Values represent the mean and standard deviation of five replicate plates.

The low mutagenic activity of BP 7,8-oxide and BP 9,10-oxide could have been due to the intrinsic inactivity of these arene oxides with the tester strains or to a much higher rate of spontaneous isomerization to phenols relative to BP 4,5-oxide. Should the latter case pertain, substantially decreased amounts of intact BP 7,8- and 9,10-oxides would reach the bacterial DNA. Similarly, the phenols could be inactive because of decomposition at a rate sufficient to preclude their interaction with DNA. We, therefore, examined the half-lives of the arene oxides and phenols in nutrient broth at 37° by extracting the compounds into ice-cold acetone/hexane (1:3) at various time intervals and chromatographing the extracts on thin-layer silica gel as described above. Ten percent triethylamine was added to the acetone/hexane mixture used for the extraction of arene oxides. All six phenols, BP, and BP 4,5-oxide showed no significant decomposition in this aqueous environment for at least 90 min. Additional studies with BP 4,5-oxide indicated that it was stable for at least 8 hr. BP 7,8- and 9,10-oxides, however, had half-lives of about 2 hr and 0.5 hr, respectively.¶ Since the pour plate procedure gives no indication of the minimum time necessary to induce mutations, an additional set of experiments was initiated to compensate for the differences in arene oxide stability (Table 2). BP 7,8- and 9,10-oxides (2.5 or 12.5 μg) were added every 20 min for 2 hr to 5 ml of nutrient broth which contained 5×10^8 *Salmonella* of strain TA1538. BP 4,5-oxide (0.625 μg) was added once at zero time. Immediately after the first addition and at the end of 2 hr, 0.1-ml samples of each incubation mixture were added to 2.0 ml of top agar, plated, and incubated for 2 days according to the usual procedure. As shown in Table 2, both the BP 7,8- and 9,10-oxides had low but significant mutagenic activity at both concentrations. However, BP 4,5-oxide was still more than 100 times as mutagenic as BP 9,10- or 7,8-oxide on a per μg basis corrected for the differences in stability of the three arene oxides. The zero time sample of BP 4,5-oxide gave the same reversion frequency as the untreated control incubation. This result indicates that the colonies reverted by the 2-hr exposure to BP 4,5-oxide did not result from the subsequent 2-day exposure to the small amount (0.03 μg) of arene oxide in the sample that was plated. None of the arene oxides or phenols that were tested showed any toxicity, as judged by the uniform background growth on the plates (17).

¶The half-lives of the three BP oxides were determined in nutrient broth, pH 7.4, and may not be the same as the half-lives in other aqueous media.

Table 3. Induction of 8-azaguanine-resistant variants by BP oxides

Addition	Concentration (μg/ml)	Plating efficiency		Mutations per 10^4 survivors	
		1 hr	3 hr	1 hr	3 hr
Acetone/NH_4OH	—	100	100	1.0	1.0
BP	10	91	92	1.1	1.2
BP 4,5-oxide	2	76	48	2.2	9.4
BP 7,8-oxide	10	90	86	1.0	1.3
BP 9,10-oxide	10	55	18	1.6	4.6

V79 Chinese hamster cells were treated with BP or BP oxides for 1 or 3 hr as described in *Materials and Methods*. Cells treated for 3 hr were washed in phosphate-buffered saline and received fresh medium and compound every hour. Plating efficiencies and mutation frequencies are expressed as described in the legend to Table 1. The absolute plating efficiencies of the 1- and 3-hr controls were 80 and 77%, respectively.

Mammalian cell culture system

Figure 1 illustrates the cytotoxic and mutagenic activity of BP 4,5-, 7,8-, and 9,10-oxides, the six corresponding phenols, and BP as a function of concentration in cultures of Chinese hamster V79 cells. BP, as previously reported (12), had little cytotoxicity and failed to alter the spontaneous mutation frequency in the V79 cells. BP 4,5-oxide was the most cytotoxic of the three arene oxides, and BP 7,8-oxide the least. As was found with the bacterial studies, BP 4,5-oxide was the most active of the derivatives in inducing mutations. A 1-hr exposure to BP 4,5-oxide (5 μg/ml) resulted in a 6-fold increase in the number of 8-AG-resistant colonies seen over those in a control culture. In contrast, neither BP 7,8- nor 9,10-oxide, at any concentration tested, induced more than a 1.5-fold change in the mutation frequency. Of the six phenols tested, none changed the spontaneous mutation frequency, but large differences in their cytotoxicity were observed. 8-HOBP was the most toxic of the phenols, while the 7-HOBP was the least. Both BP 4,5- and 9,10-oxides were more toxic than their corresponding phenols.

Since we found differences in the stability of the three arene oxides, as mentioned above, we performed an additional experiment in which the cells were exposed to each of the three BP oxides for a total of 3 hr, with fresh media and arene oxide added at hourly intervals. BP 7,8- and 9,10-oxides were added in 5-fold higher amounts than the 4,5-oxide (2 μg/ml) to compensate for differences in stability. Whereas, increased exposure time resulted in increased toxicity and increased mutation frequency for BP 4,5- and 9,10-oxides, BP 7,8-oxide was still inactive (Table 3). The mutagenicity of BP 9,10-oxide during the incubation may be overestimated because the mutation frequency is expressed as number of 8-AG-resistant colonies per 10^4 surviving cells, and some of the cell death observed after exposure to BP 9,10-oxide is most likely due to the nonmutagenic but cytotoxic 9-HOBP formed during incubation.

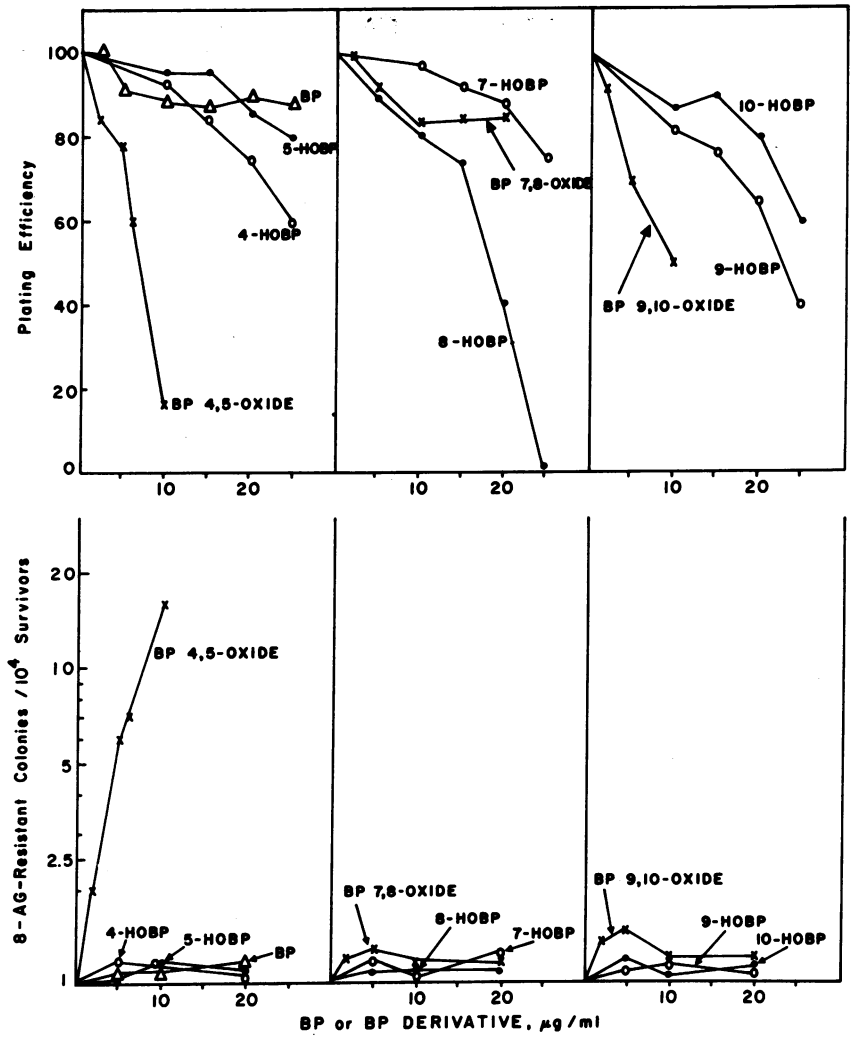

Fig. 1. Cytotoxic and mutagenic effects of BP oxides and BP phenols on Chinese hamster V79 cells. The cells were treated as described in *Materials and Methods*. Each point represents the mean of at least two experiments. In each experiment, four 60-mm tissue culture dishes were used to evaluate toxicity and six dishes were used to score 8-AG-resistant variants. Plating efficiency is expressed as a percentage of the solvent-treated controls. The absolute plating efficiencies of control cultures were between 67 and 82%. Mutation frequency is expressed as the number of 8-AG-resistant colonies observed per 10^4 surviving cells.

DISCUSSION

The carcinogenicity of BP and its widespread occurrence as an environmental pollutant have prompted numerous studies on the metabolic fate and biological activity of this hydrocarbon. Recent studies (20–22) suggest that BP 4,5-, 7,8-, and 9,10-oxides are major metabolites. The non-K-region arene oxides, BP 7,8- and 9,10-oxides, are relatively unstable and rearrange preponderantly to 7- and 9-HOBP, respectively (16). In contrast, the K-region BP 4,5-oxide is quite stable at physiological pH and would be expected to form a *trans*-dihydrodiol rather than a mixture of phenols on spontaneous solvolysis (24). Our studies indicate that BP 4,5-oxide (K-region), in contrast to the other eight derivatives of BP, is a potent mutagen in strains TA1537 and TA1538 of *Salmonella typhimurium* and in cultured V79 Chinese hamster cells. BP 4,5-oxide has been shown to bind covalently to DNA in aqueous solution (3), and it was suggested that this arene oxide was the active DNA binding metabolite formed during metabolism of BP by hamster liver microsomes (23). A recent study by Rasmussen and Wang (25) suggested that BP 4,5-oxide may have been responsible for the mutations induced in strain TA1538 of *Salmonella typhimurium* when these bacteria were incubated on petri plates with BP, NADPH, and a 9000 × *g* liver supernatant fraction. It should, however, be noted that a profile of metabolites formed under these incubation conditions has yet to be described.

The low mutagenic activity of BP 7,8- and 9,10-oxides of BP and the six BP phenols eliminate these primary compounds (many of which are BP metabolites) as mutagens in the bacterial strains and mammalian cell line used in the present study. However, primary metabolites of BP can undergo further oxidative metabolism at other sites on the hydrocarbon molecule, and recent studies indicate that such secondary metabolites of BP can interact with DNA (26, 27). With regard to our studies, it should be noted that mutagenic activity is not an absolute indication of carcinogenic activity, and certain mutagenic K-region arene oxides of polycyclic hydrocarbons are only weakly carcinogenic in mice (2, 28–30). Therefore, while we have found that BP 4,5-oxide is highly mutagenic in bacteria and in mammalian cells, additional studies are needed to determine the carcinogenicity of

these arene oxides as well as the other arene oxides and phenols described above.[‖]

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[‖] Note Added in Proof. The carcinogenicity of BP and the BP 4,5-, 7,8-, and 9,10-oxides has been evaluated by biweekly topical application to female C57 BL/6J mice for 60 weeks. BP and BP 7,8-oxide were highly carcinogenic at a dose of 0.4 μmol per application but BP 7,8-oxide, in contrast to BP, induced considerably fewer tumors at a dose of 0.1 μmol per application. BP 4,5-oxide induced few if any tumors at both doses tested, and BP 9,10-oxide was noncarcinogenic at both doses.