TUMOR INITIATING AND PROMOTING ACTIVITIES OF VARIOUS BENZO(A)PYRENE METABOLITES IN MOUSE SKIN[1]

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Running Title: TUMORIGENICITY OF BP METABOLITES

DISCLAIMER

Footnotes

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[3]The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; BP, benzo(a)-pyrene; BP 4,5-oxide, benzo(a)pyrene 4,5-oxide; BP 7,8-, 9,10-, and 11,12-oxides, other BP arene oxides; BP 7,8-diol, (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)-pyrene; BP 7β, 8α-diol-9α, 10α-epoxide or BP-diol-epoxide (anti), (±)-trans-7β, 8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; BP 7β, 8α-diol-9β, 10β-epoxide, (±)-trans-7β,8α-dihydroxy-9β, 10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; BP-tetraol 1, a mixture of the two possible tetraols from BP-7β, 8α-diol-9β, 10β-epoxide; BP-tetraol 2, a mixture of the two possible tetraols from BP-7β, 8α-diol-9α, 10α-epoxide. 1- to 12-OHBP, 1- to 12- hydroxy-benzo(a)pyrene; BP-1, 6 quinone, benzo(a)pyrene 1,6-quinone; BP-3,6-quinone, benzo(a)pyrene-3,6-quinone; BP-6,12-quinone, benzo(a)pyrene-6,12-quinone; BP-6-OH methyl, benzo(a)pyrene-6-hydroxymethyl; BP-6-methyl, benzo(a)pyrene-6-methyl; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.

INTRODUCTION

The PAH comprise a wide class of compounds, certain of which are potent carcinogens. These ubiquitous environmental pollutants have been found in the air, soil, and water as well as in the food chain. It has been suggested that the major portion of human cancers are chemically induced and that the PAH may play a significant role in their etiology. The major enzymes that metabolize PAH, drugs, steroids, pesticides and food additives are the mixed-function oxidases (10, 13). The enzyme system, AHH, is part of the complex which metabolize PAH. This enzyme complex has been found in most mammalian tissues including mouse skin, in which it is highly inducible (8, 16, 46, 49). Investigators have shown this complex to function as a detoxification system (10, 13), and Gelboin and coworkers (12, 15, 16) have proposed that this enzyme complex is also responsible for the activation of PAH to toxic and carcinogenic metabolites. Current information indicates PAH exert their toxic, mutagenic, and carcinogenic activities after they have been metabolically activated by microsomal enzymes to reactive epoxides (1, 14, 20, 27, 31). These electrophiles can then be converted to transdihydrodiols by the action of microsomal epoxide hydrase(s), rearranged spontaneously to phenols, conjugated with glutathione, or combined with cellular nucleophiles (18, 20, 40). Although most of the studies on the metabolism of BP have utilized the liver (18, 20), the skin is one of the major target organs related to BP carcinogenesis. We have recently reported that 3-OHBP, 9-OHBP and BP-7, 8-diol were the major metabolites while BP-4, 5-diol and BP-9, 10-diol were minor metabolites formed from BP metabolism in

mouse epidermis (3). One way to determine which metabolite(s) is responsible for the carcinogenic activity of BP is to assess them individually for carcinogenic activity.

Skin tumors in mice can be induced by the sequential application of a subthreshold dose of a PAH carcinogen (initiation phase) followed by repetitive treatment with a noncarcinogenic tumor promoter (2, 5). The initiation phase requires only a single application and is essentially irreversible (24), while the promotion phase is initially reversible but later becomes irreversible (5). Whenever a known skin carcinogen has been appropriately tested, it has shown initiating activity. However, urethan (36) and possibly dibenz(a, c)-anthracene (37, 47) appear to be unique in that they act as tumor initiators but are not complete carcinogens in the skin. For this reason, when a PAH is tested as a complete carcinogen, one cannot assume that all PAH and derivatives are equally effective in supplying both initiating and promoting stimuli. In a two-stage mouse skin system, initiation is the only stage that requires the presence of the carcinogen, and the measured carcinogenic potency of a chemical reflects its capacity for tumor initiation. Thus, it is possible that a carcinogen which lacks promoting ability would not be detected when tested as a complete carcinogen. Because of these considerations, we have undertaken the testing of known and potential metabolites of BP for tumor-initiating activity.

MATERIALS AND METHODS

Female CD-1 mice were purchased from Charles River Mouse Farms, North Wilmington, Mass. Mic, 7 to 9 weeks old, were shaved with surgical clippers 2 days before treatment, and only those mice in the resting phase of the hair cycle were used. Groups of 30 mice were used in the tumor experiments. The incidence of papillomas was recorded weekly and the tumors were removed at random for histological verification. BP (>99% pure) was purchased from the Aldrich Chemical Company. TPA was obtained from Dr. Peter Borchert at the University of Minnesota, Minneapols, Minnesota.

Some of the compounds (3-OH-BP, 9-OHBP, BP-4,5-diol, BP-9,10-diol, BP-7,8-diol, BP-1,6-quinone, BP-3,6-quinone, BP-6,12-quinone, BP-4,5-oxide and BP-7,8-diol-9,10-epoxide (anti) were generously supplied by the Carcinogenesis Program of NCI. BP-6-methyl and BP-6-OH-methyl were generous gifts from Dr. Flesher at the University of Kentucky. The twelve isomeric phenols of BP were obtained from Dr. Jerina of NIH and synthesized as previously described (55). BP-9,10-oxide (54), BP-11,12-oxide, the diastereomeric BP-7,8-diol-9,10-epoxides (56), BP-7,8-diol (17) and the (+) and (-) enantiomers of BP-7,8-diol (17) were synthesized as described by Jerina and coworkers. All compounds used in this study were ≥98% pure, as judged by mass spectrometry, nuclear magnetic resonance spectroscopy. and combustion analysis. The BP 7,8-diol-9,10-epoxides were applied topically in 0.2 ml of either acetone. benzene, THF or anhydrous DMSO:acetone (1:3), whereas BP 9,10-oxide, BP 11, 12-oxide, BP 7,8-dihydrodiol, and BP were applied topically in 0.2 ml of acetone:

NH_4OH (1000:1). The BP phenols were dissolved in acetone. All solutions were prepared in a darkened room immediately before use. Mice were treated with either 200 or 400 nmoles of the above compounds under subdued light. The time-lapse between preparation of the above solutions and animal treatment was < 1/2 hr. TPA was prepared in stock solutions and kept in a freezer until use. Mice received twice-weekly applications of 10 ug of TPA starting 1 week after treatment with the initiators and was continued for 30 weeks.

RESULTS AND DISCUSSION

In Table 1 are shown the skin tumor initiating activities of the twelve isomeric phenols of BP. 2-HOBP was found to be as potent a tumor initiator as BP whereas 11-HOBP was about one third as active. The remaining phenols of BP must be considered either weak or very weak tumor initiators in mouse skin when followed by twice weekly applications of 10 ug of TPA. 1-, 4-, and 8-HOBP are essentially inactive as tumor initiators since solvent vehicle controls can sometimes give rise to one tumor in a group of 30 mice. 3-, 5-, 6-, 7-, and 10-HOBP would be considered as very weak or borderline whereas 9-HOBP and 12-HOBP are definitely weak tumor initiators.

These results are in agreement with the reported carcinogenic activity in mouse skin of 2-HOBP and 11-HOBP by Wislocki et al. (50). They also found that the other 10 phenols of BP were noncarcinogenic (22). The slight tumor initiating activity of some of the other phenols of BP suggest that the two-stage system of skin tumorigenesis may be a more sensitive assay for tumorigenesis.

2-HOBP and 11-HOBP were both found to be inactive as mutagens in S. typhimurium and in Chinese hamster V-79 cells without a metabolic activating system (51). Incubation of 2-HOBP with a highly purified hepatic cytochrome P-448 monooxygenase from 3-methylcholanthrene-treated rats indicated metabolism of 2-HOBP to compound(s) that were mutagenic to S. typhimurium strain TA98 (53). Using similar incubation conditions, BP was metabolically activated to a greater extent than 2-HOBP whereas 11-HOBP was not metabolized to mutagens (53).

Bresnick et al. (7) recently found that application of 2-HOBP or 9-HOBP to mouse skin caused marked epidermal hyperplasia resembling that caused by tumor promoters, but the other ten isomeric hydroxybenzo(a)pyrenes were either less active or completely inactive. They also reported that BP was less active than 2-HOBP or 9-HOBP in causing epidermal hyperplasia (7). The ability of 2-HOBP to cause epidermal hyperplasia may be related to its strong tumor initiating and complete carcinogenic activities but the inactivity of 9-HOBP as a tumor initiator and complete carcinogen is presently not known unless it has tumor promoting activity. Since some phenols have been shown by Boutwell (6) to be promoters, we decided to test 3-OHBP and 9-OHBP for tumor-promoting activity in the two-stage system of tumorigenesis. Table 2 shows that 3-OHBP and 9-OHBP lacked tumor-promoting activity when given at a dose level of either 200 or 400 nmol twice weekly for 30 weeks. Histologically, 200 nmol of 3-OHBP and 9-OHBP had no effect on the skin, whereas 400 nmol of 9-OHBP caused a very slight inflammatory and hyperplastic response 2 days after treatment.

Studies on the metabolism of BP by liver indicates that formation of 1-, 3-, 6-, 7- and 9-HOBP (25, 38) but 2-HOBP has not been detected. 3-HOBP and 9-HOBP have also been identified as metabolites of BP in mouse skin (3). The possibility exists that 2-HOBP is formed but is very rapidly conjugated and then removed from the liver and skin.

6-HOBP is mutagenic to strains TA98 and TA100 of S. typhimurium, (51) and its mutagenic activity was increased by metabolic activation of 6-HOBP

in the presence of highly purified microsomal enzymes (53). 6-HOBP was shown to induce fibrosarcomas in rats, and to a lesser degree in mice (34), but this effect was far less potent than that of BP. 6-HOBP was found to be inactive as a skin tumor initiator in the present study and also was reported to be inactive as a complete carcinogen in mouse skin (22).

Table 3 shows the tumor initiating activity of various BP diols, BP quinones, BP-6-OH methyl and BP-6-methyl. We have previously reported that the BP-7,8-diol was approximately as potent as BP (42). Shortly afterwards, Levin et al. (26) reported that BP-7,8-diol was a potent skin carcinogen, and Chouroulinkov et al. (9) also reported that BP-7,8-diol was almost as active a tumor initiator as was BP. Recently, Malaveille et al. (29) reported that BP-7,8-diol was more active than the parent hydrocarbons in inducing his$^+$-revertant colonies of S-typhimurium TA 100 when incubated with 9,000 X g rat-liver supernatant. Additionally, Huberman et al. (19) reported that BP-7.8-diol was more mutagenic than BP in Chinese hamster V79 cells, but only when BP-metabolizing golden hamster cells were cocultivated with them. The above results plus our recent findings (42), suggest that BP-7,8-diol is a proximate carcinogen requiring further metabolism to become an ultimate carcinogen.

The BP-9,10-diol and BP-4,5-diol were both found to be weak tumor initiators when applied topically in acetone (Table 3). Chouroulinkov et al. (9) recently found that the BP-9,10-diol was a tumor initiator, but with greater activity than reported here. BP-6-OH-methyl and BP-6-methyl both were found to have moderate tumor initiating activity whereas the 1,6-, 3,6-, and 6,12 quinones of BP were weak initiators (Table 3).

The ability of optically pure (+)- and (-)-trans-7,8-diol of BP to initiate skin tumors in mice is shown in Table 4. A single application of 50-200 nmoles of (+)- or (-)-BP 7,8-diol to the backs of CD-1 mice followed by twice weekly applications of TPA revealed that the (-)-enantiomer was 5-10 fold more potent than the (+)-enantiomer as a tumor initiator at the three dosage levels tested (27). When the tumor initiating activity of the (+)- and (-)-enantiomers of BP 7,8-diol was compared to the activity of BP the (-)-enantiomer was more active while the (+)-enantiomer was considerably less active (27).

We recently reported that the BP-diol-epoxide (anti) was a weak tumor initiator when applied topically in acetone to female mice (42). We rationalized that the alleged ultimate carcinogen of BP, the diol-epoxide, was a weak initiator because of its relatively high polarity and reactivity. For this reason we tested the comparative tumor-initiating activity of BP-diol-epoxide (anti) when applied topically in either acetone, benzene or THF. As can be seen in Table 5 (also, see ref. 43) the BP-diol-epoxide (anti) was found to be a fairly effective tumor initiator when applied topically in THF. The effectiveness of the various solvents for the diol-epoxide was as follows: THF > benzene > acetone. This was true in terms of latency period, number of papillomas per mouse, percentage of mice with papillomas, and percentage of mice with carcinomas. The latency period for the diol-epoxide was 8 weeks in THF, 10 weeks in benzene, and 13 weeks in acetone. It is of interest that acetone was the best solvent for BP initiation (Table 4). The diol-epoxide applied topically in THF had about a third the tumor-initiating activity as did BP in THF.

Table 6 shows the ability of the BP-7,8-diol-9,10-epoxides, BP-4,5-oxide, BP-7,8-oxide, BP-11,12-oxide, BP-9,10-oxide and the tetraols to initiate skin tumors. The BP-7β, 8α-diol-9α, 10α-epoxide, anti) is a much more effective tumor initiator than BP-7β, 8α-diol-9β, 10β -epoxide. BP-7,8-oxide was found to be a moderate initiator whereas BP-11,12-oxide, BP-9,10-oxide and BP-4,5-oxide were weak. A mixture of the tetraols from BP-7β, -8α-diol-9β, 10β -epoxide and a mixture of the tetraols from BP 7β, 8α-diol-9α, 10α-epoxide were found to have weak tumor initiating activity. This suggests that the diol-epoxides of BP are not further metabolized to a carcinogenic intermediate(s) and the tetraols represent detoxified products.

The results obtained from in vitro and in vivo binding (4, 11, 23, 30, 32, 35, 48), mutagenicity (19, 26, 34, 51) metabolism (39, 44, 45, 57) and carcinogenicity studies (9, 26-28, 41-43) have led to the conclusion that BP-7,8-diol is the proximate carcinogenic metabolite of BP and BP-7β, 8α-diol-9α, 10α-epoxide is the ultimate carcinogenic metabolite of BP. Recent results on the strong carcinogenicity of BP 7β, 8α-diol-9α, 10α-epoxide in the newborn mouse (21) strongly indicate that it is the ultimate carcinogenic metabolite of BP. Our findings that 2-HOBP is as strong a tumor initiator as BP-7,8-diol indicate that additional studies are needed to determine whether or not 2-HOBP is formed from BP in skin or in other tissues.

CONCLUSIONS

1. The skin tumor-initiating activities of the twelve isomeric phenols of BP revealed that 2-OHBP was as potent as BP while 11-OHBP was moderately active and the others were weak or inactive. However, 2-OHBP has not been shown to be formed from BP in the skin or any other tissue.
2. The (-)-trans-7,8-diol of BP skin was found to be more active as a skin tumor initiator than BP suggesting that it is a proximal carcinogen. The data on carcinogenicity, mutagenicity and metabolism suggest that BP-7β , 8α-diol-9α, 10α-epoxide is the ultimate carcinogenic form of BP.
3. The skin tumor-initiating activities of the various BP metabolites correlate very well with their complete carcinogenic in mouse skin except for BP-7β , 8α-diol-9α, 10α-epoxide. It was found to have skin tumor initiating activity but not complete carcinogenic activity. However, BP-7β , 8α-diol-9α, 10α-epoxide was found to be a very potent complete carcinogen in newborn mice. It is possible that BP-7β , 8α -diol-9α, 10α-epoxide is only a tumor initiator in which a promoting stimulus must be supplied for carcinogenic activity. A natural tumor promoting stimulus may be present in the newborn mouse.
4. There is also a good correlation between the skin tumor initiating activities of the various BP metabolites and their mutagenic activity in the V79 mammalian cell mediated mutagenesis system.

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TABLE 1

Skin tumor initiating activities of BP and various BP phenols[a]

Initiators	No. of mice[b]	Papillomas per mouse[c]	Mice with tumors(%)[d]
BP	30	7.50	90
BP-1-OH	29	0.03	3
BP-2-OH	30	6.00	85
BP-3-OH	29	0.2	17
BP-4-OH	30	0.03	3
BP-5-OH	30	0.13	13
BP-6-OH	30	0.17	17
BP-7-OH	30	0.17	17
BP-8-OH	30	0.03	3
BP-9-OH	30	0.33	30
BP-10-OH	30	0.13	13
BP-11-OH	30	2.10	80
BP-12-OH	30	0.27	20

[a] All the compounds were applied at a dose of 400 nmol and were followed 1 week later by twice weekly application of 10 μg TPA.

[b] Surviving at the 30th week after promotion.

[c] Total number of papillomas divided by the total number of surviving mice 30 weeks after promotion.

[d] Percentage of surviving mice with papillomas 30 weeks after promotion.

TABLE 2

Skin Tumor promoting activity of BP-3-OH and BP-9-OH after DMBA initiation[a]

Promoter	Dose (nmol)	No. of mice[b]	Papillomas per mouse[c]	Mice with tumors[d] (%)
TPA	17	29	10	100
BP-3-OH	200	30	0	0
BP-3-OH	400[e]	30	0	0
BP-9-OH	200	30	0	0
BP-9-OH	400	30	0	0

[a] All mice were initiated with 200 nmol DMBA in acetone 1 week before promotion.

[b] Surviving at the 30th week after promotion.

[c] Total number of papillomas divided by the total number of surviving mice 30 weeks after promotion.

[d] Percent of surviving mice with tumors 30 weeks after promotion.

[e] No tumors also after 35 weeks.

TABLE 3

Skin tumor initiating activities of BP and various BP dihydrodiols, BP-6-OH methyl and BP-6-methyl [a]

Initiators	No of mice [b]	Papillomas per mouse [c]	Mice with tumors(%) [d]
BP	30	7.4	97
BP-4,5-diol	29	0.3	30
BP-9,10-diol	30	0.1	13
BP-7,8-diol	30	6.5	94
BP-6-OH-methyl	30	1.0	57
BP-6-methyl	27	1.6	74
BP-1,6-quinone	28	0.3	29
BP-3.6,-quinone	29	0.2	21
BP-6.12-quinone	30	0.3	29

[a] All the compounds were applied at a dose of 400 nmol and were followed 1 week later by twice weekly applications of 10 ug TPA.

[b] Surviving at the 30th week after promotion.

[c] Total number of papillomas divided by the total number of surviving mice 30 weeks after promotion.

[d] Percentage of surviving mice with papillomas 30 weeks after promotion.

TABLE 4

Tumorigenicity of benzo(a)pyrene and (+)- and (-)-benzo(a)pyrene-7,8-dihydrodiol on mouse skin

Initiator	Weeks of Promotion	Percent of mice with tumors	Papillomas Per mouse
BP	7	0	0
(-) BP 7,8-dihydrodiol	7	13	0.17
(+) BP 7,8-dihydrodiol	7	0	0
BP	11	13	0.20
(-) BP 7,8-dihydrodiol	11	37	0.77
(+) BP 7,8-dihydrodiol	11	7	0.07
BP	15	47	1.10
(-) BP 7,8-dihydrodiol	15	53	1.70
(+) BP 7,8-dihydrodiol	15	17	0.17
BP	21	77	2.6
(-) BP 7,8-dihydrodiol	21	77	3.8
(+) BP 7,8-dihydrodiol	21	23	0.43
None	21	7	0.07

Female CD-1 mice (7-8 weeks of age) were treated topically with 100 nmoles of BP, (+)-BP 7,8-dihydrodiol or (-)-BP 7,8-dihydrodiol in 200 ul of acetone: NH_4OH (1000:1). Commencing eleven days after application of the polycyclic hydrocarbon, 16 nmoles of TPA (in 200 ul of acetone) was applied to the backs of the mice twice weekly for 21 weeks. Control mice received acetone: NH_4OH followed by twice weekly applications of TPA. Each treatment group consisted of 30 mice.

TABLE 5

Skin-tumor-initiating activities of BP and BP-diol-epoxide (anti) in various solvents [a]

Initiators	Solvent	No. of mice[b]	Papillomas per mouse[c]	Mice with tumors[d] (%)
BP	acetone	30	7.5	90
BP	benzene	28	5.8	82
BP	THF	29	6.2	93
BP-diol-epoxide	acetone	29	0.3	30
BP-diol-epoxide	benzene	30	1.1	60
BP-diol-epoxide	THF	28	1.9	71

[a] All the compounds were applied at a dose of 400 nmol and were followed 1 week later by twice weekly applications of 10 ug TPA.

[b] Surviving at the 30th week after promotion.

[c] Total number of papillomas divided by the total number of surviving mice 30 weeks after promotion.

[d] Percentage of surviving mice with papillomas 30 weeks after promotion.

TABLE 6

Skin tumor initiating activities of BP, BP-4.5-oxide, BP-7.8-oxide, BP-9,10-oxide, BP-11 12-oxide, the diastereomeric BP 7,8-diol-9,10-epoxides[a] and their tetraols

Initiator	No. of mice[b]	Papillomas per mouse[c] 15 weeks	Papillomas per mouse[c] 30 weeks	% of mice with tumors[d] 15 weeks	% of mice with tumors[d] 30 weeks
BP	30	2.00	5.30	60	92
BP-4,5-oxide	30	0.10	0.24	10	24
BP-7,8-oxide	29	0.73	2.52	47	89
BP-7β, 8α-diol-9α,10α-epoxide	29	0.30	1.50	27	69
BP-7β, 8α-diol-9β,10β-epoxide	28	-	0.07	-	7
BP-11,12-oxide	30	0.20	0.45	17	38
BP-9,10-oxide	29	0.04	0.15	4	15
BP-tetraol I[e]	26	0.07	0.25	7	25
BP-tetraol II[e]	29	0.28	0.52	17	41

[a] All the compounds were applied at a dose of 200 nmoles and followed one week later by twice weekly applications of 10 ug of TPA.

[b] Surviving at the 30th week after promotion.

[c] Total number of papillomas divided by the total number of surviving mice.

[d] Percent of surviving mice with papillomas.

[e] Applied at a 400 nmole dose.