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Marked Differences in the Skin Tumor-initiating Activities of the Optical Enantiomers of the Diastereomeric Benzo(*a*)pyrene 7,8-Diol-9,10-Epoxides[1]

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ABSTRACT

The abilities of the optically pure (+)- and (−)-enantiomers of the diastereomeric 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrenes derived from the enantiomeric *trans*-7,8-dihydrodiols to initiate skin tumors in mice were determined with a two-stage system of tumorigenesis. As a tumor initiator, (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene 2 was approximately 60% as active as was benzo(*a*)pyrene, whereas (−)-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene 2 was about 2% as active as was benzo(*a*)pyrene. The racemic mixture of the above diol-epoxide, in which the 9,10-epoxide is *trans* to the 7-hydroxyl group, was 25% as active as was benzo(*a*)pyrene as a tumor initiator. (−)-7β,8α-Dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene 1 and (+)-7α,8β-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene 1, in which the 9,10-epoxide is *cis* to the 7-hydroxyl group, were found to have little or no tumorigenic activity. The tumor-initiating ability of (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene 2 was found to be greater when given daily for 6 days at a dose of 34 nmol/day than when given once at a 200-nmol dose level. Similar fractionated doses of benzo(*a*)pyrene or (−)-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene 2 did not increase their skin tumor-initiating activity. The data suggest that (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene is an ultimate carcinogenic form of benzo(*a*)pyrene.

INTRODUCTION

Chemically unreactive carcinogens such as the polycyclic aromatic hydrocarbons are thought to exert their carcinogenic effects only after metabolism to chemically reactive species (ultimate carcinogens) that bind to critical cellular constituents (8, 13, 17). Studies of the metabolism-induced binding of BP[3] and its metabolites to DNA have provided evidence that a BP 7,8-diol-9,10-epoxide was responsible for most of these bound adducts (1, 5, 18). The 2 possible diastereomers from the *trans*-BP 7,8-dihydrodiol were characterized (28), and one of them [(±)-BP 7β,8α-diol-9α,10α-epoxide] was shown to be an ultimate carcinogen in newborn mice (9, 10).

The diastereomeric BP 7,8-diol-9,10-epoxides are known to be potent mutagens in bacterial and mammalian cells (6, 15, 24, 26). Recently, substantial differences in the mutagenic activities of the optically pure (+)- and (−)-enantiomers of the diastereomeric BP 7,8-diol-9,10-epoxides were reported (25). In Chinese hamster cells, (+)-BP 7β,8α-diol-9α,10α-epoxide 2 was the most mutagenic for the 4 isomers, inducing 6 to 18 times as many variant colonies as the other 3 compounds. In contrast, (−)-BP 7β,8α-diol-9β,10β-epoxide 1 was 1.3 to 9.5 times as mutagenic as were the other 3 optical isomers in strains TA 98 and TA 100 of *Salmonella typhimurium*. Marked differences exist in the carcinogenic activities of the optical enantiomers of the diastereomeric BP 7,8-diol-9,10-epoxides in newborn mice (2). At doses of 7 and 14 nmol of hydrocarbon per mouse, only (+)-BP 7β,8α-diol-9α,10α-epoxide 2 was highly active in causing lung adenomas in newborn mice, while BP and the 3 other isomers of the BP 7,8-diol-9,10-epoxides had little or no tumorigenic activity. Skin tumor-initiating activities of the 4 optically pure stereoisomers of the BP 7,8-diol-9,10-epoxides are compared in the present study. In addition, we determined the effects of removing the keratinized portion of the skin by pressure-sensitive tape and the effects of fractionated doses of the optical enantiomers of the diastereomeric BP 7,8-diol-9,10-epoxides on their skin tumor-initiating activities.

MATERIALS AND METHODS

Female CD-1 mice were purchased from Charles River Mouse Farms, North Wilmington, Mass., and female Sencar mice (skin tumor sensitive) were raised at the Oak Ridge National Laboratory. Mice, 7 to 9 weeks old, were shaved with surgical clippers 2 to 4 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

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[3] The abbreviations used are: BP, benzo(*a*)pyrene; BP 7,8-diol-9,10-epoxide, either or both of the diastereomeric 9,10-epoxides derived from BP 7,8-dihydrodiol in which the epoxide is *cis* (diol-epoxide 1) or *trans* (diol-epoxide 2) to the benzylic 7-hydroxyl group; BP 7,8-dihydrodiol, (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene; (+)-BP 7β,8α-diol-9α,10α-epoxide 2, (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; (−)-BP 7β,8α-diol-9β,10β-epoxide 1, (−)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; (−)-BP 7α,8β-diol-9β,10β-epoxide 2, (−)-BP 7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; (+)-7α,8β-diol-9α,10α-epoxide 1, (+)-7α,8β-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene.

mice were maintained under a 12-hr light-dark cycle at a constant temperature and humidity and received water and food *ad libitum*. 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, Minneapolis, Minn.

The optical enantiomers of the diastereomeric BP 7,8-diol-9,10-epoxides were synthesized from (+)- and (−)-BP 7,8-dihydrodiol as previously described (20, 27, 29), and all compounds were greater than 98% optically pure. The diol-epoxides were greater than 96% chemically pure as determined by chromatographic analysis (20) and were stored as solids at −90° prior to use. Diastereomer 1 of the diol-epoxides has the benzylic 7-hydroxyl group *cis* to the epoxide oxygen, while in diastereomer 2 these groups are *trans*. The BP 7,8-diol-9,10-epoxides were applied topically in 0.2 ml of tetrahydrofuran (distilled over $LiAlH_4$ and stored over sodium wire). All solutions were prepared in a darkened room immediately before use. Mice were treated with 100 to 200 nmol of the above compounds under subdued light. The time lapse between preparation of the above solutions and animal treatment was <0.5 hr. Stock solutions of TPA were prepared and kept in a freezer until they were used. Mice received twice-weekly applications of 10 μg of TPA starting 1 week after treatment with the initiators.

Preshaven mice in the resting phase of the hair cycle were treated 15 times with pressure-sensitive tape in order to remove the keratinized portion of the epidermis (19). Either 1 or 2 days following this treatment, the mice received (+)-BP 7β,8α-diol-9α,10α-epoxide 2 or (−)-BP 7α,8β-diol-9β,10β-epoxide 2.

RESULTS

The skin tumor-initiating activities of the 4 optically pure stereoisomers of the BP 7,8-diol-9,10-epoxides in female Charles River CD-1 mice are shown in Table 1. The results show that (+)-BP 7β,8α-diol-9α,10α-epoxide 2 is an effective tumor initiator which showed a dose-response relationship. (+)-BP 7β,8α-diol-9α,10α-epoxide 2 possessed approximately 60% of the tumor-initiating activity of BP, whereas (−)-BP 7β,8α-diol-9β,10β-epoxide 1, (−)-BP 7α,8β-diol-9β,10β-epoxide 2, and (+)-BP 7α,8β-diol-9α,10α-epoxide 1 had little or no tumorigenic activity. Table 2 shows the effect of pressure-sensitive tape treatment of the skin on the initiating activities of (+)-BP 7β,8α-diol-9α,10α-epoxide 2 and (−)-BP 7α,8β-diol-9β,10β-epoxide 2. It should be noted that treatment of the skin with tape either 1 or 2 days before initiation increased the initiating activity of (+)-BP 7β,8α-diol-9α,10α-epoxide 2. A very slight increase in initiating activity of (−)-BP 7α,8β-diol-9β,10β-epoxide 2 following pretreatment of the skin with tape was noted, but the tumor incidence was so low that the results are probably not significant.

Chart 1 shows the tumor-initiating activities of (+)-BP 7β,8α-diol-9α,10α-epoxide 2, (−)-BP 7α,8β-diol-9β,10β-epoxide 2, the racemic mixture of BP 7,8-diol-9,10-epoxide 2, and BP during the time course of tumor induction over the 24 weeks of promotion by TPA. (+)-BP 7β,8α-diol-9α,10α-epoxide 2 was about 0.6 and 2.4 times, respectively, as active as were BP or racemic BP 7,8-diol-9,10-epoxide 2 in terms of the number of papillomas observed per mouse. When compared to (+)-BP 7β,8α-diol-9α,10α-epoxide 2, (−)-BP 7α,8β-diol-9β,10β-epoxide 2 was extremely weak as a tumor initiator. BP induced tumors in all the animals at risk, whereas (+)-BP 7β,8α-diol-9α,10α-epoxide 2 and (−)-

Table 1

Skin tumor-initiating activities of BP and the 4 optically pure isomers of the BP 7,8-diol-9,10-epoxides in female Charles River CD-1 mice

All the compounds were applied at a dose of 100 and 200 nmol and were followed 1 week later by twice-weekly applications of 10 μg of TPA.

Initiator	Dose (nmol)	No. of mice[a]	Papillomas/mouse at 24 wk[b]	% of surviving mice with papillomas at 24 wk
None	0	29	0.06	6
BP	200	29	4.3	100
	100	30	2.1	68
(+)-BP 7β,8α-diol-9α,10α-epoxide 2	200	28	2.6	76
	100	30	1.1	47
	200[c]	30	0.0	0
(−)-BP 7β,8α-diol-9β,10β-epoxide 1	200	30	0.1	10
	100	30	0.1	10
(−)-BP 7α,8β-diol-9β,10β-epoxide 2	200	29	0.1	10
	100	30	0.06	6
(+)-BP 7α,8β-diol-9α,10α-epoxide 1	200	28	0.1	7
	100	29	0.17	17

[a] Surviving at the 24th week after promotion.
[b] Total number of papillomas divided by the total number of surviving mice.
[c] The initiating dose was not followed by application of TPA.

Table 2

Effects of removing the keratinized layer of the epidermis on the skin tumor-initiating activity of (+)-BP 7β,8α-diol-9α,10α-epoxide 2 and (−)-BP 7α,8β-diol-9β,10β-epoxide 2 in female Charles River CD-1 mice

The compounds were applied at a dose of 100 nmol and followed 1 week later by twice-weekly applications of 10 μg of TPA.

Initiator	Taping (days before)[a]	No. of mice[b]	Papillomas/mouse at 24 wk[c]	% of surviving mice with papillomas at 24 wk
(+)-BP 7β,8α-diol-9α,10α-epoxide 2	0	30	1.1	47
	−1	29	1.5	66
	−2	29	1.5	60
(−)-BP 7α,8β-diol-9β,10β-epoxide 2	0	30	0.06	6
	−1	29	0.20	18
	−2	30	0.15	15

[a] The mice received 15 applications of pressure-sensitive tape across the preshaven back either 1 or 2 days before application of the diol-epoxides.
[b] Surviving at the 24th week after promotion.
[c] Total number of papillomas divided by the total number of surviving mice.

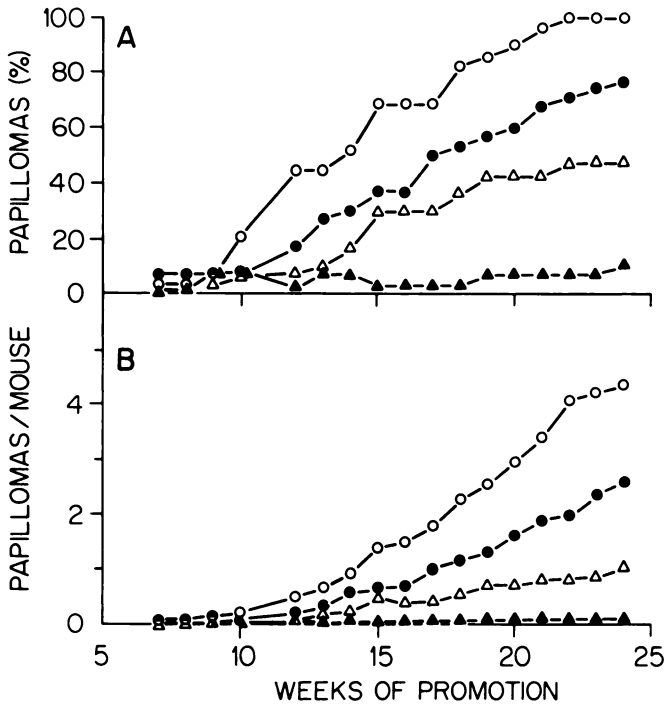

Chart 1. The skin tumor-initiating activities of BP, (+)-BP 7β,8α-diol-9α,10α-epoxide 2, (−)-BP 7α,8β-diol-9β,10β-epoxide 2, and the racemic mixture of BP 7,8-diol-9,10-epoxide 2 in female Charles River CD-1 mice. Each group consisted of 30 mice, and all mice were initiated with 200 nmol of one of the above compounds. One week after initiation, the mice were promoted twice weekly with 10 μg of TPA. *A*, percentage of mice with papillomas as a function of weeks of tumor promotion; *B*, average number of papillomas as a function of weeks of tumor promotion. ○, BP; ●, (+)-BP 7β,8α-diol-9α,10α-epoxide 2; ▲, (−)-BP 7α,8β-diol-9β,10β-epoxide 2; △, the racemic mixture of BP 7,8-diol-9,10-epoxide 2.

BP 7α,8β-diol-9β,10β-epoxide 2, respectively, induced tumors in 76 and 10% of the animals.

The skin tumor-initiating activities of the 4 optically pure stereoisomers of the BP 7,8-diol-9,10-epoxides in skin tumor-sensitive mice (Sencar) are shown in Table 3. With the exception of (+)-BP 7β,8α-diol-9α,10α-epoxide 2, which had about 70% of the skin tumor-initiating activity of BP, the other BP 7,8-diol-9,10-epoxides had little or no tumorigenic activity.

Because the BP 7,8-diol-9,10-epoxides are extremely reactive, the effects of fractionated doses of the hydrocarbons on skin tumor-initiating activities were also determined. As shown in Table 4, 6 daily topical applications of 34 nmol of (+)-BP 7β,8α-diol-9α,10α-epoxide 2 resulted in greater skin tumor-initiating activity than did a single application of 200 nmol. However, fractionated doses of either (−)-BP 7α,8β-diol-9β,10β-epoxide 2 or BP did not increase the activity of these compounds. In fact, the skin tumor-initiating activity of a fractionated dose of BP was less than when the total dose was given in one application.

DISCUSSION

The results of this investigation and those recently reported (2) indicate that (+)-BP 7β,8α-diol-9α,10α-epoxide 2 is the ultimate carcinogenic form of BP 7,8-diol-9,10-epoxide. In the present study, (+)-BP 7β,8α-diol-9α,10α-epoxide 2 possessed between 60 and 70% of the skin tumor-initiating activity of BP, whereas the 3 other possible stereoisomeric BP 7,8-diol-9,10-epoxides of BP were either very weak or essentially inactive. A recent paper (2) which reported that (+)-BP-7β,8α-diol-9α,10α-epoxide 2 was more than 30-fold as potent in inducing lung adenomas in newborn mice than was BP or the 3 other isomers of the BP 7,8-diol-9,10-epoxides established that (+)-BP 7β,8α-diol-9α,10α-epoxide 2 is an ultimate carcinogenic metabolite of BP in this model. Interestingly, (−)-BP 7β,8α-diol-9β,10β-epoxide 1 was found to be more mutagenic in strains TA 98 and TA 100 of *S. typhimurium* than were the 3 other optically pure isomers of the BP 7,8-diol-9,10-epoxides (25). However, in Chinese hamster V79 cells, (+)-BP 7β,8α-diol-9α,10α-epoxide 2 was the most mutagenic compound, inducing from 6 to 18 times as many variant colonies as did the 3 other optically pure isomers of the BP 7,8-diol-9,10-epoxides (25). These results, plus published data (6, 25), indicate that mutagenesis studies with mammalian V79 cells are more predictive of the carcinogenic activities of these diol-epoxides than are the mutagenesis studies with bacteria.

When the enantiomeric BP 7,8-diol-9,10-epoxide 2 was

Table 3

Skin tumor-initiating activities of BP and the 4 optically pure stereoisomers of the BP 7,8-diol-9,10-epoxides in female Sencar mice

All the compounds were applied at a dose of 100 nmol and were followed 1 week later by twice-weekly applications of 5 μg of TPA.

Initiator	No. of mice[a]	Papillomas/ mouse at 24 wk[b]	% of surviving mice with papillomas at 24 wk
None	29	0.1	10
BP	28	2.8	86
(+)-BP 7β,8α-diol-9α,10α-epoxide 2[c]	30	2.0	75
(−)-BP 7β,8α-diol-9β,10β-epoxide 1	29	0.13	13
(−)-BP 7α,8β-diol-9β,10β-epoxide 2	30	0.03	3
(+)-BP 7α,8β-diol-9α,10α-epoxide 1	29	0.10	10

[a] Surviving at the 24th week after promotion.
[b] Total number of papillomas divided by the total number of surviving mice.
[c] No tumors were observed without promotion by TPA.

Table 4

Comparison of a single or fractionated dose of BP, (+)-BP 7β,8α-diol-9α,10α-epoxide 2, and (−)-BP 7α,8β-diol-9β,10β-epoxide 2 on their skin tumor-initiating activities in female Charles River CD-1 mice

All the compounds were applied at either a single topical dose of 200 nmol or 6 daily applications of 34 nmol and were followed 1 week later by twice-weekly applications of 10 μg of TPA.

Initiator[a]	Dose (nmol)	No. of mice[b]	Papillomas/ mouse at 24 wk[c]	% of surviving mice with papillomas at 24 wk
BP	1 × 200	29	4.3	100
	6 × 34	30	3.1	78
(+)-BP 7β,8α-diol-9α,10α-epoxide 2	1 × 200	28	3.2	76
	6 × 34	29	4.2	95
(−)-BP 7α,8β-diol-9β,10β-epoxide 2	1 × 200	29	0.1	10
	6 × 34	28	0.13	13

[a] The initiator was applied once at a dose of 200 nmol or 6 times at a dose of 34 nmol.
[b] Surviving at the 24th week after promotion.
[c] Total number of papillomas divided by the total number of surviving mice.

given in fractionated doses, the skin tumor-initiating activity of (+)-BP 7β,8α-diol-9α,10α-epoxide 2 increased and was comparable to that of BP. The initiating activity of (−)-BP 7α,8β-diol-9β,10β-epoxide 2 did not increase when this compound was administered in fractionated doses. Notably, a fractionated dose of BP was less active in inducing skin tumors than was a single dose of the same total amount of hydrocarbon. One possible explanation for this difference between the tumor-initiating activities resulting from fractionated doses of BP and (+)-BP 7β,8α-diol-9α,10α-epoxide 2 is that BP must be metabolized to become carcinogenic. Colburn and Boutwell (3) previously reported that the skin tumor-initiating activity of β-propiolactone, a direct-acting carcinogen, was greater using fractionated doses than with a single application of the same total dose. However, the single application of β-propiolactone was highly cytotoxic to the skin, whereas the fractionated dose was much less toxic. No cytotoxic effect was observed in our study with either one application or a fractionated dose of 200 nmol of (+)-BP 7β,8α-diol-9α,10α-epoxide 2. The decreased activity of BP when given in fractionated doses may be related to the fact that BP stimulates its own metabolism (4). Thus, the first of the fractionated doses causes an enhanced rate of metabolism of subsequent doses. Inducers of polycyclic hydrocarbon metabolism have been shown to decrease the carcinogenicity of these compounds (*cf.* Ref. 22).

There is a high degree of stereospecificity in the metabolism of BP to BP 7,8-dihydrodiol and the diastereomeric BP 7,8-diol-9,10-epoxides. Liver microsomal enzymes from 3-methylcholanthrene-treated rats metabolize BP to (−)-BP 7,8-dihydrodiol in a 24-fold excess relative to (+)-BP 7,8-dihydrodiol (20, 30). The same enzyme preparation metabolizes the (−)-BP 7,8-dihydrodiol to (+)-BP 7β,8α-diol-9α,10α-epoxide 2 and (−)-BP 7β,8α-diol-9β,10β-epoxide 1 in a 6:1 ratio, while (+)-BP 7,8-dihydrodiol is metabolized to (+)-BP 7α,8β-diol-9α,10α-epoxide 1 and (−)-BP 7α,8β-diol-9β,10β-epoxide 2 in a ratio of 22:1 (20, 21). (−)-BP 7,8-dihydrodiol is 5- to 10-fold more potent as a tumor initiator on mouse skin than is (+)-BP 7,8-dihydrodiol (12). Adducts of both (+)-BP 7β,8α-diol-9α,10α-epoxide 2 and (+)-BP 7α,8β-diol-9α,10α-epoxide 1 have been found bound to the RNA of mouse skin after topical application of BP (11, 14). In addition, (+)-BP 7β,8α-diol-9α,10α-epoxide 2 has been found bound to the RNA and DNA of bronchial explants (7, 16, 23). These results indicate that the major metabolic pathway of BP leading to the production of an ultimate carcinogen involves the formation of (−)-BP 7,8-dihydrodiol which is then converted predominantly to (+)-BP 7β,8α-diol-9α,10α-epoxide 2.

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