Evidence That Benzo(a)anthracene 3,4-Diol-1,2-Epoxide Is an Ultimate Carcinogen on Mouse Skin


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ABSTRACT

Benzo(a)anthracene (BA) and several benzo-ring derivatives of BA were tested for their tumor-initiating activity on mouse skin. A single topical application of 0.1 to 2.0 μmol of hydrocarbon was followed 7 days later by twice-weekly applications of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate for 20 weeks. Comparisons of the percentage of mice with papillomas and the number of papillomas observed per mouse indicated that both diastereomeric 3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydrobenzo(a)anthracenes, in which the epoxide oxygen is either cis (diol-epoxide 1) or trans (diol-epoxide 2) to the benzylic 4-hydroxyl group, were 10- to 40-fold more tumorigenic (papillomas/mouse) than was the parent hydrocarbon, benzo(a)anthracene. Diol-epoxide 2 was 2- to 3-fold more tumorigenic than was diol-epoxide 1 after 20 weeks of promotion. The immediate metabolic precursor of the 3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydrobenzo(a)anthracenes, trans-3,4-dihydroxy-3,4-dihydrobenzo(a)anthracene, had intermediate tumorigenic activity compared to diol-epoxide 1 and diol-epoxide 2 and was approximately 20-fold more tumorigenic (papillomas/mouse) than was BA. Resolution of racemic trans-3,4-dihydroxy-3,4-dihydrobenzo(a)anthracene into the (−) and (+)-enantiomers and assignment of their absolute stereochemistry revealed that the (−)-(3R,4R) isomer was at least 5-fold more active as a tumor initiator than was the (+)-(3S,4S) isomer. 1,2-Dihydrobenzo(a)anthracene and 3,4-dihydrobenzo(a)anthracene were also tested for tumorigenic activity. 3,4-Dihydrobenzo(a)anthracene was the most potent tumorigenic compound used in this study. 1,2-Dihydrobenzo(a)anthracene was a very weak tumor initiator, with activity comparable to BA. These results support the concept that a diol-epoxide in which the epoxide on a saturated, angular benzo ring forms part of a “bay region” of the hydrocarbon is a prime candidate for an ultimate carcinogenic metabolite of the hydrocarbon. In the case of BA, this metabolite is either or both of the diastereomeric 3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydrobenzo(a)-anthracenes.

INTRODUCTION

The pioneering efforts of Miller and Miller (17, 18) have brought wide acceptance of the concept that highly reactive metabolic intermediates, designated ultimate carcinogens, are responsible for the carcinogenicity of many cancer-causing chemicals, including the polycyclic aromatic hydrocarbons. The polycyclic aromatic hydrocarbons are a particularly intriguing class of carcinogens that, due to their structural similarities, represent a group of compounds in which chemical and, presumably, biological activities should vary in a predictable fashion. For the alternant polycyclic aromatic hydrocarbons, a variety of parameters have been calculated by quantum mechanical methods in the anticipation that one of these parameters would correlate with carcinogenic activity (2, 20). These theoretical calculations, such as the “K-region theory” of Pullman and Pullman (20), have had value in their ability to predict carcinogenicity in terms of chemical reactivity at different positions of the molecule, even though exceptions to this theory have been found. More recently, on the basis of the compelling evidence that a BP 7,8-diol-9,10-epoxide is an ultimate carcinogenic metabolite of BP (5, 9-11, 14, 16, 19, 22, 27, 30, 32, 35), Jerina et al. (6-8) have proposed a unifying concept, termed the “bay-region” theory, that attempts to explain and predict the carcinogenicity of polycyclic aromatic hydrocarbons. This theory contends that an epoxide on a saturated, angular benzo ring that forms part of a bay region of the hydrocarbon is a prime candidate as an ultimate carcinogenic metabolite of the hydrocarbon (6-8). Confirmation of this hypothesis depends on the demonstration that the appropriate bay-region diol-epoxides of a number of carcinogenic polycyclic hydrocarbons possess unique biological activity. Toward this end, we have recently shown that the bay-region 3,4-diol-1,2-epoxides of BA are at least 15 to 125 times more mutagenic to Salmonella typhimurium strain TA100 and Chinese hamster V79 cells than are the diastereomeric BA 1,2-diol-3,4-epoxides (31). These results, together with the metabolic activation of BA 3,4-dihydrodiol to a potent bacterial mutagen(s) (34) and the high tumorigenicity of BA 3,4-dihydrodiol relative to BA or the 4 other metabolically possible dihydrodiols of BA (29, 33), have implicated a BA

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3,4-diol-1,2-epoxide as an ultimate carcinogenic metabolite of BA. In this report we describe the results obtained from the testing of several benzo-ring derivatives of BA as tumor initiators on mouse skin. In addition to the diastereomeric BA 3,4-diol-1,2-epoxides, we have examined the tumorigenicity of 1,2-H₂BA, 3,4-H₂BA, and the resolved (+)- and (-)-enantiomers of BA 3,4-dihydrodiol. The structures of BA and some of its benzo-ring derivatives are shown in Chart 1.

MATERIALS AND METHODS

Chemicals. BA was purchased from Sigma Chemical Co., St. Louis, Mo. Analytically pure samples of racemic BA 3,4-dihydrodiol, H₂BA 3,4-diol, 1,2-H₂BA, 3,4-H₂BA, and the diastereomeric BA 3,4-diol-1,2-epoxides were obtained by unequivocal chemical synthesis as described (12, 13). (-)-Methoxytrifluoromethylphenylacetic acid (3) and (-)-menthoxycetic acid (21) (Aldrich Chemical Co., Milwaukee, Wis.) were converted into their acid chlorides, as described in the references cited. 12-O-Tetradecanoylphorbol-13-acetate was purchased from the Department of Laboratory Medicine and Pathology at the University of Minnesota.

Resolution of (±)-BA 3,4-Dihydrodiol. Since the mono- and bis(-)-MTPA esters of BA 3,4-dihydrodiol had low stability, a mixture of (±)-BA 3,4-dihydrodiol (247 mg) and an equal molar amount of (-)-menthoxycetic chloride were allowed to react in dry pyridine at 4° for 12 hr. After removal of the pyridine, 65 mg of unreacted dihydrodiol were separated from the mixture of mono and bis esters by passage over a silica gel column eluted with 50% tetrahydrofuran in hexane. Analytical HPLC (Chart 2) indicated that about one-half of the starting dihydrodiol had been converted into the pair of diastereomeric esters that were not as readily separated as were the diastereomeric pairs of monoesters at positions 3 and 4 of the dihydrodiol. Preparative separation of the sample (38 injections) was achieved with a Whatman Partisil-10 column (50 cm x 9.2 mm) eluted with 1% isopropyl alcohol and 3% tetrahydrofuran in hexane at a flow rate of 20 ml/min. The bis esters (260 mg; cf. Chart 2, Peak a) were isolated without any attempt to separate this pair of diastereomers. After chro- matography of Component b (cf. Chart 2), Components b to e were obtained in >98% purity. Proton magnetic resonance established that Components b and c were the pair of diastereomeric esters at position 3 while Components d and e were the pair at position 4. The 4 components

![Chart 2. HPLC separation of the 3- and 4-monoesters (Peaks b and c and Peaks d and e, respectively) and the 3,4-bis(-)-menthoxycetic ester (Peak a) of (+)- and (-)-BA 3,4-dihydrodiol. A Du Pont Zorbax SIL column (25 cm x 6.2 mm) eluted with 1% isopropyl alcohol and 3% tetrahydrofuran in hexane at a flow rate of 12 ml/min was used to achieve the separation.](image-url)
were hydrolyzed separately with methanol:1 N NaOH: tetrahydrofuran for 2 hr at 4°C under argon. The (+)-enantio-
momer of BA 3,4-dihydrodiol was formed from Components c and e ([α]D +225°, tetrahydrofuran: λmax,3,4-DH = 69,000 based
on εmax,3,4-DH = 104,450 1 M−1 in methanol). In this
manner, 20 mg of each enantiomer were obtained with
optical purity > 98%.

Absolute Stereochemistry of (+)- and (−)-BA 3,4-Dihydro-
diol. Assignment of absolute stereochemistry to the (+)- and (−)-enantiomers of the BA 3,4-dihydrodiols was
achieved through an exciton chirality experiment (4) on the
bis-p-N,N-dimethylaminobenzoate of an enantiomer of H4-
BA 3,4-diol. Racemic H4-BA 3,4-diol was quantitatively con-
verted (1 day at 4°C in pyridine) into its diastereomeric bis
esters with (−)-methoxytrifluoromethylenephenoxyacetic
acid chloride [(+)-H4-BA 3,4-diol bis-(−)-MTPA ester and (−)-H4-
BA 3,4-diol bis-(−)-MTPA ester]. Although the pair of dia-
stereomers had very similar retention times, they were
separated by analytical HPLC on a Du Pont Zorbax SIL
column (25 cm x 6.4 mm) eluted with 30% hexane in
methylene chloride (k' = 5.0 and k" = 5.8). A sample of 0.8
g of the mixture of diastereomers was injected into a Waters
PrepLC/System 500 equipped with 2 cartridges. The sample
was eluted with 25% hexane in methylene chloride. After 9
recycles and selective shaving of the early and late portions
of the sample peak, 105 mg of the less polar component
and 112 mg of the more polar component were isolated (85
and 70% diastereomeric purity, respectively). Proton mag-
netic resonance spectra (100 MHz, CDCl3) distinguished
between the 2 diastereomers since the doublets (J,4 = 5.0
Hz) for H4 had different chemical shifts. The less polar
isomer with k' = 5.0 had H4 at 6.488, while the more polar
isomer with k" = 5.8 had H4 at 6.408. The less polar
diastereomer, after crystallization from cyclopentane:
methanol, was greater than 98% diastereomerically pure.
HPLC on a Du Pont Zorbax ODS column (25 cm x 6.4 mm)
eluted with 0.5% methanol in water to 100% methanol at a rate of change of 1%/min after a 1-min delay (retention time, 35.0 min for this
diastereomer and 34.3 min for the other diastereomer).

Although the diastereomers were completely separated on
this column, the loading capacity was too low for prepara-
tive separations. The less polar diastereomer was hydrolyzed
cf. Ref. 36) to (−)-H4-BA 3,4-diol ([α]D −55.7°, tetra-
hydrofuran), which was converted to its bis-4-N,N-dimethy-
aminobenzoate ester [retention time, 44 min on a Du Pont
ODS column (25 cm x 6.4 mm) eluted with 1.2 ml/min
with a gradient of 70% methanol in water to 100% methanol at a rate of change of 1%/min after a 1-min delay] by reaction with an
excess of 4-N,N-dimethylaminobenzoyl chloride (3) at 80°C
in pyridine for 3 days with a trace of 4-N,N-dimethylamino-
pyridine as catalyst. The absolute stereochemistry of this
derivative was assigned from its circular dichroism spec-
trum as described in "Results."

In order to interrelate absolute stereochemistry between the
BA 3,4-dihydrodiol enantiomers and those of the H4-BA
3,4-diol, (+)-BA 3,4-dihydrodiol was reduced at the 1,2-
position with 1 atmosphere of hydrogen (in tetrahydrofuran
with 5% of PtO2 as catalyst, 15 min). The product was (−)-
H4-BA 3,4-diol by chromatography of its bis-(−)-MTPA ester
on the ODS column. Thus, (+)-BA 3,4-dihydrodiol, (−)-H4-
BA 3,4-diol, and its bis-(−)-MTPA ester (which has the
shorter retention time on the Du Pont SIL column and the
longer retention time on the Du Pont ODS column) all have
the same absolute stereochemistry.

Animals. Female CD-1 mice (7 to 9 weeks old) were
obtained from Charles River Breeding Laboratory, North
Wilmington, Mass. The mice were anesthetized with ether
and shaved on the dorsal surface with electric clippers 3
days before treatment. Each treatment group consisted of
30 mice, except for the group of animals receiving the high
dose (2.0 μmol) of 1,2-H4-BA, which contained 20 mice. The
BA derivatives in 200 μl of acetone: NH4OH (1000:1) were
applied once to the dorsal surface of the mice. Control

Chart 3. Skin tumor-initiating activity of BA and benzo-ring derivatives of BA. Each group consisted of 30 mice, and all mice received a single topical
application of the compounds (0.4 μmol) in 200 μl of acetone:NH4OH (1000:1). Twice-weekly applications of the tumor promoter 12-O-tetradeca-
nyloxyphorbol-13-acetate (16 nmol/application) commenced 7 days after
the hydrocarbon treatment. At least 28 of the original 30 mice in each group
were alive after 20 weeks of promotion. A, percentage of mice with tumors;
B, tumors observed per mouse during 20 weeks of promotion.
mice were treated with the solvent. All mice received twice-weekly applications of TPA (16 nmol/200 μl acetone), beginning 7 days after treatment with the BA derivative or acetone:NH₂OH. The development of skin papillomas was recorded once every 2 to 3 weeks, and papillomas greater than 2 mm in diameter were included in the cumulative total if they persisted for 2 weeks or longer. Gross examination of the mice at 25 weeks of promotion indicated that none of the papillomas had developed into a squamous cell carcinoma.

RESULTS

The development of skin papillomas in CD-1 mice after a single topical application of 0.4 μmol BA or several benzo-ring derivatives of BA is shown in Chart 3. The most potent tumor initiator was 3,4-H BA, which was followed in activity by diol-epoxide 2 of BA 3,4-dihydrodiol, BA 3,4-dihydrodiol, and diol-epoxide 1 of BA 3,4-dihydrodiol. At 20 weeks of promotion with 12-O-tetradecanoylphorbol-13-acetate, 86% of the animals treated with 3,4-H BA had developed papillomas, and an average of 4.5 papillomas/mouse was observed with this hydrocarbon. Diol-epoxide 2, in which the 1,2-epoxide is trans to the benzylic 4-hydroxyl group, had approximately 80 and 35%, respectively, of the activity of 3,4-H BA when the data were expressed as percentage of mice with papillomas and papillomas observed per mouse. Diol-epoxide 1 had approximately 40% of the tumor-initiating activity of diol-epoxide 2. BA 3,4-dihydrodiol was more tumorigenic than diol-epoxide 1 but was less active than diol-epoxide 2. At a low dose of hydrocarbon (0.4 μmol), BA and 1,2-H BA did not produce a significant number of papillomas compared to that of control animals (2 of 30 animals; 0.07 papilloma/mouse). However, when the dose of BA was increased to 2.0 μmol, 21% of the treated animals developed papillomas (Table 1). At the higher dose of hydrocarbon, 1,2-H BA had tumorigenic activity comparable to that of BA, but both compounds still had less than one-fifth of the activity of 3,4-H BA, BA 3,4-dihydrodiol, or diol-epoxide 2.

For determination of the role of absolute stereochemistry on the tumor-initiating activity of the BA 3,4-dihydrodiols, the (+)- and (-)-enantiomers were resolved as their monooesters with (-)-menthoxacyl chloride as described in "Materials and Methods." Although absolute stereochemistry of these dihydrodiols might be assigned based on an isolated excitation chirality interaction between 2 N,N-di-methylaminobenzoil chromophores in such a diester of BA 3,4-dihydrodiol based on the "dibenzole chirality rule" (4), the bis-4-N,N-dimethylaminobenzoate esters of BA 3,4-dihydrodiol were found to be difficult to prepare and unstable. In contrast, the bis-4-N,N-dimethylaminobenzoates of (+)- and (-)-H BA 3,4-diol were much more stable and easily prepared. The bis ester of the H BA 3,4-diol with stereochemistry related to (+)-BA 3,4-dihydrodiol was found to have a circular dichroism spectrum that readily allowed assignment of its absolute stereochemistry. A pair of symmetrical Cotton effects (positive at 326 nm with Δε 45.1 and negative at 302 nm with Δε 47.8 based on an ε of 206,000 1 M⁻¹ cm⁻¹ for H BA 3,4-diol at 255 nm) that pass through 0 at 315 nm (cf. Chart 4) had about 5 times the activity of the (+)-BA 3,4-dihydrodiol and (-)-H BA 3,4-diol. A comparison of the tumor-initiating activities of the optical enantiomers of BA 3,4-dihydrodiol revealed that the (-)-(3R,4R)-enantiomer (cf. Chart 4) had about 5 times the activity of the (+)-(3S,4S)-enantiomer at the 0.4-μmol dose (Table 1). When the dose of the enantiomeric BA 3,4-dihydrodiols was decreased to 0.1 μmol, the (-)-enantiomer still produced a high tumor incidence while the (+)-enantiomer had no tumorigenic activity compared to control animals. This would seem to indicate that the difference in tumorigenic activity of the optical enantiomers of BA 3,4-dihydrodiol is even greater than 5-fold. Racemic BA 3,4-dihydrodiol had tumorigenic activity that was between the (+)- and (-)-enantiomers, indicating that there was no significant synergism or inhibition of activity with the mixture of optical enantiomers.

DISCUSSION

At a low dose of BA (0.4 μmol) that was not significantly tumorigenic, the diastereomeric bay region diol-epoxides of BA produced tumors in 43 (Isomer 1) to 70% (Isomer 2) of the animals. When the dose of BA was increased 5-fold to 2.0 μmol (Table 1), the tumorigenic activity of this compound was still less than that observed with the low dose

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Table 1

<table>
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<th>Initiator</th>
<th>Dose (μmol)</th>
<th>% of mice with tumors</th>
<th>Tumors/mouse</th>
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<td>7</td>
<td>0.07</td>
</tr>
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<td>7</td>
<td>0.07</td>
</tr>
<tr>
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<td>7</td>
<td>0.07</td>
</tr>
<tr>
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<td>35</td>
<td>0.50</td>
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<td>86</td>
<td>4.50</td>
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<tr>
<td>(±)-BA</td>
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<td>45</td>
<td>1.30</td>
</tr>
<tr>
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<td>3</td>
<td>0.07</td>
</tr>
<tr>
<td>(-)-BA</td>
<td>0.1</td>
<td>43</td>
<td>1.00</td>
</tr>
</tbody>
</table>

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Chart 4. Absolute stereochemistry of the more tumorigenic (-)-(3R,4R)-BA 3,4-dihydrodiol and (-)-(7R,8R)-BP 7,8-dihydrodiol.
The metabolism of the (-)-BP 7,8-dihydrodiol to predominantly found to be more tumorigenic than was the (+)-enantiomer. Isomer 2 of the enantiomer has been attributed to the stereoselective metabolism of the (-)-BP 7,8-dihydrodiol as tumor initiators on mouse skin. These results provide evidence that a BA 3,4-diol-1,2-epoxide, in which the epoxide oxygen is in the bay region of the hydrocarbon, is an ultimate carcinogenic metabolite of BA.

The very high tumorigenic activity of 3,4-H₂-BA in this study further supports the bay-region hypothesis. The presumed active tumorigenic metabolite of this hydrocarbon (which is not a metabolite of BA) is 1,2-epoxy-1,2,3,4-tetrahydro-BA, which has an epoxide group in the bay region and is an even more potent mutagen than the diastereomeric BA 3,4-diol-1,2-epoxides (31). The low tumorigenic activity of 1,2-H₂-BA relative to 3,4-H₂-BA is consistent with the high biological activity of tetrahydroepoxides, in which the epoxide oxygen forms part of the bay region of the molecule (31, 35). With both BP and BA, we have shown that a principal role of the diol group in diol-epoxides is to provide a metabolic pathway to the aliphatic epoxide (31, 35). The diol-epoxides are simple aliphatic epoxides that, as opposed to arene oxides, are significantly more susceptible to attack by nucleophiles relative to their rates of isomerization to ketones (1). This was confirmed by the observation that the BP 7,8-diol-9,10-epoxides hydrolyze to mixtures of tetroalts (5, 26, 39), with only trace amounts of the 7,8-dihydroxy-9-keto isomerization product produced in the acidic to basic pH range (28, 39). However, the 9,10-arene oxide of BP, although highly reactive in aqueous medium, yields only phenolic isomerization products upon hydrolysis (38).

The high tumorigenic activity of the bay-region diol-epoxides of BA on mouse skin relative to the parent hydrocarbon was somewhat surprising, based on the results obtained with the bay-region diol-epoxides of BP. Although there is overwhelming evidence to support the concept that a BP 7,8-diol-9,10-epoxide is an ultimate carcinogenic metabolite of BP, the diastereomeric diol-epoxides of BA are far less carcinogenic on mouse skin than is the parent hydrocarbon (15, 23, 24). However, the BP 7,8-diol-9,10-epoxides are considerably more mutagenic and are more reactive towards nucleophiles (12, 31, 37, 39) than are the BA 3,4-diol-1,2-epoxides. These results lend support to the proposal (15) that the low tumorigenic activity of the bay-region diol-epoxides of BP on mouse skin is due to their high reactivity and low stability, which may prevent their penetration within the cell to critical receptors that are important for tumor initiation.

In analogy to the results obtained with the optical enantiomers of BP 7,8-dihydrodiol as tumor initiators on mouse skin (14), the (-)-enantiomer of BA 3,4-dihydrodiol was found to be more tumorigenic than was the (+)-enantiomer. In the case of BP 7,8-dihydrodiol, the more potent tumorigenic activity of the (-)-enantiomer relative to the (+)-enantiomer has been attributed to the stereoselective metabolism of the (-)-BP 7,8-dihydrodiol to predominantly (+)-diol-epoxide 2, while the (+)-enantiomer is stereoselectively metabolized to (+)-diol-epoxide 1 (25, 36). Isomer 2 of BP 7,8-diol-9,10-epoxide is a more potent carcinogen than isomer 1 in the newborn mouse (9, 10), and (+)-diol-epoxide 2 is 6 to 18 times more potent as a mutagen in Chinese hamster V79 cells when compared to (-)-diol-epoxide 2, (-)-diol-epoxide 1, and (+)-diol-epoxide 1 of BP (32). In accord with these observations, diol-epoxide 2 is bound to a greater extent to DNA, RNA, and protein of mouse skin than is diol-epoxide 1 after topical application of BP (11). The metabolism of BA to BA 3,4-dihydrodiol and the subsequent metabolism of this dihydrodiol to the BA 3,4-diol-1,2-epoxides has not yet been investigated for possible stereospecificity of these enzymatic reactions. It is clear, however, that the (-)-(3R,4R) enantiomer of BA 3,4-dihydrodiol is a more potent tumor initiator than is the (+)-(3S,4S)-enantiomer on mouse skin. Interestingly, the more tumorigenic BA and BP dihydrodiols both have the same absolute stereochemistry at the benzylic and nonbenzylic hydroxyl groups [(R,R)], as shown in Chart 4.

In summary, the results presented here provide direct evidence for our previous proposal (29, 31, 33, 34) that a BA 3,4-diol-1,2-epoxide is an ultimate carcinogenic metabolite derived from BA. Interestingly, both diastereomeric BA 3,4-diol-1,2-epoxides are more potent tumor initiators than is BA on mouse skin. Further studies will be required to determine the relative proportion of diol-epoxide 1 to diol-epoxide 2 formed during the metabolism of BA.

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