CARCINOGENICITY OF BENZO[a]PYRENE DERIVATIVES: THE BAY REGION THEORY

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INTRODUCTION

The polycyclic aromatic hydrocarbon benzo[a]pyrene (BP) is one of the most prevalent environmental carcinogens to which man is exposed. As little as 0.10 µmol to 0.15 µmol of BP applied once every two weeks to the backs of C57BL/6J mice produces a 100% incidence of Squamous cell carcinoma on the skin of the animals while one 0.4 µmol dose followed twice weekly by a promoting agent for 20–30 weeks causes a 90% tumor incidence in the animals, and the tumor yield is 4–8 papillomas per mouse. In the United States alone, over 1300 tons of this carcinogen are released into the environment each year as an unfortunate by-product of numerous combustion processes (Ref. 1). Since BP is relatively inert, it is thought to exert its carcinogenic activity through a metabolically formed reactive intermediate(s) which covalently interacts with cellular macromolecules such as DNA, RNA, and protein (Ref. 2). BP, like other carcinogenic and noncarcinogenic aromatic hydrocarbons (Ref. 3 & 4), is first converted to arene oxides at several positions by the cytochrome P-450 mixed function oxidases in the endoplasmic reticulum of cells (cf. Ref. 5). Once formed, these arene oxides are subject to spontaneous isomerization to phenols (Ref. 6), chemical or enzymatic conjugation with the thiol group in glutathione (Ref. 7), enzymatic hydration to trans-dihydrodiols by the action of epoxide hydrase (Ref. 8 & 9), and covalent interaction with nucleophiles within the cell (Fig. 1). Formation of arene oxides at the 4,5-, 7,8-, and 9,10-positions are known to represent the substantial precentages of the total metabolism of BP, and these arene oxides can isomerize spontaneously to phenols. The very unstable 1,2- and 2,3-arene oxides probably account for the 1- and 3-hydroxy-BP (1- and 3-HOBP) known to be metabolites. In addition, 6-HOBP is formed and autoxidized to quinones involving the 6-position (Ref. 10 & 11). Since the reactive metabolite(s) responsible for the biological activity of BP may represent only a very small percentage of the total metabolites and since the reactive species may not be directly detectable due to its reactivity, metabolism studies may be of limited value in attempting to identify the suspect molecule. For this reason, our laboratories undertook a systematic evaluation of all the known and potential metabolites of BP which could be readily synthesized (Ref. 12) in an attempt to identify the metabolic pathway(s) responsible for the carcinogenic activity. Although other approaches to the identification of ultimate carcinogens from BP, such as mutagenicity, cell transformation, and covalent binding to nucleic acid, have been undertaken by other laboratories as well as our own (cf. Ref. 13), only carcinogenicity data will be considered here.
CARCINOGENICITY OF BP DERIVATIVES

Carcinogenicity of BP and BP 4,5-, 7,8-, 9,10-, and 11,12-oxides has been tested by application of each compound once every two weeks to the backs of C57BL/6J mice for 60 weeks (Ref. 14 & 15). Doses of 0.1 μmol and 0.4 μmol were selected (Fig. 2) in order to be able to detect carcinogens which are significantly less active than BP, since the dose of 0.1 μmol causes tumors in nearly all of the animals treated with BP. Both BP 9,10- and 11,12-oxides were inactive at the doses tested, and BP, 4,5-oxide produced only one skin tumor among the 30 mice tested. In contrast, BP 7,8-oxide was a potent carcinogen at the high dose, although clearly less active than BP when tested at the lower dose. Histological examination established that almost all of the tumors produced by BP and BP 7,8-oxide were squamous cell carcinomas. Thus, both BP and BP 7,8-oxide are complete carcinogens on mouse skin. Preliminary data (unpublished) are now available on the carcinogenic activity of these four arene oxides upon intraperitoneal injection into newborn mice. As in the skin model, BP 7,8-oxide is much more tumorigenic (pulmonary adenomas) than the 4,5-, 9,10-, and 11,12-oxides of BP. These arene oxides have also been tested for their skin tumor initiating abilities. In these experiments, a single dose of 0.2 μmol of each compound was applied once to the backs of CD-1 mice followed by twice weekly applications of 10 μg of the promoting agent 12-O-tetradecanoylphorbol-13-acetate (16,17). The data parallel the chronic studies on skin and the experiments in the newborn in that BP 7,8-oxide had appreciable but lower tumor-initiating activity than BP while the other three arene oxides were much weaker in activity.
Carcinogenicity of phenols
Several experiments have been done to determine whether BP 7,8-oxide acts directly as a carcinogen. Since non-K-region arene oxides readily isomerize to phenols (6), the twelve possible isomeric phenols of BP were tested as complete carcinogens by chronic application to mouse skin at the 0.4 µmol dose (Fig. 3, Ref. 15,18) with the result that 2-HOBP was equipotent to BP, 11-HOBP was weakly active and the remainder of the phenols were inactive. Similar results were obtained from initiation-promotion experiments (19) where 11-HOBP was moderately active, BP and 2-HOBP were strong tumor initiators, and the remaining phenols had less than 5% of the activity of BP. Clearly, the activity of BP 7,8-oxide cannot be due to its phenolic isomerization products. Although 6-HOBP has been considered as a possible ultimate carcinogen because of its facile conversion to 6-oxy-BP (Ref. 20 & 21), this possibility seems unlikely in light of the present data and its lack of activity in the newborn mouse (unpublished). The surprising high activity of 2-HOBP, which is even more active than BP in the newborn mouse (unpublished), is under further study.
Carcinogenicity of dihydrodiols

Since BP 7,8-oxide is the metabolic precursor of BP 7,8-dihydrodiol (Fig. 1), the dihydrodiol was tested as a complete carcinogen by chronic application to mouse skin (Fig. 4, Ref. 22). At a dose of 0.15 μmol once every two weeks, both BP and BP 7,8-dihydrodiol caused a 100% incidence of tumors in the animals. At the same dose, less than 20% of the animals developed tumors when treated with BP 7,8-oxide. BP 9,10-dihydrodiol is practically inactive as a carcinogen under similar conditions (unpublished). Decreased activity of the labile arene oxide relative to the dihydrodiol may be due in part to isomerization of the arene oxide to inactive phenols. Since BP 7,8-dihydrodiol is too stable chemically to be considered as a reactive metabolite, both BP 7,8-oxide and BP 7,8-dihydrodiol must be considered as proximate carcinogens. Complete inactivity of 7,8,9,10-tetrahydro BP 7,8-epoxide and 7,8,9,10-tetrahydro BP 7,8-diol as carcinogens (Fig. 4, Ref. 22,23) indicates that the 9,10-double bond is important in their sequential metabolic transformation into ultimate carcinogens. If BP 7,8-dihydrodiol is a proximate carcinogen from BP, it should be significantly more active than BP as a carcinogen. When BP and the 7,8-dihydrodiol were compared for tumorigenicity in the dose range of 0.025-0.10 μmol, the dihydrodiol was indeed more active but only to a small extent (Ref. 23). Attempts to demonstrate higher activity for the dihydrodiol by initiation-promotion experiments have indeed shown BP 7,8-dihydrodiol to be a potent initiator although the dihydrodiol was found to be either equal to (Ref. 16,17) or slightly less active (Ref. 24) than BP. Since all of the dihydrodiols produced from BP by liver microsomes are highly optically active (25), the optical enantiomers of BP 7,8-dihydrodiol were examined in an initiation-promotion experiment (26). The data indicate the the (−)-enantiomer is 5- to 10-fold more potent than the (+)-enantiomer as a tumor initiator (Fig. 5) and that the (−)-enantiomer caused about 50% more papillomas per mouse than did BP at an initiating dose of 0.1 μmol. The initiation-promotion study which found BP 7,8-dihydrodiol slightly less active than BP (Ref. 24) is somewhat surprising in light of the fact that the dihydrodiol used was biosynthetic. Since this dihydrodiol was probably the (−)-enantiomer (Ref. 25), much higher activity would be expected.

Fig. 4. Skin tumors produced by application of BP 7,8-dihydrodiol and related compounds once every two weeks to the backs of male C57BL/6J mice.
Carcinogenicity of 7,8-diol-9,10-epoxides

During the course of the above tumor studies, Borgen et al. (Ref. 27) found that liver microsomal metabolism of BP 7,8-dihydrodiol resulted in more extensive binding to added DNA than did metabolism of BP or several other metabolites of BP. Secondary oxidative metabolism of all primary oxidative metabolites of BP was reported (Ref. 28), a "diol oxide" was suggested (Ref. 3) as one of several secondary oxidative metabolites which might account for the metabolism-induced binding of BP 7,8-dihydrodiol to DNA, and Sims et al. (Ref. 29) provided evidence that a 7,8-diol-9,10-epoxide was the metabolite responsible for the binding. Metabolism of BP 7,8-dihydrodiol could produce either or both diastereomERICALLY related diol epoxides in which the epoxide oxygen is either cis (isomer 1 series) or trans (isomer 2 series) to the benzylic hydroxyl group at position-7 (Fig. 5). The stereochemical situation
is of particular interest since the close proximity of the 7-hydroxyl group to the epoxide oxygen in isomer 1 causes markedly enhanced chemical reactivity relative to isomer 2, presumably due to anemic assistance to attack on the epoxide (Ref. 30,31). When the BP 7,8-diol-9,10-epoxide isomers 1 and 2 were tested as complete carcinogens on mouse skin by application of 0.02 μmol to 0.40 μmol of each compound once every two weeks for 60 weeks (Table 1, Ref. 23), isomer 1 was inactive while isomer 2 caused tumors in only 13% of the animals compared to a 100% tumor incidence for BP at the high dose. Similarly, isomer 2 had only 20–30% of the initiating activity of BP while isomer 1 had only 12% (Ref. 16 & 17). Failure of the diol epoxides to be more active than the parent hydrocarbon, a requirement for proof of an ultimate carcinogen, in this tumor model may be a consequence of their high chemical reactivity. Although the diol epoxide isomers are potent hyperplastic agents, presumably through interaction at the surface of skin cells (Ref. 32), they may not be effective in reaching receptors for tumor initiation deep within the cells. The newborn mouse has provided a very useful model for studies of the tumorigenic activity of BP 7,8-dihydrodiol and the 7,8-diol-9,10-epoxides (Table 2, Ref. 33 & 34). At a total dose of 1400 nmol, BP caused about 6 lung adenomas per animal while the 7,8-dihydrodiol produced about 75 lung adenomas per animal. At the very low dose of 28 nmol (Ref. 34), animals treated with BP, BP 7,8-dihydrodiol, and BP 7,8-diol-9,10-epoxide-2 developed 0.24, 1.77, and 4.42 pulmonary adenomas per mouse, respectively. This sequential increase in tumorigenic activity clearly establishes BP 7,8-diol-9,10-epoxide-2 as an ultimate carcinogen in this tumor model and provides the first example of an ultimate carcinogen in the polycyclic aromatic hydrocarbon series based on tumor data. Due to very high toxicity, the tumorigenic activity of BP 7,8-diol-9,10-epoxide-1 has yet to be accurately assessed (Ref. 33).

<table>
<thead>
<tr>
<th><strong>Compound</strong></th>
<th><strong>Dose every two weeks (μmol)</strong></th>
<th><strong>Percent of mice with tumors</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>0.40</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>4</td>
</tr>
<tr>
<td>BP 7,8-diol-9,10-epoxide-1</td>
<td>0.40</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0</td>
</tr>
<tr>
<td>BP 7,8-diol-9,10-epoxide-2</td>
<td>0.40</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE 1. Carcinogenicity of benzo[a]pyrene and benzo[a]pyrene 7,8-diol-9,10-epoxides by application once every two weeks to the backs of C57BL/6J mice for 60 weeks.

<table>
<thead>
<tr>
<th><strong>Compound</strong></th>
<th><strong>Total dose (μmol)</strong></th>
<th><strong>No. of animals autopsied</strong></th>
<th><strong>Percent of mice with malignant lymphomas</strong></th>
<th><strong>Lung adenomas per animal</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>48</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>BP</td>
<td>1400</td>
<td>45</td>
<td>0</td>
<td>84</td>
</tr>
<tr>
<td>BP 7,8-dihydrodiol</td>
<td>1400</td>
<td>50</td>
<td>70</td>
<td>74</td>
</tr>
<tr>
<td>BP 7,8-diol-9,10-epoxide-2</td>
<td>28</td>
<td>37</td>
<td>0</td>
<td>81</td>
</tr>
</tbody>
</table>

aCompounds were administered in three injections on day 1, 8, and 15 of life.
bThe experiment was terminated when the animals were 24 weeks of age.
THE BAY REGION THEORY

In an attempt to generalize the concept of BP 7,8-diol-9,10-epoxides as ultimate carcinogens from BP to other polycyclic aromatic hydrocarbons, we proposed a concept (Ref. 35) now known as the "bay-region" theory. The foundation of this theory rests on our initial observations (Ref. 30) of remarkably high chemical reactivity for the epoxide ring in the BP diol epoxides. The fundamental question then became one of identifying those factors responsible for this unusual reactivity. A unique structural feature of the 7,8-diol-9,10-epoxides is that the epoxide ring forms part of a "bay-region" in the hydrocarbon. For BP, the "bay-region" is the hindered area between the 10- and 11-positions. The term "bay-region" originates from the field of proton magnetic resonance where hydrogens in such positions have been noted to resonate below the usual aromatic envelope due to edge deshielding by the proximate aromatic ring (Ref. 36). The simplest example of a "bay-region" is the hindered area between the 4- and 5-positions of phenanthrene. The basic hypothesis of the "bay-region" theory is that a diol epoxide be formed on a saturated, angular benzo-ring such that the oxirane ring forms part of a "bay-region" of the hydrocarbon. A principle role of the dihydrodiol as a precursor is to provide a metabolic pathway to an aliphatic epoxide rather than an arene oxide since aliphatic epoxides are more susceptible to nucleophilic attack relative to other solvolytic processes (Ref. 37). This point is illustrated by the fact that the 7,8-diol-9,10-epoxides of BP hydrolyze to mixtures of tetraols with only trace amounts of the 7,8-diol-9,10-keto isomerization product produced in the acidic to basic pH range (Ref. 31). Initial biological support for the "bay-region" theory came from reinterpretation of existing carcinogenicity data which indicated that alkyl or halogen substitution on the critical benzo-ring markedly reduced carcinogenicity relative to the parent hydrocarbon (Ref. 35, 38 & 39). Such substitution is expected to substantially reduce the rate of enzymatic epoxidation at the formal double bond to which the substituent is attached.

The fundamental reason for unusually high chemical reactivity of an epoxide on a saturated, angular benzo-ring which forms part of a "bay-region" of the hydrocarbon derives from the electronic nature of the \( \pi \)-skeleton which remains after saturation of the benzo-ring. Perturbational molecular orbital calculations by the method of Dewar (Ref. 40) have been used to estimate the ease of carbonium ion formation from all of the possible isomeric diol epoxides on terminal-rings of a large number of polycyclic hydrocarbons (Ref. 41 & 42). The calculations estimate the energy change in the \( \pi \)-electron system for the conversion

\[
\text{Ar} - \text{ArCH}_2^+.
\]

as a model for the reactivity of diol epoxides. Application of these calculations to the hydrocarbon benzo[a]anthracene is illustrated in Fig. 7 (see next page), where larger values of \( \Delta E_{\text{deloc}}/\beta \) signify greater ease of carbonium ion formation. As is shown for benzo[a]anthracene, formation of a carbonium ion on a saturated, terminal ring is always easiest when this carbonium ion forms part of a "bay-region." The calculations also allow a qualitative ranking of hydrocarbons for carcinogenicity (Fig. 7) based on the ease of formation of the "bay-region" carbonium ion (Ref. 39, 41 & 42).

TUMORIGENICITY OF BENZO[a]ANTHRACENE DIHYDRODIOLS

The hydrocarbon benzo[a]anthracene (BA) was selected as the first compound to be examined as a test of the "bay-region" theory. Even though BA is considered to be a rather weak carcinogen, the perturbational molecular orbital calculations indicate the ease of carbonium ion formation in the "bay-region" is quite high (\( \Delta E_{\text{deloc}}/\beta = 0.766 \)) when compared to the more potent carcinogen dibenzo[a,h]anthracene (\( \Delta E_{\text{deloc}}/\beta = 0.738 \), Fig. 7, see next page). Thus, the metabolic precursor of BA 3,4-diol-1,2-epoxides was anticipated to show strong biological activity. Furthermore, BA provides an excellent model for studies on the much more potent carcinogens 7-methylbenzo[a]anthracene, 7,12-dimethylbenzo[a]anthracene, and 3-methylcholanthrene since all four hydrocarbons share the same \( \pi \)-electron skeleton. The five metabolically probable dihydrodiols (Fig. 8) and most of the corresponding diol epoxides were synthesized for biological testing (Ref. 43 & 44). Initial experiments on the ability of the cytochrome P-450 system to metabolically activate BA and the dihydrodiols to mutagens (Ref. 45) and on the mutagenic activity of the diol epoxides toward bacterial and mammalian cells (Ref. 46) provided strong support for the "bay-region" theory in that the BA 3,4-dihydrodiol and the diastereomeric BA 3,4-diol-1,2-epoxides had the highest activity.
Fig. 7. Comparisons of the predicted ease of carbonium ion formation for the four possible benzylic carbonium ions from benzo[a]anthracene (right) and for the benzylic "bay region" carbonium ions for several hydrocarbons (left).
BA and the five dihydriodols (Fig. 8) were tested for their ability to initiate skin tumors in CD-1 female mice (Ref. 47). Eighteen days after a single topical application of 0.4–2.0 mmol of each compound, the mice were treated twice weekly with the skin promoter 12-0-tetradecanoylphorbol-13-acetate. After twenty weeks, comparisons of latency period, percent of mice with tumors, and number of papillomas per mouse all indicated that the BA 3,4-dihydrodiol was 10– to 20-fold more tumorigenic than the parent hydrocarbon which was more active than the other four dihydriodols (Ref. 47, Table 3). Experiments in the newborn mouse were even more dramatic (Ref. 48). Animals were given a total dose of 2800 mmol of either BA or each of the dihydriodols. The BA 3,4-dihydrodiol caused 30-fold more pulmonary adenomas than did BA while the other dihydriodols had little or no activity. In addition, 28% of the mice treated with the 3,4-dihydrodiol developed malignant lymphomas. Both the promotion experiments (Ref. 47) and the experiments in newborn mice (Ref. 48) provide clear evidence that the 3,4-dihydrodiol is a proximate carcinogen derived from BA. Preliminary results of initiation-promotion experiments with the BA 3,4-diol-1,2-epoxides on the skin of CD-1 mice indicate that these diol epoxides are more active than BA in this tumor model (unpublished). These tumor experiments and the mutagenicity experiments on the diol epoxides (Ref. 46) implicate the BA 3,4-diol-1,2-epoxides as ultimate carcinogens from BA and provide strong support for the "bay-region" theory.
TABLE 3. Tumor induction in CD-1 mice by benzo[a]anthracene and benzo[a]anthracene dihydrodiols after 20 weeks of promotion with 12-0-tetradecanoylphorbol-13-acetate.

<table>
<thead>
<tr>
<th>Compound (2.0 μmol)</th>
<th>Surviving animals</th>
<th>Tumor animals</th>
<th>Total tumors</th>
<th>Tumors per survivor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>1</td>
<td>1</td>
<td>0.03</td>
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<tr>
<td>BA</td>
<td>30</td>
<td>7</td>
<td>9</td>
<td>0.30</td>
</tr>
<tr>
<td>BA 1,2-dihydrodiol</td>
<td>30</td>
<td>2</td>
<td>2</td>
<td>0.07</td>
</tr>
<tr>
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<td>30</td>
<td>24</td>
<td>144</td>
<td>4.80</td>
</tr>
<tr>
<td>BA 5,6-dihydrodiol</td>
<td>30</td>
<td>6</td>
<td>6</td>
<td>0.02</td>
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<tr>
<td>BA 8,9-dihydrodiol</td>
<td>29</td>
<td>5</td>
<td>6</td>
<td>0.21</td>
</tr>
<tr>
<td>BA 10,11-dihydrodiol</td>
<td>29</td>
<td>4</td>
<td>4</td>
<td>0.14</td>
</tr>
</tbody>
</table>

CONCLUSIONS

Since definitive proof for the identification of an ultimate carcinogen can only come from carcinogenicity studies, there are only two examples of polycyclic aromatic hydrocarbons for which ultimate carcinogens are known; the 7,8-diol-9,10-epoxides from BP and the 3,4-diol-1,2-epoxides of BA where the epoxide forms part of a "bay-region" in each case. Additional indirect support is, however, available in support of the "bay-region" theory. Studies on the metabolic activation of chrysene and its three metabolically probable dihydrodiols (Fig. 9) to a compound mutagenic to bacteria have shown that the 1,2-dihydrodiol which has a "bay-region" double bond produced a 20-fold higher mutagenic response than chrysene or the other two dihydrodiols (Ref. 49). Interestingly, attempts to form mutagens from trans-1,2-dihydroxy-1,2,3,4-tetrahydrochrysene were without success thereby suggesting 1,2-diol-3,4-epoxides as the probable mutagens formed from the 1,2-dihydrodiol. Studies on the ability of drug metabolizing enzymes to convert 7-methylbenzo[a]anthracene and its isomeric dihydrodiols into bacterial mutagens (Ref. 50) and into compounds which transform cells in culture (Ref. 51) have demonstrated that the 3,4-dihydrodiol with the "bay-region" double bond produces the most activity. Furthermore, studies of the metabolism induced binding of 7,12-dimethylbenzo[a]anthracene to the DNA of cultured cells have shown a bound
The bay region theory

The bay region theory adduct which has a 1,2,3,4-tetrahydro-7,12-dimethylbenzo[a]anthracene chromophore (Ref. 52). The 3,4-diol-1,2-epoxides of the parent hydrocarbons (Fig. 10) are probably the active agents in each case. These latter studies are particularly important in that they suggest that the "bay-region theory applies to substituted as well as unsubstituted polycyclic aromatic hydrocarbons."

REFERENCES