Enhancing Effect of a Bay Region Methyl Group on Tumorigenicity in Newborn Mice and Mouse Skin of Enantioisomeric Bay Region Diol Epoxides Formed Stereoselectively from Methylchrysenes in Mouse Epidermis

Stephen S. Hecht, Shantu Amin, Keith Huie, Assieh A. Melikian, and Ronald G. Harvey

ABSTRACT

The stereochernistry of diol epoxide formation in mouse epidermis upon topical application of [3H]-1R,2R-dihydroxy-1,2-dihydro-5-methylchrysene (5-MeC-1,2-diol) and [3H]-6-MeC-1,2-diol, and the tumorigenicity in mouse skin and in newborn mice of the R,S,R,S enantiomers of 1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydro-5-methylchrysenes (5-MeC-1,2-diol-3,4-epoxide), 5-MeC-7,8-diol-9,10-epoxide, and 6-MeC-1,2-diol-3,4-epoxide were examined. Analysis of tetaols and their derived tetracetates present in mouse epidermis, 2 h after application of [3H]-5-MeC-1,2-diol or [3H]-6-MeC-1,2-diol, demonstrated greater than 90% stereoselectivity in formation of 5-MeC-1,2,5-diol-3,5,4-epoxide and 6-MeC-1,2,5-diol-3,5,4-epoxide. Taken together with previous data, these results demonstrate that there is a high degree of stereoselectivity for formation of R,S,R,S enantiomers of 5-MeC- and 6-MeC-1,2-diol-3,4-epoxides in mouse skin. The results of the tumorigenicity studies in mouse skin and in newborn mice clearly demonstrated that 5-MeC-1R,2S-diol-3,5,4-epoxide was the most tumorigenic of the diol epoxide enantiomers tested; 6-MeC-1R,2S-diol-3,5,4-epoxide was inactive. The results of this study show that the high tumorigenacity of 5-MeC compared to 6-MeC is due to the remarkable tumorigenic activity of 5-MeC-1,2,5-diol-3,5,4,4-epoxide which, in contrast to 6-MeC-1,2,5-diol-3,5,4,4-epoxide, has a methyl group in the same bay region as the epoxide ring. We propose that such methyl bay region diol epoxides of other carcinogenic methylated polynuclear aromatic hydrocarbons will also show unique tumorigenic properties.

INTRODUCTION

A major pathway in the metabolic activation of the strong carcinogen 5-MeC is 5-MeC→5-MeC-1,2-diol—5-MeC-1,2-diol-3,4-epoxide (1). In mouse epidermis, the analogous pathway is observed in the metabolism of the weak carcinogen 6-MeC (2). The low carcinogenicity of 6-MeC compared to 5-MeC might potentially be due to differences in the stereochernistry of the diols and diol epoxides formed in this activation pathway, or to differences in the tumorigenicity of the diols and diol epoxides. In a recent study, we demonstrated that both 5-MeC and 6-MeC were converted with high stereoselectivity to the corresponding 1R,2R-diols (3). 5-MeC-1R,2R-diol was highly tumorigenic in mouse skin but 6-MeC-1R,2R-diol was essentially inactive (3). In the present study, we have investigated the stereochernistry of diol epoxide formation from the 1R,2R-diols of 5-MeC and 6-MeC and have compared the tumorigenic activities in mouse skin and newborn mice of the R,S,R,S and S,R,S,R enantiomers of 5-MeC-1,2-diol-3,4-epoxide, 5-MeC-7,8-diol-9,10-epoxide, and 6-MeC-1,2-diol-3,4-epoxide (Fig. 1).

MATERIALS AND METHODS

Apparatus. HPLC was carried out with a system composed of a Rhodyne Model 7125 injection loop (Rhodyne Inc., Cotati, CA), 2 Model 510 pumps (Millipore, Waters Division, Milford, MA), an automated gradient controller (Waters), a Model 116 UV Detector (Gilson, Middleton, WI), and a Flo-one/Beta radioactive flow detector (Radiometric Instruments, Tampa, FL). The following columns and programs were used: System 1, a 10 × 250-mm Vydac 211TP1010 10-μm column (Separations Group, Hesperia, CA) programmed with a linear gradient from 20% methanol in H2O to 100% methanol in 60 min at 3 ml/min; System 2, a 4 × 250-mm Lichrosorb Si 65 μm column (EM Reagents, Cincinnati, OH) eluted with 30% tetrahydrofuran in hexane at 2 ml/min.

UV spectra were run on a Hewlett Packard Model 8452A diode array spectrophotometer. Mass spectra were determined with a Hewlett Packard Model 5988A instrument. NMR spectra were obtained at Hunter College, City University of New York, on a Jeol JNM-GX 400 FT-NMR.

Chemoicals. Racemic syn- and anti-5-MeC-1,2-diol-3,4-epoxide and racemic syn-5-MeC-7,8-diol-9,10-epoxide were synthesized (4). For preparation of the R,S,R,S and S,R,S,R-diol epoxides, 5-MeC-1,2-diol (4), 5-MeC-1,2,5-diol (4, 6-MeC-1,2-diol (5), and 6-MeC-1,2-diol (7) were resolved into their R,R- and S,S-enantiomers by chiral stationary phase HPLC as previously described (3). The appropriate diol enantiomer (3 mg) was dissolved in tetrahydrofuran (5 ml) at 0°C under N2, and m-chloroperbenzoic acid (30 mg) was added with stirring. The mixture was stirred at 0°C for 1 h, then allowed to come to room temperature while stirring was continued for 4 h. Ether was added and the reaction mixture was washed twice with 1% cold aqueous NaOH, twice with H2O, and dried (K2CO3). The organic extracts were concentrated in vacuo to give the diol epoxides which were >99% pure according to analysis by HPLC with the use of System 2 and by thin layer chromatography on silica gel pretreated with 2% triethylamine in CH2Cl2, with elution by tetrahydrofuran:ethyl acetate:CH3Cl (43:33:33).

Tetraols were prepared by hydrolysis of 5 mg of either racemic syn- or anti-5-MeC-1,2-diol-3,4-epoxide or racemic syn- or anti-6-MeC-1,2-diol-3,4-epoxide. The diol epoxides were dissolved in tetrahydrofuran and added to 10 ml of 10 mM sodium cacodylate buffer. The mixture was incubated at pH 7.0, 37°C, for 24 h. The tetraols were extracted with ethyl acetate:tetrahydrofuran (90:10) and separated by HPLC with the use of System 1. The retention times are given in Table 1. Each tetraol was collected and its UV spectrum determined; all were similar (K2CO3). This organic extract was then added to 10 ml of 10% sodium cacodylate buffer. The mixture was incubated at 37°C, 0°C, for 24 h. The tetraols were extracted with ethyl acetate:tetrahydrofuran (90:10) and separated by HPLC with the use of System 2. The retention times are given in Table 1. Each tetraol was collected and its UV spectrum determined; all were similar.
concentrated to dryness and mixed with 3 ml of dry pyridine containing 0.5 ml of acetic anhydride. The mixture was stirred at room temperature overnight and the solvent was removed at reduced pressure. The residues were analyzed by HPLC with the use of System 1. The retention times of the tetracetates are given in Table 1. The trans-anti and trans-syn tetracetates from 5-MeC-1,2-diol-3,4-epoxide were characterized by their UV spectra which were similar to those of the tetraols, and by their mass spectra, m/e (relative intensity), trans-anti 478 (3, M+), 436 (4, M—CH2=CH2CO2H), 315 (14, M—2CH3CO2H + CH2=CH2CO2H), 274 (100, M—3CH3CO2H + CH2=CH2CO2H + CH2=CH—O); trans-syn 436 (2), 358 (2), 316 (12), 274 (84), 256 (100).

The stereochemistry of epoxidation of 5-MeC-1,2-diol and 6-MeC-1,2-diol in mouse skin was investigated by analyzing the tetracols formed upon hydrolysis of the 1,2-diol-3,4-epoxides. Standard tetracols were prepared by hydrolysis of the racemic anti- or syn-isomers of 5-MeC-1,2-diol-3,4-epoxide and 6-MeC-1,2-diol-3,4-epoxide (Fig. 2). The HPLC retention times and relative yields of the hydrolysis products are given in Table 1. The trans- and cis-ring opening products were assigned by analogy to previous studies on the hydrolysis of the racemic anti- and syn-isomers of chrysene-1,2-diol-3,4-epoxide in which similar product distributions and HPLC retention times were observed (9). The NMR spectra of the trans-anti- and trans-syn products are shown in Fig. 2.

RESULTS

The stereochemistry of epoxidation of 5-MeC-1,2-diol and 6-MeC-1,2-diol in mouse skin was investigated by analyzing the tetracols formed upon hydrolysis of the 1,2-diol-3,4-epoxides. Standard tetracols were prepared by hydrolysis of the racemic anti- or syn-isomers of 5-MeC-1,2-diol-3,4-epoxide and 6-MeC-1,2-diol-3,4-epoxide (Fig. 2). The HPLC retention times and relative yields of the hydrolysis products are given in Table 1. The trans- and cis-ring opening products were assigned by analogy to previous studies on the hydrolysis of the racemic anti- and syn-isomers of chrysene-1,2-diol-3,4-epoxide in which similar product distributions and HPLC retention times were observed (9). The NMR spectra of the trans-anti- and trans-syn products are shown in Fig. 2.
syn-tetraols formed upon hydrolysis of anti- and syn-5-MeC-1,2-diol-3,4-epoxide were consistent with these assignments, although not in themselves definitive (10). These trans-anti- and trans-syn-tetraols could not be separated by using a variety of different HPLC conditions. Therefore they were converted to the corresponding tetraacetates which were readily separated as indicated in Table 1.

The tetroal standards were used as markers for analysis of products formed in mouse epidermis upon application of either \([\text{H}]\)-5-MeC-1R,2R-diol or \([\text{H}]\)-6-MeC-1R,2R-diol. These diol enantiomers are formed stereoselectively in mouse skin from 5-MeC and 6-MeC, respectively (3). HPLC analysis of the tetrools formed, 2 h after topical application of \([\text{H}]\)-5-MeC-1R,2R-diol, demonstrated that the epoxidation proceeded >90% anti, giving predominantly \([\text{H}]\)-6-MeC-1R,2S-diol-3S,4R-epoxide (Fig. 3). HPLC analysis of the metabolites of \([\text{H}]\)-5-MeC-1R,2R-diol gave a chromatogram similar to that shown in Fig. 3, except that the trans-anti- and trans-syn-isomers were not separated. These results indicated that the epoxidation proceeded mainly anti, since little radioactivity coeluted with the cis-syn-tetraol which is the major one produced in the hydrolysis of syn-5-MeC-1,2-diol-3,4-epoxide. However, it was possible that the stereoselectivity of ring opening of syn-5-MeC-1,2-diol-3,4-epoxide was different in buffer versus in mouse epidermis. Therefore, the peak corresponding in retention time to the trans-anti and trans-syn tetrools formed metabolically from \([\text{H}]\)-5-MeC-1R,2R-diol was collected and converted to tetraacetates. Analysis of the tetraacetates demonstrated that the epoxidation did proceed >90% anti, giving \([\text{H}]\)-5-MeC-1R,2S-diol-3S,4R-epoxide in these experiments, >95% of the radioactivity recovered from the epidermis was unchanged diol, and several metabolites other than tetrools were observed.

The half-life of anti-6-MeC-1,2-diol-3,4-epoxide in pH 7.0 cacyclate buffer at 37°C was 78 min compared to 59 min for anti-5-MeC-1,2-diol-3,4-epoxide under identical conditions.

Since the metabolic studies demonstrated predominant conversion of the methylchrysene diols to anti-diol epoxides, the tumorigenic activities of the \(R,S,S,R\) and \(S,R,R,S\) diol epoxides illustrated in Fig. 1 were assessed on mouse skin and in newborn mice. Racemic syn-5-MeC-1,2-diol-3,4-epoxide and syn-5-MeC-7,8-diol-9,10-epoxide were included in the mouse skin assay because these compounds were not available at the time of our previous bioassay of racemic 5-MeC diol epoxides (8). The results which are summarized in Tables 2 and 3 clearly demonstrate that 5-MeC-1R,2S-diol-3S,4R-epoxide was the most tumorigenic of the diol epoxides tested. None of the other diol epoxides showed high activity, although 5-MeC-1S,2R-diol-3R,4S-epoxide and 5-MeC-7R,8S-diol-9S,10R-epoxide induced a significant incidence of tumors in the lungs of female newborn mice. In the newborn mouse assay, females were significantly (\(P = 0.03\)) more sensitive than males to lung tumor induction by 5-MeC-1R,2S-diol-3S,4R-epoxide, but males were significantly (\(P < 0.001\)) more sensitive to liver tumor induction.

**DISCUSSION**

Based on our previous studies, we developed the hypothesis that the high tumorigenicity of 5-MeC compared to 6-MeC...
TUMORIGENICITY OF BAY REGION DIOL EPOXIDES

Table 3  Tumorigenicity of methylchrysene diol epoxide enantiomers in newborn mice

ICR/Ha mice were given i.p. injections of each compound (total dose, 56 nmol) in DMSO on the first, eighth, and 15th days of life. Mice were weaned at the age of 21 days, separated by sex, and sacrificed at 35 weeks.

<table>
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<th>Compound</th>
<th>Effective no. of mice</th>
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<th>Hepatic tumors</th>
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<td>Sex</td>
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</tr>
<tr>
<td>5-MeC-1R,2S-diol-3S,4R-epoxide</td>
<td>65</td>
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<tr>
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</tr>
<tr>
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<td></td>
<td></td>
<td>Total</td>
<td>59</td>
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</table>

* P < 0.001 versus control.
° P < 0.005 versus control.

Fig. 4. Major pathways of stereoselective metabolism of 5-MeC and 6-MeC to 1,2-diols, 3,4-epoxides in mouse epidermis. The R,S,S,R-enantiomer is formed with high stereoselectivity from both 5-MeC and 6-MeC. The amounts formed from 6-MeC exceed those from 5-MeC. Tumor multiplicity for each compound on mouse skin or in newborn mouse lung (numbers in parentheses) is indicated. The results demonstrate that the intrinsic tumorigenicity of 5-MeC-1R,2S-diol-3S,4R-epoxide is responsible for the high tumorigenicity of 5-MeC compared to 6-MeC.

resulted from differences in the tumorigenicity of their 1,2-diols, 3,4-epoxides. This hypothesis was based on the following observations. (a) 5-MeC and 6-MeC were both metabolized in mouse epidermis with high stereoselectivity from both 5-MeC and 6-MeC. The amounts formed from 6-MeC exceed those from 5-MeC. Tumor multiplicity for each compound on mouse skin or in newborn mouse lung (numbers in parentheses) is indicated. The results demonstrate that the intrinsic tumorigenicity of 5-MeC-1R,2S-diol-3S,4R-epoxide is responsible for the high tumorigenicity of 5-MeC compared to 6-MeC.

Comparison of the present results with those obtained in our study of the tumorigenicity of the racemic 5-MeC diol epoxides indicates that the tumorigenic properties of the racemic mate-
rial are due virtually exclusively to the \( R,S,S,R \)-enantiomer. There was no indication of synergy between the enantiomers. The sex differences observed in the present study were also observed previously (8).

The higher tumorigenicity of 5-MeC-1R,2S-diol-3S,4R-epoxide than of 5-MeC-1S,2R-diol-3R,4S-epoxide is consistent with previous studies on several unsubstituted PAH including chrysene, benz(a)anthracene, benzo(c)phenanthrene, and benzo(a)pyrene, which have shown that \( R,S,S,R \) diol epoxides are more tumorigenic on mouse skin and in newborn mice than the \( S,R,R,S \)-enantiomers (12–16). This is the first report on the tumorigenicity of methylated PAH diol epoxide enantiomers. The results demonstrate that the selective tumorigenicity of the \( R,S,S,R \)-enantiomer is retained in the methyl bay region diol epoxide.

In the present study we did not test the syn-diol epoxide enantiomers because the metabolic experiments did not indicate that they were formed to a major extent. In addition, our previous bioassays had shown that racemic syn-5-MeC-1,2-diol-3,4-epoxide was inactive in newborn mice. The results in Table 2 demonstrate that neither racemic syn-5-MeC-1,2-diol-3,4-epoxide nor syn-5-MeC-7,8-diol-9,10-epoxide had high tumorigenic activity in mouse skin. Thus, the syn-bay region diol epoxides of 5-MeC do not appear to play a significant role in its metabolic activation. In previous bioassays of PAH diol epoxides, high tumorigenicity has been observed only for the syn-bay region diol epoxides of benzo(c)phenanthrene, in mouse skin (16). DNA binding studies have suggested a likely role for syn-diol epoxides in the metabolic activation of 7,12-dimethylbenz(a)anthracene (17).

The stereoselective pathways summarized in Fig. 4 for 5-MeC and 6-MeC are similar to those which have been reported for the formation of bay region diol epoxide from phenanthrene, chrysene, benz(a)anthracene, benzo(c)phenanthrene, and benzo(a)pyrene, in studies carried out predominantly with rat liver preparations. These investigations have shown that the \( R,S,S,R \)-diol epoxide enantiomer is frequently formed to the greatest extent (18, 19). Methyl substitution can affect the stereoselectivity of diol formation in PAH systems (20). The results summarized in Fig. 4 indicate that methyl substitution in the chrysene system has little effect on the stereochemistry of enzymatic formation of 1,2-diol-3,4-epoxides. As in the unsubstituted PAH, the most tumorigenic bay region diol epoxide enantiomer is the one which is formed to the greatest extent metabolically.

The reason for the enhancing effect on bay region diol epoxide tumorigenicity of a bay region methyl group is not known. 5-MeC-1R,2S-diol-3S,4R-epoxide, 6-MeC-1R,2S-diol-3S,4R-epoxide, and 5-MeC-7R,8S-diol-9S,10R-epoxide comprise a unique set of compounds to investigate this effect since the only difference among them is the position of the methyl group. The rates of solvolysis of the racemic diol epoxides do not correlate with their tumorigenic activities. The half-life of racemic anti-5-MeC-7,8-diol-9,10-epoxide is 17.5 min, while those of anti-5-MeC-1,2-diol-3,4-epoxide and anti-6-MeC-1,2-diol-3,4-epoxide are 59 and 78 min, respectively. Therefore it does not appear that the differences in tumorigenicity can be explained by ease of epoxide ring opening, in the absence of catalysis.

At least two hypotheses seem plausible to account for the differences in tumorigenicity among these diol epoxides. First, selective detoxification by glutathione conjugation of 6-MeC-1R,2S-diol-3S,4R-epoxide or 5-MeC-7R,8S-diol-9S,10R-epoxide compared to 5-MeC-1R,2S-diol-3S,4R-epoxide seems possible because the bay region methyl group may inhibit the reaction. The formation of glutathione conjugates of bay region diol epoxides is well established (21, 22). Second, and in our view more likely, is selective reaction with DNA of 5-MeC-1R,2S-diol-3S,4R-epoxide. Previous studies with the enantiomers of anti-benzo(a)pyrene-7,8-diol-9,10-epoxide have shown that the DNA interactions of the highly tumorigenic \( R,S,S,R \)-enantiomer are different from those of the inactive \( S,R,R,S \)-enantiomer (23). This has been attributed in part to differences in the preliminary intercalation of each enantiomer in a specific DNA receptor site (24, 25). The conformations of the adducts formed from the two enantiomers are different (25). Stereochimistry appears to be the dominant factor controlling these processes. The results of the present study suggest that the bay region methyl group helps to draw the diol epoxide into the receptor site and may affect the configuration of the resulting adduct. We are presently testing this hypothesis by comparative studies of the DNA binding of 5-MeC-1R,2S-diol-3S,4R-epoxide and 6-MeC-1R,2S-diol-3S,4R-epoxide.

A bay region methyl group adjacent to an unsubstituted angular ring enhances tumorigenicity in a number of PAH systems including methylphenanthrene, 15,16-dihydrocyclopenta(a)phenanthren-17-one, benz(a)anthracene, methylcholanthrene, benzo(a)pyrene, dibenz(a,h)anthracene, and dibenz(a,j)anthracene (1–16). Metabolism studies carried out in some of these systems strongly implicate methyl bay region diol epoxides as ultimate tumorigens (17, 32–35). It will be important to determine whether methyl bay region diol epoxides are more tumorigenic than other bay region diol epoxides in these PAH systems. We predict that the enhancing effect observed in the present study will be a general phenomenon for methylated PAH.

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