SHORT COMMUNICATION

8,9-Dihydroxy-8,9-dihydridibenzo[a,l]pyrene is a potent morphological cell-transforming agent in C3H10T1/2Cl8 mouse embryo fibroblasts in the absence of detectable stable covalent DNA adducts

Stephen Nesnow1, Christine Davis, William T. Padgett, Linda Adams, Michele Yacopucci and Leon C. King

Environmental Carcinogenesis Division, National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, MD-68, Research Triangle Park, NC 27711, USA

1To whom correspondence should be addressed
Email: nesnow.stephen@epa.gov

The comparative genotoxic effects of racemic trans-8,9-dihydroxy-8,9-dihydridibenzo[a,l]pyrene (trans-DB[a,l]P, 8,9-diol), the metabolic K-region dihydrodiol of dibenzo[a,l]pyrene (DB[a,l]P) (dibenzo[def,p]chrysene) and DB[a,l]P in transformable mouse embryo C3H10T1/2Cl8 (C3H10T1/2) fibroblasts was investigated. The C3H10T1/2 mouse embryo morphological cell-transforming activities of these polycyclic aromatic hydrocarbons (PAHs) were assayed using concentration–response studies. At concentrations of 33 nM and above both trans-DB[a,l]P, 8,9-diol and DB[a,l]P produced significant (and similar) numbers of type II and III foci per dish and numbers of dishes with type II and II foci. Concomitant cytotoxicity studies revealed a reduction in colony survival of ~25% up to 198 nM for both PAHs. DNA adducts of trans-DB[a,l]P, 8,9-diol and DB[a,l]P in C3H10T1/2 cells were analyzed by a 3P-postlabeling TLC/HPLC method. No adducts were observed in the DNA of C3H10T1/2 cells treated with trans-DB[a,l]P, 8,9-diol at concentrations that induced morphological cell transformation. Under the same exposure and chromatographic conditions, DNA adducts of deoxyadenosine and deoxyguanosine derived from the fjord region anti-DB[a,l]P, 11,12-diol, 13,14-epoxide and syn-DB[a,l]P, 11,12-diol, 13,14-epoxide were observed in the DNA of DB[a,l]P-treated cells. These results indicate that trans-DB[a,l]P, 8,9-diol has intrinsic genotoxic activity equal to that of DB[a,l]P, based on morphological cell transformation of mouse embryo fibroblasts. The activity of trans-DB[a,l]P, 8,9-diol is apparently not associated with the formation of observable stable covalent DNA adducts. These results suggest that under appropriate conditions, trans-DB[a,l]P, 8,9-diol may serve as an intermediate in the genotoxicity of DB[a,l]P.

K-region dihydrodiols of polycyclic aromatic hydrocarbons (PAHs) have been considered to be detoxification products resulting from the metabolic transformation of PAHs by cytochrome P450 and epoxide hydrolase (1,2). Recently, we reported that the metabolic K-region dihydrodiol of dibenzo[a,l]pyrene (DB[a,l]P) (dibenzo[def,p]chrysene) formed by β-naphthoflavone-induced rat liver microsomes: two diastereomers of trans, trans-DB[a,l]P, 8,9; 11,12-bis-diol; two diastereomers of cis, cis-DB[a,l]P, 8,9; 11,12-bis-diol; two diastereomers of trans, cis-DB[a,l]P, 8,9; 13,14-bis-diol. These results suggested that trans-DB[a,l]P, 8,9-diol, might possess intrinsic genotoxic activity. Since DB[a,l]P, a product of the incomplete combustion of fossil fuels, is environmentally abundant (4) and is a highly potent mouse skin (5), mouse lung (6) and rat mammary carcinogen (5), we pursued the question of the potential genotoxicity of this K-region dihydrodiol in mammalian cells. In previous investigations, using morphological cell transformation of mouse embryo C3H10T1/2Cl8 (C3H10T1/2) fibroblasts, we found DB[a,l]P to be 3–10 times as potent as benzo[a]pyrene (B[a]P), making DB[a,l]P the most potent non-methylated PAH evaluated to date in C3H10T1/2 fibroblasts (7). The toxicological activity of trans-DB[a,l]P, 8,9-diol was evaluated on mouse skin, producing a marginal response as a mouse skin tumor initiator using female Sencar mice (5). In a limited investigation, mouse skin papillomas induced by trans-DB[a,l]P, 8,9-diol were found to bear A→T transversions in codon 61 of the Ha-ras proto-oncogene (8), suggesting that it had the potential to alter DNA.

Here we present evidence that trans-DB[a,l]P, 8,9-diol is as potent as DB[a,l]P in the induction of morphological cell transformation of C3H10T1/2 cells. Furthermore, we find no evidence for the formation of stable covalent trans-DB[a,l]P, 8,9-diol–DNA adducts in C3H10T1/2 cells. These DNA adduct results, in contrast to our previous observation of stable trans-DB[a,l]P, 8,9-diol–DNA adducts from incubation of recombiant human P450 1A1 microsomes, epoxide hydrolase and CT-DNA, suggest that major differences exist between the metabolic activation of trans-DB[a,l]P, 8,9-diol by mouse embryo cells and human P450 microsomes.

DB[a,l]P (99% pure) was obtained from the NCI Chemical Carcinogen Reference Standard Repository. Racemic trans-DB[a,l]P, 8,9-diol was synthesized by previously reported methods and determined to be 99.9% pure (3). C3H10T1/2 mouse embryo cells (passage 9) were grown in a 5% CO2 in air atmosphere at 37°C and 85% humidity in a complete medium consisting of Eagle’s basal medium with Earle’s salts and L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, NY). C3H10T1/2 cells were seeded for transformation studies at 1000 cells/60 mm plastic Petri dish in 5 ml of complete

© Oxford University Press

1253

K-region dihydrodiols of polycyclic aromatic hydrocarbons (PAHs) have been considered to be detoxification products resulting from the metabolic transformation of PAHs by cytochrome P450 and epoxide hydrolase (1,2). Recently, we reported that the metabolic K-region dihydrodiol of dibenzo[a,l]pyrene (DB[a,l]P) (dibenzo[def,p]chrysene) formed by β-naphthoflavone-induced rat liver microsomes: two diastereomers of trans, trans-DB[a,l]P, 8,9; 11,12-bis-diol; two diastereomers of cis, cis-DB[a,l]P, 8,9; 11,12-bis-diol; two diastereomers of trans, cis-DB[a,l]P, 8,9; 13,14-bis-diol. These results suggested that trans-DB[a,l]P, 8,9-diol, might possess intrinsic genotoxic activity. Since DB[a,l]P, a product of the incomplete combustion of fossil fuels, is environmentally abundant (4) and is a highly potent mouse skin (5), mouse lung (6) and rat mammary carcinogen (5), we pursued the question of the potential genotoxicity of this K-region dihydrodiol in mammalian cells. In previous investigations, using morphological cell transformation of mouse embryo C3H10T1/2Cl8 (C3H10T1/2) fibroblasts, we found DB[a,l]P to be 3–10 times as potent as benzo[a]pyrene (B[a]P), making DB[a,l]P the most potent non-methylated PAH evaluated to date in C3H10T1/2 fibroblasts (7). The toxicological activity of trans-DB[a,l]P, 8,9-diol was evaluated on mouse skin, producing a marginal response as a mouse skin tumor initiator using female Sencar mice (5). In a limited investigation, mouse skin papillomas induced by trans-DB[a,l]P, 8,9-diol were found to bear A→T transversions in codon 61 of the Ha-ras proto-oncogene (8), suggesting that it had the potential to alter DNA.

Here we present evidence that trans-DB[a,l]P, 8,9-diol is as potent as DB[a,l]P in the induction of morphological cell transformation of C3H10T1/2 cells. Furthermore, we find no evidence for the formation of stable covalent trans-DB[a,l]P, 8,9-diol–DNA adducts in C3H10T1/2 cells. These DNA adduct results, in contrast to our previous observation of stable trans-DB[a,l]P, 8,9-diol–DNA adducts from incubation of recombiant human P450 1A1 microsomes, epoxide hydrolase and CT-DNA, suggest that major differences exist between the metabolic activation of trans-DB[a,l]P, 8,9-diol by mouse embryo cells and human P450 microsomes.

DB[a,l]P (99% pure) was obtained from the NCI Chemical Carcinogen Reference Standard Repository. Racemic trans-DB[a,l]P, 8,9-diol was synthesized by previously reported methods and determined to be 99.9% pure (3). C3H10T1/2 mouse embryo cells (passage 9) were grown in a 5% CO2 in air atmosphere at 37°C and 85% humidity in a complete medium consisting of Eagle’s basal medium with Earle’s salts and L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, NY). C3H10T1/2 cells were seeded for transformation studies at 1000 cells/60 mm plastic Petri dish in 5 ml of complete

© Oxford University Press

1253
medium (24 dishes/concentration) and 24 h later the dishes were treated with the PAHs dissolved in acetone (0.5% by volume) for 24 h (7). One week after treatment, the cytotoxicity study dishes were fixed with methanol and stained with Giemsa. At confluence, the medium (containing 5% fetal bovine serum) was changed weekly in the morphological transformation study dishes. At the end of 6 weeks, the dishes were fixed, stained with Giemsa and scored for morphological cell transformation according to published criteria (9). Statistical analyses were performed on the morphological cell transformation incidence data using a χ² test with Yates correction and on the transformation multiplicity data using Dunn’s multiple comparison method after a Kruskal–Wallis one-way ANOVA on ranks (SigmaStat; Jandel, San Rafael, CA).

For DNA adduct studies, C3H10T1/2 fibroblasts in mid-log growth (~70% confluent) were treated with the PAHs dissolved in acetone (0.5% by volume). After a 24 h exposure, the cells were washed with Dulbecco’s phosphate-buffered saline (three times), trypsinized and collected by centrifugation. Cells treated with acetone served as controls. DNA was isolated from C3H10T1/2 cells using a chloroform/isoamyl alcohol/phenol method (10). DNA adducts were analyzed by the 32P-post-labeling assay (11) with nuclease P1 enhancement (12) and the HPLC modification (13). Briefly, the DNA (50 µg) from each treatment was digested to mononucleotides with micrococcal nuclease and spleen phosphodiesterase and enriched by nuclease P1 treatment (13). The samples were incubated with 50 µCi [γ-32P]ATP (3000 Ci/mmol) and T2 polynucleotide kinase (3’-phosphatase free). The total incubates were applied to 10×10 cm polyethyleneimine (PEI)–cellulose sheets and separated using a TLC system (D1 direction only) (14). The remaining spot at the origin was excised, extracted with 4 M pyridinium formate (pH 4) and the samples reduced to dryness in vacuo. Each sample was spiked with the UV marker cis-9,10-dihydroxy-9,10-dihydrophenanthrene and the volume adjusted with a mixture of MeOH and 0.3 M NaH₂PO₄ buffer (pH 2) (9:1). Separation of 32P-labeled nucleoside 3’5’-bisphosphate adducts was carried out using a 5 µm, 4.6×250 mm Zorbax phenyl-modified column (MAC-MOD Analytical Inc., Chadds Ford, PA) (15). The radiolabeled adducts were eluted using a previously described gradient system (3) and were detected by an in-line flow-through scintillation counter.

C3H10T1/2 cells were used to evaluate the morphological cell-transforming activities of trans-DB[a,j]P-8,9-diol and to compare them with those of the parent PAH, DB[a,j]P. Colony survival assays were undertaken to assess the cytotoxic effects of these agents. In triplicate studies and over a concentration range of 0–200 nM both PAHs gave similar concentration-related toxicity curves. At 300 nM, trans-DB[a,j]P-8,9-diol was more cytotoxic than DB[a,j]P (58 versus 82% survival; Figure 2A). In morphological cell transformation replicate studies, trans-DB[a,j]P-8,9-diol and DB[a,j]P produced statistically significant (P < 0.05, compared with the acetone control) numbers of dishes with type II or III foci at all concentrations evaluated (Figure 2B). For each agent at 89–99 nM and above, over 80% of the treated dishes exhibited at least one transformed focus. Both trans-DB[a,j]P-8,9-diol and DB[a,j]P produced statistically significant (P < 0.05, compared with the acetone control) numbers of type II and III foci per dish at every concentration evaluated (Figure 2C). Near linear concentration-related responses were recorded up to 179–199 nM. Maximal transformation multiplicity was observed at 179–199 nM, giving 2.3–2.6 type II and III foci per dish. Statistically, the morphological cell transformation incidence results for trans-DB[a,j]P-8,9-diol and DB[a,j]P were virtually

**Fig. 1.** Structures of DB[a,j]P and its K-region dihydrodiol, trans-DB[a,j]P-8,9-diol

**Fig. 2.** Morphological cell transformation of C3H10T1/2 cells treated with trans-DB[a,j]P-8,9-diol and DB[a,j]P in the concentration range 0–331 nM. All treatment groups were treated for 24 h. Cytotoxicity was scored after 2 weeks and morphological cell transformation scored after 6 weeks. In each study, and at each concentration, 24 individual dishes were plated and scored. All treated groups were statistically different from the acetone control group (P < 0.05). The error bars represent the standard deviation. (A) Cytotoxicity of trans-DB[a,j]P-8,9-diol and DB[a,j]P measured as the reduction in colony formation. Means and standard deviations from replicate studies. (B) Morphological cell transformation by trans-DB[a,j]P-8,9-diol and DB[a,j]P expressed as a percentage of dishes exhibiting a type II or III focus. Means and standard deviations from replicate studies. (C) Morphological cell transformation by trans-DB[a,j]P-8,9-diol and DB[a,j]P expressed as the number of type II and III foci per dish.
indistinguishable, as were the morphological cell transformation multiplicity results.

DNA adduct analyses of DB[a,l]P and trans-DB[a,l]P-8,9-diol were performed using a 32P-post-labeling TLC-coupled HPLC technique (3). The technique and elution solvent systems used were designed to separate fjord region anti-DB[a,l]P-11,12-diol-13,14-epoxide (anti-DB[a,l]PDE) and syn-DB[a,l]P-11,12-diol-13,14-epoxide (syn-DB[a,l]PDE) adducts and the more polar trans-DB[a,l]P-8,9-diol–DNA adducts. HPLC chromatograms obtained from the DNA of C3H10T1/2 cells exposed to DB[a,l]P and trans-DB[a,l]P-8,9-diol for 24 h (at concentrations that induced morphological cell transformation) were compared with DNA adduct standards obtained from metabolic activation of DB[a,l]P by recombinant human P450 1A1 microsomes with human epoxide hydrolase and CT-DNA. DNA (50 µg) from C3H10T1/2 cells treated with DB[a,l]P (331 nM) produced a group of DNA adducts (Figure 3A). The total level of adduction of these DNA adducts was 7.85 fmol/µg DNA, as reported earlier (3). The level of adduction was concentration dependent and similar patterns of DNA adducts were observed at lower concentrations (data not shown). These adducts had been previously identified as anti-DB[a,l]PDE–dAdo, anti-DB[a,l]PDE–dGuo, syn-DB[a,l]PDE–dAdo and syn-DB[a,l]PDE–dGuo (3). No polar DNA adducts were observed. Under similar exposure and chromatographic conditions, the DNA (50 µg) from C3H10T1/2 cells treated with trans-DB[a,l]P-8,9-diol (298 nM) contained no observable DNA adducts in each of four replicate investigations (Figure 3B). C3H10T1/2 cells treated with trans-DB[a,l]P-8,9-diol at 198 nM also contained no observable DNA adducts (data not shown). The limit of sensitivity for detection of DNA adducts by this method was 0.05 fmol/µg DNA (RAL = 1.7 × 10^{-6}) (13). Incubation of DB[a,l]P with CT-DNA and recombinant human P450 1A1 microsomes with human epoxide hydrolase gave the same pattern of DB[a,l]P-DNA adducts as reported earlier (3) (Figure 3C). The peaks eluting at 60–65 min represented the syn- and anti-DB[a,l]PDE adducts of dGuo and dAdo. The peak eluting at 47 min has been reported to be derived, in part, from further metabolic activation of trans-DB[a,l]P-8,9-diol. No adducts were observed in the DNA from cells treated with acetone (data not shown) (7). The DNA adduct patterns presented here for DB[a,l]P–DNA adducts in C3H10T1/2 cells differ slightly from the pattern published earlier (7). In the HPLC system used in that study, five individual peaks were observed which were identified as syn- and anti-DB[a,l]PDE adducts of dGuo and dAdo. The HPLC system here was designed to retard early eluting polar adducts and accelerate less polar DNA adducts. Thus, the pattern of DB[a,l]P–DNA adducts in C3H10T1/2 cells in Figure 3A show a compressed elution pattern of all of the syn- and anti-DB[a,l]PDE adducts.

There are a number of competing hypotheses that describe routes of metabolic activation of carcinogenic PAHs. One hypothesis proposes the formation of stable PAH fjord region diol epoxide–DNA adducts, through the initial

---

**Fig. 3.** 32P-post-labeling analyses by TLC/HPLC of DNA adducts from C3H10T1/2 cells treated with trans-DB[a,l]P-8,9-diol or DB[a,l]P and CT-DNA adducts from metabolic activation of DB[a,l]P by recombinant human P450 1A1 microsomes with human epoxide hydrolase. C3H10T1/2 cells in mid-log growth were treated for 24 h with each PAH. After enzymatic hydrolysis of 50 µg of DNA, adducts were enriched by nuclease P1 treatment, 5'–32P-labeled and chromatographed on 10×10 cm PEI–cellulose sheets to remove unmodified nucleotides (D1 direction only). The remaining spot at the origin was excised, eluted with pyridinium formate, the solvent evaporated and the residue analyzed by reverse phase HPLC. (A) DNA adduct chromatogram of C3H10T1/2 cells treated with trans-DB[a,l]P-8,9-diol (331 nM). (B) DNA adduct chromatogram of C3H10T1/2 cells treated with trans-DB[a,l]P-8,9-diol (298 nM). Similar results were obtained with 198 nM trans-DB[a,l]P-8,9-diol (data not shown). (C) DNA adduct chromatogram of CT-DNA incubated with DB[a,l]P and recombinant human P450 1A1 microsomes and epoxide hydrolase. The group of DNA adducts in the bracket at 55–75 min represent a mixture of syn- and anti-DB[a,l]PDE adducts of dGuo and dAdo. The adducts eluting between 35 and 50 min are not related to syn- and anti-DB[a,l]PDE adducts of dGuo and dAdo. The peak in the bracket at 47 min co-elutes with CT-DNA adducts produced by metabolic activation of trans-DB[a,l]P-8,9-diol using recombinant human P450 1A1-containing microsomes and epoxide hydrolase.
formation of dihydrodiols and their subsequent epoxidation (2), while another proposes the formation of unstable bay region or fjord region diol epoxide–DNA adducts (16,17) that lead to apurinic sites (8). Unstable PAH–DNA adducts can also be derived from PAH radical cations (18). The further metabolism of dihydrodiols to bis-diols and their epoxidation to bis-diol epoxides, which form stable DNA adducts (that are more polar than those arising from diol epoxides), has also been proposed (19,20). The predominant microsomal enzymes involved in the formation of PAH dihydrodiols, diol epoxides and bis-diol epoxides are P450 1A1, P450 1B1 and epoxide hydrolase (15,21,22). Another hypothesis proposes that the soluble enzyme dihydrodiol dehydrogenase converts dihydrodiols to their corresponding quinones (23). These quinones can either bind to DNA to form both stable and unstable DNA adducts, or induce reactive oxygen species that alter soluble enzyme dihydrodiol dehydrogenase converts dihydrodiols to their corresponding quinones (23). These quinones can either bind to DNA to form both stable and unstable DNA adducts, or induce reactive oxygen species that alter DNA adducts or undergo redox cycling producing reactive oxygen species that bind to DNA. Many of the DNA adducts identified with each of these pathways are associated with mutations in dominant proto-oncogenes that have been measured in the tumors induced by PAHs (24).

To date, the only reported mechanism of metabolic activation of PAHs in C3H10T1/2 cells is their conversion via dihydrodiols to DNA-reactive bay region and fjord region diol epoxides and, for cyclopenta-PAHs, their conversion to DNA-reactive arene oxides. This has been demonstrated for a number of PAHs, including DB[a]P, in both metabolism (25–27) and DNA adduct studies (7,28–30). The enzymes responsible for metabolic activation of PAHs to genotoxic intermediates in mouse embryo C3H10T1/2 cells are microsomal epoxide hydrolase and a predominantly induced microsomal cytochrome P450, P450 1B1 (31–33). Other metabolic enzymes have also been detected in these cells; the soluble enzymes glucuronyl transferase and PAPS sulfotransferase (25,26). The metabolism and metabolic activation of trans-DB[a]P-8,9-diol by mouse P450 1B1 have not yet been studied. While the human analog of P450 1B1 (as well as human P450 1A1) expressed in V79 cells metabolized trans-DB[a]P-8,9-diol to trans-DB[a]P-8,9-diol-derived bis-diols (34) and formed stable polar trans-DB[a]P-8,9-diol–DNA adducts (35), mouse P450 1B1 has been reported to possess different catalytic and regiospecific properties compared with human P450 1B1 and P450 1A1 (27,36). These differences might explain the detection of stable polar trans-DB[a]P-8,9-diol–DNA adducts in V79 cells expressing human P450 1B1 (and P450 1A1) and the absence of detectable stable polar trans-DB[a]P-8,9-diol–DNA adducts in C3H10T1/2 cells that express mouse P450 1B1.

Several quantitative linear relationships between genotoxic events in B[a]P-treated C3H10T1/2 cells have been described. B[a]P–DNA adduct levels and B[a]P-induced morphological cell transformation are linearly related (30). Similarly, B[a]P-induced ouabain resistance (mutation) and morphological cell transformation are also linearly related (37). Furthermore, B[a]P-induced sister chromatid exchange and chromosomal aberrations in C3H10T1/2 cells have similar dose–response characteristics (38). For PAHs in vivo there are also linear quantitative relationships between DNA adduct levels and mouse skin (39) and mouse lung tumors (40). While it is not known whether the genotoxic events of DNA adduction, mutation, SCE and chromosomal aberrations are in parallel with or sequential to the induction of morphological cell transformation, the predominance of mechanistic studies with genotoxic carcinogens in vivo suggests that DNA damage is a dominant mechanism of action for heritable changes in mammalian cells, including morphological cell transformation. Consequently, the lack of detectable stable DNA adducts in C3H10T1/2 cells treated with trans-DB[a]P-8,9-diol implies either that DNA adducts are formed but are not detectable by our methods or that other types of DNA damage leading to morphological cell transformation have occurred in these cells. There are a number of possible explanations for these events. (i) Unstable DNA (depurinating or depyrimidinating) adducts may be formed that create apurinic sites. Apurinic sites have been observed after DB[a]P administration to mouse skin (8,41). (ii) Dihydrodiol dehydrogenase could activate a potential trans-DB[a]P-8,9-diol bis-diol metabolite [e.g. trans-DB[a]P-8,9:11,12-bis-diol], thus forming a quinone intermediate that could either bind to DNA, generating unstable DNA adducts, or induce reactive oxygen species that alter DNA (e.g. 8-oxo-dGuo). This scheme has been described for trans-B[a]P-7,8-diol (23). (iii) trans-DB[a]P-8,9-diol could induce chromosomal damage in C3H10T1/2 cells.

We conclude that trans-DB[a]P-8,9-diol is a potent morphological cell-transforming agent in C3H10T1/2 cells in the absence of significant cytotoxicity. Its activity approximately equals that of DB[a]P and it gives concentration-related responses. While its mechanism of action is unknown, we find no evidence for the further metabolic activation of trans-DB[a]P-8,9-diol to detectable stable DNA adducts. The observation that trans-DB[a]P-8,9-diol is also an extremely active morphological cell-transforming agent (approximately equal to DB[a]P) warrants further investigations into its mechanisms of action in these cells.

Acknowledgements

We thank the NCI Chemical Carcinogen Reference Repository and Dr David H. Phillips (Haddow Laboratories, The Institute of Cancer Research) for their assistance with research materials. The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

References


Received October 7, 1999; revised February 8, 2000; accepted February 17, 2000.

DB[a]/JP/8,9-diol-induced transformation