Metabolic Activation of Dibenzo[*a*,*l*]pyrene by Human Cytochrome P450 1A1 and P450 1B1 Expressed in V79 Chinese Hamster Cells

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Metabolic activation of the strongly carcinogenic polycyclic aromatic hydrocarbon (PAH) dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P) and its *trans*-8,9-dihydrodiol (*trans*-8,9-diol) catalyzed by human cytochromes P450 (P450) 1A1 and 1B1 was investigated. DNA binding of DB[*a*,*l*]P in mammalian cell lines has previously been shown to be preferentially mediated by fjord region DB[*a*,*l*]P-11,12-dihydrodiol 13,14-epoxides (DB[*a*,*l*]PDE). In order to elucidate different capabilities of both P450 enzymes for metabolic activation of DB[*a*,*l*]P V79 Chinese hamster cells, stably expressing human P450s 1A1 or 1B1 have been exposed to the parent PAH or its racemic *trans*-8,9-diol. For this purpose, synthesis and spectroscopic characterization of the *trans*-DB-[*a*,*l*]P-8,9-diol and its individual enantiomers have been achieved. Both human P450-expressing cell lines were capable of transforming DB[*a*,*l*]P to its fjord region DB[*a*,*l*]PDE, but the extent of metabolism to DB[*a*,*l*]PDE catalyzed by human P450 1B1 was higher compared to human P450 1A1 at all times measured. On the other hand, cytotoxicity studies performed with the same incubation systems emerged stronger effects by DB[*a*,*l*]P and its enantiomeric *trans*-11,12-diols in human P450 1A1-expressing cells. Both human P450 enzymes stereospecifically catalyzed the formation of the (−)-DB[*a*,*l*]P-11,12-diol with *R*,*R*-configuration, whereas only the human P450 1A1-expressing cells form small amounts of the K-region *trans*-8,9-diol with high excess of the (+)-(8*R*,9*R*)-enantiomer. Application of *trans*-DB[*a*,*l*]P-8,9-diol in metabolism studies revealed that this compound is converted by human P450s 1A1 and 1B1 to several diol phenols and *bis*-diols. However, and even at concentrations as high as 10 μM, in both cell lines the *trans*-DB[*a*,*l*]P-8,9-diol showed no cytotoxicity at all, suggesting that an activation of DB[*a*,*l*]P via further oxidation of the K-region *trans*-8,9-diol plays a minor role.

Introduction

Metabolic activation of polycyclic aromatic hydrocarbons (PAH)[1] is predominantly catalyzed by members of the endoplasmatically bound superfamily of cytochrome P450 (P450) enzymes which belong to the group of monooxygenases (*1*−*4*). Biotransformation of PAH by P450 enzymes displays, in general, different regio- and stereoselectivities depending on the particular P450 enzyme and the structural properties of the aromatic hydrocarbon under consideration (*5*, *6*). It has been widely demonstrated that P450 1A1, irrespective of the species of origin, is one of the most important enzymes involved in metabolic activation of various carcinogenic PAH to bay or fjord region diol epoxides (*7*−*10*). Although P450 1A1 is virtually inactive in human tissues in the absence of inducers (*4*, *11*), it may play an important role in long-term induction of tumorigenicity in different human organs due to its inducibility by various PAH (*12*, *13*).

The view that P450 1A1 has an outstanding role for the biotransformation of PAH to proximate or ultimate carcinogens is in need for partial revision since P450 1B1, another member enzyme of the P450 family recently discovered and detected in rodent and human tissues (*14*−*18*), has been characterized regarding its enzymatic activity toward a wide range of PAH (*19*). After transfection of human P450 1B1 into V79 Chinese hamster cells and subsequent verification of its stable expression

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[1] Abbreviations: B[*a*]A, benz[*a*]anthracene; B[*a*]P, benzo[*a*]pyrene; B[*c*]Ph, benzo[*c*]phenanthrene; CSP, chiral stationary phase; DB[*a*,*h*]A, dibenz[*a*,*h*]anthracene; DB[*a*,*l*]P, dibenzo[*a*,*l*]pyrene; (+)-*syn*-DB[*a*,*l*]-PDE, (+)-*syn*-dibenzo[*a*,*l*]pyrene-(11*S*,12*R*)-dihydrodiol (13*S*,14*R*)-epoxide; (−)-*anti*-DB[*a*,*l*]PDE, (−)-*anti*-dibenzo[*a*,*l*]pyrene-(11*R*,12*S*)-dihydrodiol (13*S*,14*R*)-epoxide; DB[*a*,*l*]P-*bis*-MAA-ester, *trans*-11,12-*bis*[(−)-menthoxyacetoxy]-11,12-dihydrodibenzo[*a*,*l*]pyrene; (*r*,*t*,*c*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol, *r*-11,*t*-12,*c*-13,*c*-14-tetrahydroxy-11,12,13,14-tetrahydrodibenzo[*a*,*l*]pyrene; (*r*,*t*,*t*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol, *r*-11,*t*-12,*t*-13,*c*-14-tetrahydroxy-11,12,13,14-tetrahydrodibenzo[*a*,*l*]pyrene; DDQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone; diol, dihydrodiol; DMBA, 7,-12-dimethylbenz[*a*]anthracene; DMEM, Dulbecco Vogt's modified Eagle's medium; FD, field desorption; GC, gas chromatography; G418, geneticin sulfate; HPLC, high-performance liquid chromatography; MAA-Cl, (−)-menthoxyacetyl chloride; MC, 3-methylcholanthrene; MS, mass spectra; *O*-TMS, *O*-trimethylsilyl; P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon(s).

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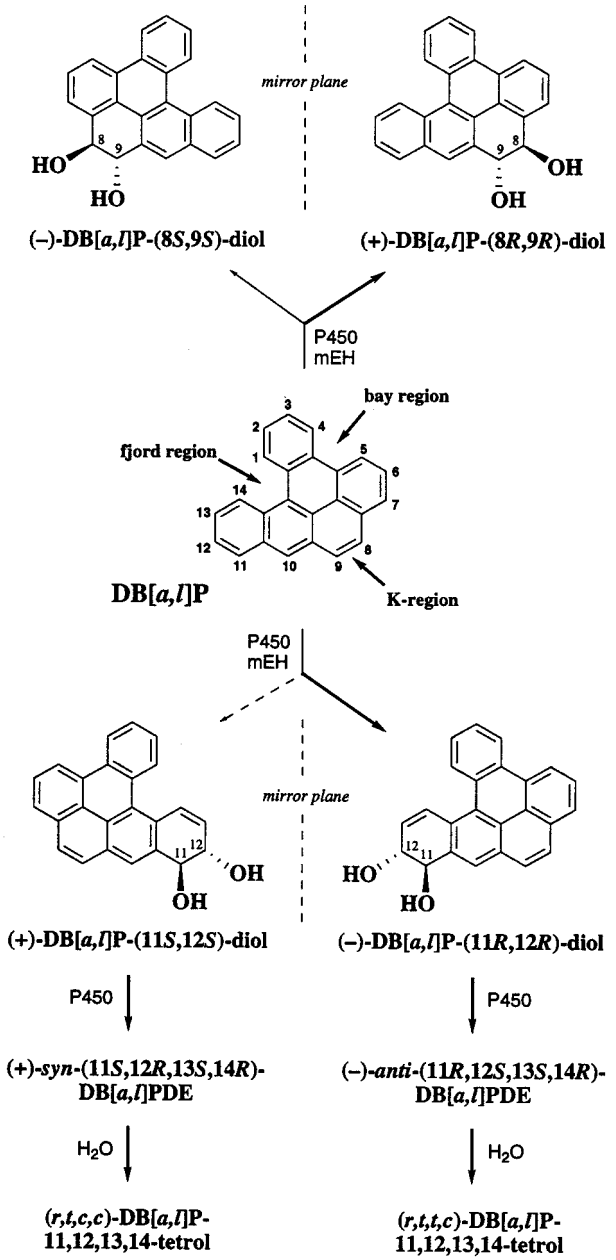
Scheme 1. Stereoselective Bioactivation of Dibenzo[*a,l*]pyrene in Its Fjord and K-Region, Catalyzed by Cytochromes P450 (P450) and Microsomal Epoxide Hydrolase (mEH)[a]



[a] The scheme includes activation of DB[*a,l*]P to the diastereomeric fjord region (+)-*syn*- and (−)-*anti*-DB[*a,l*]PDE via the corresponding metabolic precursors, the (+)- and (−)-*trans*-DB[*a,l*]-P-11,12-diol, respectively. Detection of the electrophilically reactive DB[*a,l*]PDE was performed after acidic workup of the incubation mixture (pH 3) via measurement of the corresponding hydrolysis products, the (*r,t,c,c*)- and (*r,t,t,c*)-DB[*a,l*]P-11,12,13,14-tetrols, as outlined previously (*34, 36*). The scheme also includes K-region metabolism leading to enantiomeric (+)- and (−)-*trans*-DB[*a,l*]P-8,9-diols.

(*20*), it was demonstrated that this enzyme plays a crucial role in the metabolic activation of benzo[*c*]phenanthrene (B[*c*]Ph), the prototypic PAH with a fjord region (*10*). Using the ^{33}P-postlabeling assay in conjunction with HPLC separation techniques, it was also demonstrated that both human P450s 1A1 and 1B1 are mainly responsible for DNA binding of the very potent carcinogenic hexacyclic dibenzo[*a,l*]pyrene (DB[*a,l*]P, Scheme 1) (*20*). DB[*a,l*]P is stereoselectively metabolized by human P450 1B1 with the same stereoselectivity as previously found in human mammary carcinoma MCF-7 cells (*21, 22*): only (+)-(1*S*,12*R*,13*S*,14*R*)- and (−)-(11*R*,12*S*,13*S*,14*R*)-DB-[*a,l*]P-11,12-dihydrodiol 13,14-epoxides [(+)-*syn*- and (−)-*anti*-DB[*a,l*]PDE] were catalytically generated via their respective precursors, the (+)-DB[*a,l*]P-(11*S*,12*S*)- and (−)-DB[*a,l*]P-(11*R*,12*R*)-dihydrodiols [(+)- and (−)-*trans*-DB[*a,l*]P-11,12-diols], respectively (Scheme 1). In contrast, human P450 1A1-expressing V79 cells were competent to produce both the well-known DB[*a,l*]PDE-adducts as well as several highly polar adducts as yet unidentified (*20*).

DB[*a,l*]P has been characterized as the strongest carcinogen among all PAH tested to date in mouse skin and rat mammary gland (*23*−*26*). High tumor rates obtained after direct administration of the (−)-*trans*-DB-[*a,l*]P-11,12-diol on mouse skin (*27*) and the finding that exclusively (−)-*anti*-DB[*a,l*]PDE-DNA-adducts could be detected in mouse skin after application of the parent compound (*28*) reasonably suggest the assumption that tumor initiation by DB[*a,l*]P is mainly mediated by metabolic formation of the fjord region (−)-*anti*-DB[*a,l*]-PDE (Scheme 1). Most importantly, due to its presence in various environmental sources (*29*−*33*) and its exceptional high carcinogenicity, any exposure to DB[*a,l*]P has to be considered as a serious risk for human health.

In order to follow up our goal to clarify the role of individual human P450 enzymes during toxification of DB[*a,l*]P and to supplement our findings on the DNA binding of this PAH (*20*), human P450 1A1- and 1B1-expressing V79 Chinese hamster cell lines have been exposed to DB[*a,l*]P and its racemic and enantiomeric *trans*-8,9- and *trans*-11,12-diols. The different patterns of metabolites formed from DB[*a,l*]P and its *trans*-8,9-diol as well as the corresponding cytotoxicity emerged during incubation have been analyzed and quantified. Therefore, the results presented may contribute to a better understanding of the toxification of the very potent carcinogen DB[*a,l*]P by P450 1A1 and 1B1 in man.

Experimental Procedures

Caution: *All derivatives of DB[a,l]P used in this study are potential carcinogenic agents and should be handled in an appropriate manner.*

Instrumentation and Chemicals. Melting points were determined on a Büchi 510 apparatus using unsealed capillary tubes and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AM 400 operating at 400 MHz. Chemical shifts (δ) are reported as ppm downfield from tetramethylsilane. UV spectra were obtained with a Shimadzu MPS-2000 spectrophotometer. Field desorption (FD) mass spectra (MS) were run on a Finnigan MAT 90 mass spectrometer. Specific optical rotations ($[\alpha]_D^{20}$) were obtained with a Perkin-Elmer polarimeter (Model 241) at 20 °C. CD spectra were recorded on an Instruments S. A. Jobin Yvon CD6-Dichrograph. Preparative high-performance liquid chromatography (HPLC) purifications were conducted on a Du Pont 830 using a silica gel (LiChrosorb Si 60, 5 μm) column (16 × 250 mm). MS of the reference compounds and metabolites separated by preparative HPLC were run on a Varian MAT 112 S instrument after derivatization into their *O*-trimethylsilyl-(*O*-TMS)-ethers. Gas chromatography (GC) separations were carried out on a Perkin-Elmer PE 8320 instrument with FID-detector or in conjunction with the above-mentioned Varian MAT 112 S mass spectrometer operating at 70 eV. This instrument was adapted to the aforementioned GC device using the same operating conditions. Analytical HPLC separation of

the metabolites was performed on a Hewlett-Packard HP 1090 instrument coupled to a diode array detector.

Coronene (99.8%) and DB[*a,l*]P (99.8%) were purchased from Promochem GmbH (Wesel, Germany). Trisil and *N,O-bis*(trimethylsilyl)trifluoroacetamide used for the derivatization of hydroxyl group-containing metabolites were obtained from Pierce (Rockford, IL). Solvents used for cleanup and for HPLC were freshly distilled or of high grade purity for spectroscopy. 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) was obtained from Aldrich (Steinheim, Germany). (−)-Menthoxyacetyl chloride [(−)-MAA-Cl] was synthesized by the reaction of (−)-MAA (Aldrich; $[\alpha]_D^{20} = -92.5°$) with thionyl chloride (Fluka, Neu-Ulm, Germany) as described (*34*). Synthesis of the racemic and enantiomerically pure *trans*-DB[*a,l*]P-11,12-diols (*34*, *35*) and preparation of the diastereomeric DB[*a,l*]P-11,12,13,14-tetrols (*36*) were described elsewhere.

Synthesis of DB[*a,l*]P-8,9-quinone. DB[*a,l*]P (350 mg; 1.16 mmol) was dissolved in pyridine (50 mL) and 294 mg (1.16 mmol) of OsO_4 was added. The mixture was stirred at room temperature for 6 days until the reaction was finished as checked by TLC. Following the addition of 10% $NaHSO_3$ (17 mL), the mixture was stirred for an additional hour at room temperature and then poured into 500 mL of water. After precipitation, the crude product was filtered off, washed with water, and dried in a desiccator over $CaCl_2$. Then 310 mg (0.92 mmol) of the crude *cis*-DB[*a,l*]P-8,9-diol was reacted with DDQ (0.91 g, 4.0 mmol) in 1,4-dioxane (35 mL) under an argon atmosphere for 7 days. Then the mixture was concentrated under reduced pressure and dissolved in $CHCl_3$. Purification was achieved by successive extractions with 10% Na_2CO_3 (3 times) and water. The organic layer was dried over $MgSO_4$, and the solvent was removed under vacuum. Pure 8,9-quinone (245 mg; 64% yield) was obtained as orange crystals by washing with ice-cold Et_2O, mp = 315-317 °C (decomp): UV, λ_{max} [nm, ϵ (cm^2 $mmol^{-1}$)] 258 (17 100), 282 (19 600), 292 (17 600), 331 (7100) 345 (6800); ^1H NMR ($CDCl_3$), δ (ppm) 8.99 (d, 1, H-14, $J_{13,14}$ = 7.3 Hz), 8.95 (s, 1, H-10), 8.92 (d, 1, H-1, $J_{1,2}$ = 8.6 Hz), 8.86 (d, 1, H-4, $J_{3,4}$ = 8.2 Hz), 8.74 (d, 1, H-5, $J_{5,6}$ = 8.4 Hz), 8.57 (dd, 1, H-7, $J_{6,7}$ = 7.5 Hz, $J_{5,7}$ = 1.1 Hz), 8.17 (d, 1, H-11, $J_{11,12}$ = 8.1 Hz), 7.84 (pseudo-t, 1, H-6, $J_{5,6}$ = 8.0 Hz, $J_{6,7}$ = 7.7 Hz), 7.81−7.77 (m, 2, H-12, H-13), 7.75−7.67 (m, 2, H-2, H-3); FD-MS, *m/z* (relative intensity) 333 ($[M + H]^+$, 21), 332 ($[M]^+$, 45), 305 (333 − CO, 21), 304 (332 − CO, 100).

Synthesis of (±)-*trans*-DB[*a,l*]P-8,9-diol. Reduction of DB-[*a,l*]P-8,9-quinone (166 mg, 0.5 mmol) dissolved in ethanol/water (50 mL, 9/1, v/v) was performed with $NaBH_4$ (189 mg, 5 mmol) in suspension (*35*, *37*) by vigorously stirring of the mixture in an open vessel for 3 days. After complete reduction (checked by TLC), 20 mL of water was added, and the mixture was concentrated under reduced pressure. To complete the precipitation, 150 mL of water was added and the product was isolated by filtration and dried in a desiccator over $CaCl_2$. Purification was accomplished by flash chromatography on silica gel using $CHCl_3/CH_3OH$ (9/1, v/v; R_f = 0.48) as eluent. Removal of the solvent under vacuum and subsequent sonication of a suspension in CH_3OH provided 160 mg (95% yield) of *trans*-(+)-DB-[*a,l*]P-8,9-diol as pure colorless crystals of mp = 208−210 °C (decomp): UV, λ_{max} [nm, ϵ (cm^2 $mmol^{-1}$)] 206 (76 200), 278 (56 400), 288 (59 000), 310 (10 100), 323 (10 700), 336 (8000); ^1H NMR (acetone-d_6, DMSO-d_6), δ (ppm) 8.97−8.89 (m, 3, H-1, H-4, H-14), 8.75 (d, 1, H-5, $J_{5,6}$ = 8.2 Hz), 8.29 (s, 1, H-10), 8.14 (dd, 1, H-11, $J_{11,12}$ = 7.2 Hz, $J_{11,13}$ = 2.1 Hz), 8.00 (d, 1, H-7, $J_{6,7}$ = 7.3 Hz), 7.78 (pseudo-t, 1, H-6, $J_{5,6}$ = 8.0 Hz, $J_{6,7}$ = 7.6 Hz), 7.77−7.71 (m, 2, H-12, H-13), 7.70−7.62 (m, 2, H-2, H-3), 5.74 (d, 1, −OH, J = 4.7 Hz), 5.66 (d, 1, −OH, J = 5.0 Hz), 5.06 (dd, 1, H-8/H-9, $J_{8,9}$ = 9.8 Hz, $J_{CH,OH}$ = 3.6 Hz), 4.93 (dd, 1, H-8/H-9, $J_{8,9}$ = 9.7 Hz, $J_{CH,OH}$ = 4.7 Hz); FD-MS, *m/z* (relative intensity) 337 ($[M + H]^+$, 23), 336 ($[M]^+$, 100).

Synthesis of Enantiomeric *trans*-DB[*a,l*]P-8,9-diols. The synthesis of the (+)- and (−)-*trans*-DB[*a,l*]P-8,9-diols was accomplished via chromatographic separation of the diastereomeric *bis*-(−)-MAA-esters (*34*). To a solution of (+)-DB[*a,l*]P-8,9-diol (81.4 mg, 0.242 mmol) in pyridine (30 mL) was added (−)-MAA-Cl (342 mg, 1.45 mmol), and the reaction mixture was stirred under argon at 4 °C. After 24 h, the reaction was completed as monitored by TLC, and the solvent was removed by distillation under reduced pressure. The crude product was subjected to flash chromatography on silica gel with $CHCl_3$ as mobile phase (R_f = 0.78) to give 150 mg of a mixture of the diastereomeric *bis*-(−)-MAA-esters (85% yield) as a colorless solid. HPLC separation of the two diastereomeric esters was achieved on a LiChrosorb Si 60 (5 μm) column (16 × 250 mm) using cyclohexane/diethyl ether (95/5, v/v, 8 mL/min) as eluent (*34*).

Early eluting diastereomeric ester (−)-*trans*-(8*R*,9*R*)-*bis*-[(−)-menthoxyacetoxy]-8,9-dihydrodibenzo[*a,l*]pyrene [(−)-DB-[*a,l*]P-(8*R*,9*R*)-*bis*-MAA-ester] (57 mg; 38% yield): retention time 28 min; mp = 75−76 °C; $[\alpha]_D^{20} = -335°$ (*c* 0.0274, THF); UV, λ_{max} [nm, ϵ (cm^2 $mmol^{-1}$)] 213 (38 900), 268 (47 900), 278 (58 400), 288 (60 200), 322 (12 000), 335 (10 500); ^1H NMR ($CDCl_3$), δ (ppm) 8.95−8.93 (m, 2, H-1, H-14, $J_{1,2} = J_{13,14}$ = 8.3 Hz), 8.75 (dd, 1, H-4, $J_{3,4}$ = 8.1 Hz, $J_{2,4}$ = 1.3 Hz), 8.72 (d, 1, H-5, $J_{5,6}$ = 7.6 Hz), 8.12 (s, 1, H-10), 8.02 (d, 1, H-11, $J_{11,12}$ = 7.7 Hz), 7.80 (d, 1, H-7, $J_{6,7}$ = 6.8 Hz), 7.74−7.60 (m, 5, H-2, H-3, H-6, H-12, H-13), 6.55 (d, 1, H-8/H-9, $J_{8,9}$ = 3.8 Hz), 6.50 (d, 1, H-8/H-9, $J_{8,9}$ = 3.8 Hz), 3.91 (s, 2, $-OCH_AH_BCO_2-$, J_{gem} = 0 Hz), 3.89 (s, 2, $-OCH_AH_BCO_2-$, J_{gem} = 0 Hz), 2.99−0.59 (m, 38, $H_{menthoxy-}$); FD-MS, m/z (relative intensity) 730 ($[M + H]^+$, 45), 729 ($[M]^+$, 100).

Late eluting diastereomeric ester (+)-*trans*-(8*S*,9*S*)-*bis*-[(−)-menthoxyacetoxy]-8,9-dihydrodibenzo[*a,l*]pyrene [(+)-DB[*a,l*]P-(8*S*,9*S*)-*bis*-MAA-ester] (46 mg; 31% yield): retention time 36 min; mp = 110−111 °C; $[\alpha]_D^{20} = +417°$ (*c* 0.0424, THF); UV, λ_{max} [nm, ϵ (cm^2 $mmol^{-1}$)] 213 (41 200), 268 (51 500), 278 (63 200), 288 (65 500), 322 (12 800), 335 (10 900); ^1H NMR ($CDCl_3$), δ (ppm) 8.96−8.94 (m, 2, H-1, H-14, $J_{1,2} = J_{13,14}$ = 8.1 Hz), 8.75 (d, 1, H-4, $J_{3,4}$ = 7.9 Hz), 8.72 (d, 1, H-5, $J_{5,6}$ = 8.4 Hz), 8.11 (s, 1, H-10), 8.02 (d, 1, H-11, $J_{11,12}$ = 7.7 Hz), 7.79 (d, 1, H-7, $J_{6,7}$ = 7.2 Hz), 7.74−7.60 (m, 5, H-2, H-3, H-6, H-12, H-13), 6.54 (d, 1, H-8/H-9, $J_{8,9}$ = 3.8 Hz), 6.47 (d, 1, H-8/H-9, $J_{8,9}$ = 3.8 Hz), 3.92 (AB-system, 2, $-OCH_AH_BCO_2-$, J_{gem} = 16.5 Hz), 3.91 (AB-system, 2, $-OCH_AH_BCO_2-$, J_{gem} = 16.5 Hz), 2.98−0.55 (m, 38, $H_{menthoxy}$); FD-MS, *m/z* (relative intensity) 730 ($[M + H]^+$, 59), 729 ($[M]^+$, 100).

The (+)- and (−)-*trans*-DB[*a,l*]P-8,9-diol*s* were obtained by methanolysis of the (−)- and (+)-DB[*a,l*]P-8,9-*bis*-MAA-ester, respectively. Thereby the *bis*-MAA-ester (40 mg, 0.055 mmol) was dissolved in THF (30 mL), and a solution of sodium methoxide (7.1 mg, 0.13 mmol) in 10 mL methanol was added. The mixture was stirred at room temperature until the reaction had been shown by TLC to be completed (about 4 h). Then 10 mL of ice-cold water was added, and the organic solvent was removed under vacuum. The crude mixture was poured into 40 mL of water, and the separated product was filtered, washed with water, and dried over silica gel. The residue was purified by flash chromatography on silica gel using $CHCl_3/CH_3OH$ (9/1, v/v) as mobile phase (R_f = 0.48) to remove some trace amounts of DB[*a,l*]P-8,9-quinone formed during work-up. Subsequently the compounds were dissolved again in THF and poured dropwise into ice-cold water. The enantiomerically pure (+)- or (−)-*trans*-DB[*a,l*]P-8,9- diols were obtained as colorless crystals after filtration, washing with water, and drying of the precipitate.

Specific optical rotations of (+)-DB[*a,l*]P-(8*R*,9*R*)-diol (5.3 mg obtained; 29% yield) and (−)-DB[*a,l*]P-(8*S*,9*S*)-diol (10.4 mg obtained, 56% yield) were measured as $[\alpha]_D^{20} = +173°$ (*c* 0.01565, THF) and $[\alpha]_D^{20} = -191°$ (*c* 0.02769, THF), respectively.

Cell Lines, Cell Culture, and Enzyme Activities. The parental V79MZ cell line (*38*) and the genetically engineered cell lines V79MZ-h1A1 (*39*) and V79MZ-h1B1 (*20*) expressing human P450 1A1 and 1B1, respectively, were cultivated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 4.5 g of glucose/L, 1 mM sodium pyruvate, 4 mM L-glutamine, 10% fetal calf serum, 100 units of penicillin/mL, and

100 μg of streptomycin/mL at 37 °C, 7% CO_2 and 90% saturated atmospheric humidity. The V79 cell clones used in the present study were V79Mz-h1A1 and the human P450 1B1-expressing cell clone #6 with an ethoxyresorufin *O*-deethylation activity of 22.0 ± 0.9 and 5.11 ± 0.36 pmol min^{-1} mg^{-1}, respectively (*20*). Genetically engineered cells were cultivated in the presence of 500 μg of geneticin sulfate (G418)/mL, which was not present during metabolism studies. Homogenization of cells and measurement of protein concentrations and P450 activities were carried out as described previously (*10*).

Cytotoxicity Assay. Approximately 500 cells per well were seeded in a 96 well microtiter plate and cultivated in G418-free DMEM medium for 24 h. Then, the medium was replaced by fresh medium containing DB[*a*,*l*]P or the racemic or enantiomeric *trans*-DB[*a*,*l*]P-8,9- or *trans*-DB[*a*,*l*]P-11,12-diols (Scheme 1) in eight replicate wells at concentrations between 0.01 nM and 10 μM. After 72 h of exposure, cell viability was analyzed using the Neutral Red assay as described (*10*).

Metabolism Studies. Human P450 1A1- and 1B1-expressing cell lines were used to study the metabolism of DB[*a*,*l*]P and the racemic *trans*-DB[*a*,*l*]P-8,9-diol. Approximately 10^6 cells were seeded and cultivated for 24 h prior to treatment. Then, cells were exposed to 1 μM DB[*a*,*l*]P or to 10 μM of its racemic *trans*-8,9-diol. Incubations were performed in glass tissue culture flasks for different time periods (6 and 12 h). After incubation, the supernatant medium was centrifuged to eliminate cell debris and kept at −20 °C until further analysis.

Supernatant medium of the cell cultures and cell homogenates obtained by sonication was adjusted to pH 3.0 with acetic acid, 4 μM coronene dissolved in toluene was added as internal standard, and the mixture was extracted twice with ethyl acetate. The extract was dried over sodium sulfate after addition of 50 μg of 2,6-di-*tert*-butyl-*p*-cresol in 1 mL of 2-propanol as antioxidant, and was cautiously evaporated to near dryness, leaving a volume of 0.2 mL. This was purified by column chromatography on 0.7 g of Sephadex LH 20, using 2.5 mL of 2-propanol as eluent to remove lipids. DB[*a*,*l*]P and its metabolites were eluted from this column with 25 mL of methanol. The effluent was evaporated under vacuum at 200 mbar and reduced to a total volume of 1.5 mL using a vacuum controller. The sample was concentrated to a volume of approximately 50 μL in a nitrogen stream and was subjected to HPLC analysis. Metabolites were derivatized for GC and GC/MS analysis with Trisil and *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide as described (*40*).

HPLC analysis of the DB[*a*,*l*]P metabolites was performed on a Bakerbond PAH 16 plus column (3.0 × 250 mm) by a methanol/water gradient starting with 20/80 (v/v) for 1 min isocratically, then changing the gradient to 30/70 (v/v) within 2 min and finally to 100% methanol within 90 min at 0.4 mL/min. Alternatively, a gradient from 20/80 (v/v), 5 min isocratically, to 100% methanol within 90 min has been applied. After measurement of individual UV-response factors (external standardization), DB[*a*,*l*]P and its metabolites were identified by comparison of their relative HPLC retention times to those of the authentic reference compounds compiled in Table 1. The amount of *syn*- or *anti*-DB[*a*,*l*]PDE formed during incubation could be indirectly quantified by measurement of the corresponding hydrolysis products, the (*r*,*t*,*c*,*c*)- and (*r*,*t*,*t*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrols, as outlined previously (*34*, *36*) (cf. Scheme 1). Metabolites for which no authentic reference compounds were available have been characterized by their UV spectra which displayed the typical absorption maxima of the *trans*-DB[*a*,*l*]P-8,9- and -11,12-diols in the case of the *trans*-DB[*a*,*l*]-P-8,9-diol phenols I−V and *trans*-DB[*a*,*l*]P-11,12-diol phenol, respectively, listed in Table 1. Quantitation was performed by integrating the area under the curve. For further characterization or identification of the metabolites formed from either DB-[*a*,*l*]P or the *trans*-DB[*a*,*l*]P-8,9-diol, the material underlying each peak in the HPLC run was collected, derivatized to the corresponding *O*-TMS-ether, and analyzed by GC/MS (Table 2). The GC separation was carried out with a Nordion 25 m × 0.32 mm NB-54 coated silica capillary (HNU-Nordion, Helsinki, Finland). After injection of the sample at 100 °C, the following temperature program was applied: (i) 100-120 °C with 30 °C/min followed by 3 min isothermal heating; (ii) 120−150 °C with 10 °C/min; and (iii) 150−275 °C with 3 °C/min. GC was coupled to the mass spectrometer which operated at 70 eV. Comparison of the GC retention times and the characteristic fragmentation patterns observed in the mass spectra with those of *O*-TMS-ethers of the reference metabolites (Table 2) allowed the identification of the 8,9- and 11,12-diols and the characterization of individual phenols, diol phenols, and tetrols of DB[*a*,*l*]P. For analysis of the enantiomeric composition of the metabolically formed *trans*-DB[*a*,*l*]P-*8*,*9*- and *trans*-DB[*a*,*l*]P-11,12-diols, the collected peaks from the reversed-phase HPLC runs representing these individual species were subjected to chiral stationary phase (CSP) HPLC. Enantiomers of both diols were resolved on a Daicel Chiralcel ODH[cellulose−tris-*N*-(3,5-dimethylphenyl)carbamate on 5 μm silica gel particles] column (4.6 × 250 mm) with a pre-column (4.6 × 50 mm) (Baker, Gross-Gerau, Germany) by isocratic elution with ethanol/*n*-heptane (1/9, v/v) (*41*). The enantiomers of the *trans*-DB[*a*,*l*]P-8,9-diol were recorded at 290 (±10) nm, whereas a window of 305 (±5) nm was used to detect the enantiomers of the *trans*-DB[*a*,*l*]P-11,12-diol.

Table 1. Relative HPLC Retention Times Related to (i) Coronene as Internal Standard (RRT_{COR}) and to (ii) Dibenzo[*a*,*l*]pyrene ($RRT_{DB[a,l]P}$) = 1.00 as Well as Response Factors (RF_{COR}) Related to Coronene

compound	RRT_{COR}	$RRT_{DB[a,l]P}$	RF_{COR}[a]
coronene	1.00	1.28	1.00
DB[*a*,*l*]P	0.78	1.00	1.68
DB[*a*,*l*]P-7-phenol[b]	0.64	0.82	1.68[c]
trans-DB[*a*,*l*]P-8,9-diol	0.36	0.46	5.71
trans-DB[*a*,*l*]P-11,12-diol	0.43	0.55	2.93
cis-DB[*a*,*l*]P-11,12-diol	0.41	0.53	2.91
(*r*,*t*,*c*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol	0.24	0.31	6.17
(*r*,*t*,*t*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol	0.16	0.21	6.20
trans-DB[*a*,*l*]P-11,12-diol phenol	0.22	0.28	2.93[c]
trans-DB[*a*,*l*]P-8,9-diol phenol I	0.25	0.32	5.71[c]
trans-DB[*a*,*l*]P-8,9-diol phenol II	0.26	0.33	5.71[c]
trans-DB[*a*,*l*]P-8,9-diol phenol III	0.28	0.36	5.71[c]
trans-DB[*a*,*l*]P-8,9-diol phenol IV	0.29	0.37	5.71[c]
trans-DB[*a*,*l*]P-8,9-diol phenol V	0.30	0.38	5.71[c]
trans-DB[*a*,*l*]P-8,9:*n*,*m*-*bis*-diol I[d]	0.12	0.15	5.71[c]
trans-DB[*a*,*l*]P-8,9:*n*,*m*-*bis*-diol II[d]	0.14	0.18	5.71[c]
trans-DB[*a*,*l*]P-8,9:*n*,*m*-*bis*-diol III[d]	0.15	0.19	5.71[c]
trans-DB[*a*,*l*]P-8,9:*n*,*m*-*bis*-diol IV[d]	0.16	0.20	5.71[c]

[a] Recorded at 305 ± 10 nm. [b] Tentatively identified by comparison of retention times to those published (*43*). [c] Calculated on the basis of RF_{COR} of DB[*a*,*l*]P, *trans*-DB[*a*,*l*]P-8,9-diol, and *trans*-DB[*a*,*l*]P-11,12-diol. [d] *trans*-DB[*a*,*l*]P-8,9:*n*,*m*-*bis*-diols possess in addition to the 8,9-diol group one further vicinal diol increment with $m = n + 1$.

Results

Preparation of Enantiomerically Pure *trans*-DB-[*a*,*l*]P-8,9-diols. The method described by Bushman et al. (*42*) for the synthesis of K-region diols has been adapted for preparation of *trans*-DB[*a*,*l*]P-8,9-diol. The parent PAH was reacted with OsO_4 in pyridine to provide the *cis*-DB[*a*,*l*]P-8,9-diol. Dehydrogenation of the crude *cis*-DB[*a*,*l*]P-8,9-diol with DDQ furnished the orange-colored *o*-DB[*a*,*l*]P-8,9-quinone in 64% yield. Subsequent reduction of the DB[*a*,*l*]P-8,9-quinone with sodium borohydride in the presence of oxygen as described (*35*, *37*) produced stereospecifically *trans*-DB[*a*,*l*]P-8,9-diol in high yield. The structure of the *trans*-DB[*a*,*l*]P-8,9-diol was unequivocally verified by its 400-MHz ^{1}H NMR spectrum (Experimental Procedures). Mass and UV spectra obtained were fully in accordance with those of the metabolically generated *trans*-DB[*a*,*l*]P-8,9-diol reported by

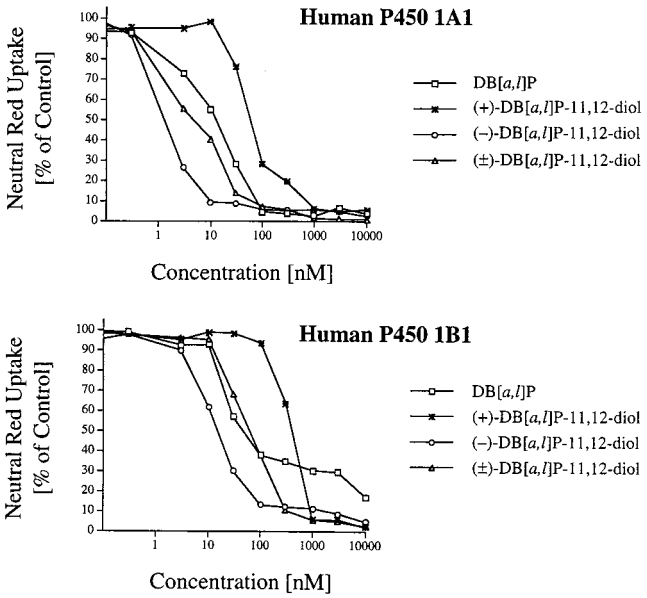


Figure 1. Cytotoxicity of DB[*a,l*]P and its racemic or enantiomeric *trans*-11,12-diols in V79 Chinese hamster cells stably expressing human P450 1A1 or 1B1. The values given represent the mean out of two independent experiments, and deviations of each individual value were found within the range of ≤21%. The Neutral Red uptake test was performed as described under Experimental Procedures.

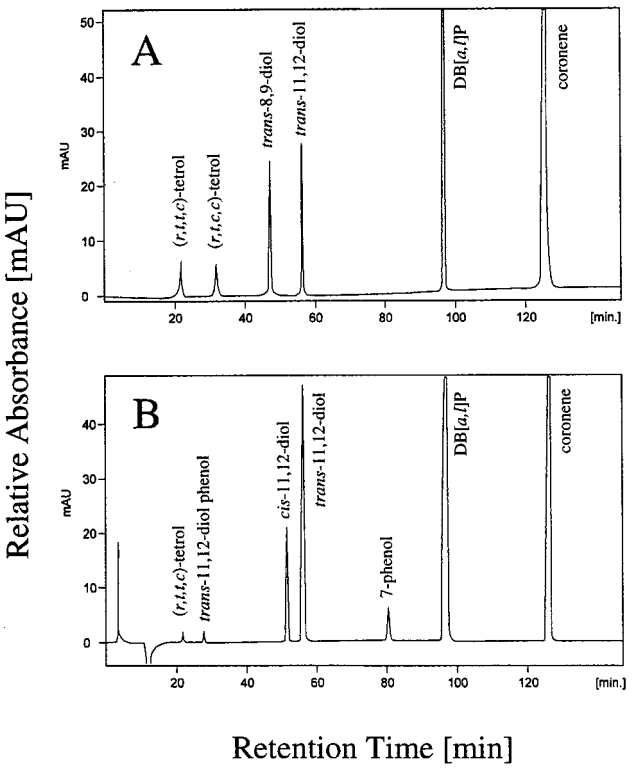


Figure 2. HPLC separation of DB[*a,l*]P, its metabolites, and coronene (used as internal standard). Panel A shows chromatographic separation of all authentic reference compounds obtained by synthetic preparation. Panel B represents a typical HPLC pattern obtained after incubation of DB[*a,l*]P with V79 Chinese hamster cells stably expressing human P450 1B1. Details of chemical synthesis and HPLC separation are given under Experimental Procedures.

Devanesan et al. (*43*). In order to determine the enantiomeric composition of the *trans*-DB[*a,l*]P-8,9-diol formed during metabolic transformation of the parent PAH, separation of the individual enantiomers via chromatography of their diastereomeric *bis*-(−)-*MAA* esters was performed. This method, already successfully applied for the separation of the enantiomeric *trans*-DB[*a,l*]P-11,12-diols (*34*) and *trans*-diols of other PAH (*44*−*46*), has been found to provide sufficient amounts of the (+)- and (−)-*trans*-DB[*a,l*]P-8,9-diol. In contrast to the 11,12-isomers of DB[*a,l*]P (*34*), methanolysis of the diastereomeric (−)- and (+)-DB[*a,l*]P-8,9-*bis*-MAA-esters furnished in each case the *trans*-DB[*a,l*]P-8,9-diol with an optical rotation of opposite sign: the early eluting (−)-DB[*a,l*]P-(8*R*,9*R*)-*bis*-MAA-ester provided the (+)-*trans*-DB[*a,l*]P-8,9-diol with *R,R*-configuration, and the late eluting (+)-DB[*a,l*]-P-(8*S*,9S)-*bis*-MAA-ester provided the (−)-*trans*-DB[*a,l*]P-8,9-diol with *S,S*-configuration. Therefore, absolute configuration and optical behavior showed the same relationship as reported earlier for the enantiomeric K-region *trans*-diols of benzo[*a*]pyrene (B[*a*]P), benz[*a*]anthracene (B[*a*]A), B[*c*]Ph, and several other PAH (*44*, *47*).

Cytotoxicity Studies. The results obtained in cytotoxicity studies with V79 cells expressing human P450s 1A1 or 1B1 are illustrated in Figure 1. Measurement of the Neutral Red uptake relative to cells of the solvent-treated control group revealed that the (−)-*trans*-DB-[*a,l*]P-11,12-diol elicits considerably stronger cytotoxic effects in both cell lines compared to the parent PAH. In contrast, the corresponding effects of the (+)-*trans*-DB-[*a,l*]P-11,12-diol were lower compared to DB[*a,l*]P. As expected, the cytotoxicity of the racemic *trans*-DB[*a,l*]P-11,12-diol has been found in between the cytotoxicity of the individual enantiomers (Figure 1). Comparison of both V79 cell lines shows that DB[*a,l*]P and its *trans*-11,12-diols, irrespective of their absolute configuration, are considerably more cytotoxic in human P450 1A1-expressing cells than in human P450 1B1-expressing cells (Figure 1). While 50% of human P450 1A1-expressing V79 cells lost their viability after treatment with 18 nM DB[*a,l*]P, 48 nM DB[*a,l*]P was required for a similar effect on human P450 1B1-expressing V79 cells.

Incubation the (±)-*trans*-DB[*a,l*]P-8,9-diol with both V79 cell lines revealed no cytotoxic effects at all (data not shown). Cell viabilities were not affected in comparison to the Me_2SO-treated control group after treatment of both cell lines with (±)-*trans*-DB[*a,l*]P-8,9-diol in a dose range between 0.01 nM and 10 μM.

Metabolic Activation of DB[*a,l*]P. Metabolism data obtained after incubation of human P450 1A1- and 1B1-expressing V79 cells with DB[*a,l*]P are summarized in Table 3. The metabolites formed were separated by HPLC (Figure 2) and unequivocally identified by comparison of their UV spectra, their relative HPLC retention times (RRT, Table 1, Figure 2), and their mass spectra with those of the reference compounds (Table 2).

The metabolic turnover obtained with both cell clones used in the present study is directly comparable because P450 activities were found to be of the same order of magnitude (cf. Experimental Procedures) taking into account that the specific activity of human P450 1A1 for ethoxyresorufin is approximately 7-fold higher than for human P450 1B1 (*48*). Both human P450 1A1 and 1B1 were capable for catalyzing the formation of fjord region 11,12-diol 13,14-epoxides of DB[*a,l*]P via intermediate formation of the *trans*-DB[*a,l*]P-11,12-diol. In P450 1B1-expressing cells, the proportion of the *trans*-DB[*a,l*]P-11,12-diol was increasing with extended incubation times (Table 3). As indicated by the levels of *trans*-DB[*a,l*]P-

Table 2. Relative Abundance (%) of Selected Ions (*m*/*z*) Observed in the Mass Spectra (Recorded at 70 eV) of *O*-Tetramethylsilyl(*O*-TMS)-Ethers of Various Metabolites of Dibenzo[*a*,*l*]pyrene

	m/*z*														
diols	73	147	191	300	302	316	319	359	375	377	390	391	449	465	480
trans-8,9-diol-*O*-TMS	66	29	0.5	8	8	3	3	5	5	1	32	31	1	18	100
trans-11,12-diol-*O*-TMS	96	12	100	13	10	2	1	1	–	38	13	16	–	8	23
cis-11,12-diol-*O*-TMS	100	23	83	22	6	6	3	2	–	61	28	17	–	11	22

	m/*z*														
trans-diol phenols	73	147	191	284	300	302	375	377	390	406	463	478	479	553	568
8,9-diol phenol I-*O*-TMS	97	14	3	8	1	–	3	6	11	3	3	33	39	14	100
8,9-diol phenol II-*O*-TMS	56	18	1	4	3	1	–	3	3	–	1	15	18	9	100
8,9-diol phenol III-*O*-TMS	83	21	2	10	3	–	2	2	10	10	10	21	28	10	100
8,9-diol phenol IV-*O*-TMS	97	23	2	5	3	2	–	5	3	3	2	17	22	11	100
8,9-diol phenol V-*O*-TMS	61	11	–	4	1	1	3	2	4	3	4	17	19	9	100
11,12-diol phenol-*O*-TMS	91	52	100	17	9	13	22	–	9	13	–	19	26	22	65

	m/*z*																		
11,12,13,14-tetrols and *bis*-diols[a]	73	147	191	265	289	300	302	377	378	390	454	465	466	479	553	568	569	643	658
(*r*,*t*,*c*,*c*)-tetrol-*O*-TMS	100	20	24	5	–	12	2	1	1	31	38	–	1	4	–	–	2	–	2
(*r*,*t*,*t*,*c*)-tetrol-*O*-TMS	100	15	62	4	–	2	4	1	1	2	47	4	1	1	1	2	–	–	4
8,9:*n*,*m*-*bis*-diol-*O*-TMS[b]	64	11	100	1	1	1	1	–	2	1	–	–	4	3	4	4	–	2	9

[a] Tetrols possess four hydroxy groups which belong to a single benzo ring, whereas two different benzo rings are substituted with vicinal *trans*-diol increments in *bis*-diols. In *bis*-diols, $m = n+1$. [b] Data set for one out of four *trans*-DB[*a*,*l*]P-8,9:*n*,*m*-*bis*-diol isomers is shown only (cf. Table 1).

Table 3. Metabolic Transformation of Dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P) in V79 Chinese Hamster Cells Expressing Human P450 1A1 and 1B1[a]

	human P450 1A1		human P450 1B1	
metabolite	6 h	12 h	6 h	12 h
trans-DB[*a*,*l*]P-8,9-diol[b]	–	–	–	–
trans-DB[*a*,*l*]P-11,12-diol	1.6	1.2	8.3	13.8
cis-DB[*a*,*l*]P-11,12-diol	–	–	1.3	3.7
DB[*a*,*l*]P-7-phenol	0.4	1.0	1.6	2.2
trans-DB[*a*,*l*]P-11,12-diol phenol	1.0	1.2	0.8	1.0
(*r*,*t*,*t*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol	2.0	2.8	4.2	10.7
DB[*a*,*l*]P	84.6	50.4	52.7	37.2
recovery	89.6	56.6	68.9	68.6

[a] Human P450 1A1- and 1B1-expressing V79 cells were exposed to 1 μM DB[*a*,*l*]P during incubation. All values given are reported in percent of the substrate added. [b] The K-region *trans*-DB[*a*,*l*]P-8,9-diol has been detected only after prolonged incubation times (48 h) in P450 1A1-expressing V79 cells with a relative proportion of 0.9%. No *trans*-DB[*a*,*l*]P-8,9-diol could be detected in P450 1B1-expressing V79 cells during any incubation period up to 48 h.

11,12-diol and (*r*,*t*,*t*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol (Table 3), during exposure to the parent PAH the human P450 1B1-expressing V79 cell clone used in this study metabolically catalyzed the formation of fjord region diol epoxides to a higher extent than the human P450 1A1-expressing clone. Thereby, the lack of any detectable (*r*,*t*,*c*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol in the incubation mixtures of both cell lines indicates stereoselective formation of *anti*-DB[*a*,*l*]PDE and absence of *syn*-DB[*a*,*l*]PDE (cf. Scheme 1).

The *trans*-DB[*a*,*l*]P-11,12-diol formed during incubation was collected and subjected to CSP HPLC analysis using a Daicel Chiralcel ODH column (cf. Experimental Procedures) to determine its enantiomeric composition. As shown in Table 4, metabolic turnover of both human P450 enzymes was enantiospecific: only the enantiomer with *R*,*R*-configuration could be detected. Exclusive formation of (−)-DB[*a*,*l*]P-(11*R*,12*R*)-diol in conjunction with the presence of the (*r*,*t*,*t*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol and the absence of any (*r*,*t*,*c*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol indicate that both human P450 1A1 and 1B1 stereospecifically catalyzed the formation of the (−)-*anti*-DB[*a*,*l*]PDE isomer with *R*,*S*,*S*,*R*-configuration (Scheme 1).

Table 4. Enantioselective Formation of the *trans*-DB[*a*,*l*]P-8,9- and *trans*-DB[*a*,*l*]P-11,12-Diols during Incubation of DB[*a*,*l*]P in Genetically Engineered V79 Cells Expressing Human P450 1A1 and 1B1[a]

	trans-DB[*a*,*l*]P-diols			
	8,9-isomer[b]		11,12-isomer	
P450 isoform	(−)-*S*,*S*	(+)-*R*,*R*	(+)-*S*,*S*	(−)-*R*,*R*
human P450 1A1	10.4	89.6	0	100
human P450 1B1	–	–	0	100

[a] Numbers were calculated from separations of enantiomers by chiral stationary phase HPLC (for details cf. Experimental Procedures). All values given are reported in percent of the total diol formed. [b] The K-region *trans*-DB[*a*,*l*]P-8,9-diol has been detected only after prolonged incubation times (48 h) in P450 1A1-expressing V79 cells with a relative proportion of 0.9%. No *trans*-DB[*a*,*l*]P-8,9-diol could be detected in P450 1B1-expressing V79 cells during any incubation period up to 48 h.

In addition to the conversion of DB[*a*,*l*]P to its fjord region DB[*a*,*l*]PDE, human P450 1A1- and 1B1-expressing V79 cells also catalyzed oxidation reactions at other molecule regions. As shown in Table 3, both enzymes generated a considerable amount of the DB[*a*,*l*]P-7-phenol and a second phenolic metabolite derived from the *trans*-11,12-diol (*trans*-DB[*a*,*l*]P-11,12-diol phenol). While

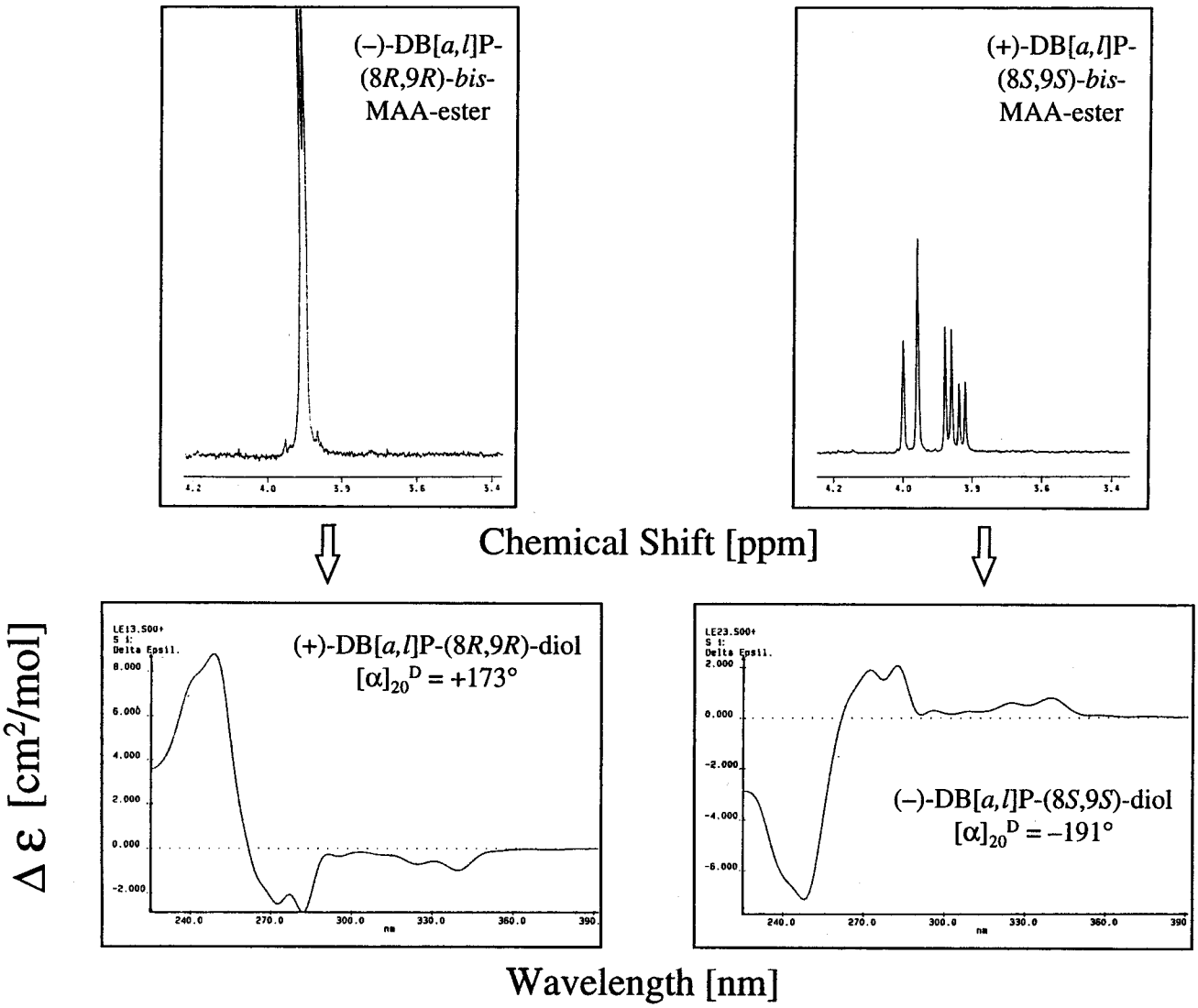


Figure 3. Partial proton NMR spectra (400 MHz, $CDCl_3$) of diastereomeric *bis*-(−)-MAA-esters of enantiomeric *trans*-DB[*a*,*l*]P-8,9-diols (top panels). Chemical shifts and signal patterns of the $-OCH_AH_BCO_2-$ methylene protons are shown in the spectra between 4.2 and 3.4 ppm. The bottom panels show the CD spectra and specific optical rotations of the corresponding enantiomeric *trans*-DB[*a*,*l*]P-8,9-diols (dissolved in THF) released from its respective ester precursor by methanolysis as described under Experimental Procedures

its principle nature could be verified by recording the UV spectrum and mass spectrum of the *O*-TMS-ether derivative (Table 2), the exact position of the hydroxy group in this diol phenol remains unclear.

K-Region metabolism leading to the *trans*-DB[*a*,*l*]P-8,9-diol (cf. Scheme 1) could be observed after incubation of DB[*a*,*l*]P only with the human P450 1A1-expressing V79 cell line and a prolonged incubation time up to 48 h (cf. footnote in Table 3). In contrast, human P450 1B1 failed to produce the *trans*-DB[*a*,*l*]P-8,9-diol. Subsequent analysis of the enantiomeric composition of this metabolite by CSP HPLC revealed a high preference of human P450 1A1 to form the (+)-(*R*,*R*)-enantiomer of the *trans*-DB[*a*,*l*]P-8,9-diol (Scheme 1). The ratio of the (+)- and the (−)-*trans*-DB[*a*,*l*]P-8,9-diol was approximately 9 to 1 (Table 4). Therefore, both *trans*-DB[*a*,*l*]P-8,9- and -11,12-diols metabolically formed either enantioselectively or enantiospecifically by human P450 1A1 possess the same absolute configuration.

HPLC patterns obtained after incubation of DB[*a*,*l*]P with human P450 1B1-expressing V79 cells usually showed one additional peak eluting between those representing the *trans*-DB[*a*,*l*]P-8,9- and the *trans*-DB[*a*,*l*]-P-11,12-diol (Figure 3). After collection and derivatization of the compound eluting within this peak, the UV and MS spectra showed the same characteristics as those of the *trans*-11,12-diol (Table 2). Based on the available data, the structure of the unknown compound was assigned as *cis*-DB[*a*,*l*]P-11,12-diol, which is expected to be slightly more polar than the *trans*-isomer in line with the observed shorter retention time. Control experiments with the *trans*-isomer indicate that the *cis*-DB[*a*,*l*]P-11,12-diol is not enzymatically formed, but rather formed during work-up procedure by a *trans*→*cis*-isomerization reaction. Small amounts of *cis*-DB[*a*,*l*]P-11,12-diol have also been detected after exposure of human P450 1A1-expressing V79 cells to DB[*a*,*l*]P for 48 h (data not shown). This isomerization has also been found with other PAH diols, e.g., B[*a*]P-7,8-diol.[2]

Metabolic Activation of (±)-*trans*-DB[*a*,*l*]P-8,9-diol. Metabolism data obtained from incubation of human P450 1A1- and 1B1-expressing V79 cells with the

[2] J. Jacob et al., unpublished observations.

Table 5. Metabolism of Racemic *trans*-DB[*a,l*]P-8,9-Diol in V79 Chinese Hamster Cells Expressing Human P450 1A1 and 1B1[a]

metabolite	RRT_{COR}[b]	P450 isoform	
		human 1A1	human 1B1
trans-DB[*a,l*]P-8,9-diol		67.7	22.4
trans-DB[*a,l*]P-8,9-diol phenols			
I	0.25	–	1.7
II	0.26	2.1	2.1
III	0.28	0.8	3.6
IV	0.29	3.8	2.7
V	0.30	7.9	30.5
trans-DB[*a,l*]P-8,9:*n,m-bis*-diols[c]			
I	0.12	1.2	4.8
II	0.14	3.3	9.9
III	0.15	2.7	5.4
IV	0.16	10.5	16.9
recovery		100	100

[a] Human P450 1A1- and 1B1-expressing cells were exposed to 10 μM *trans*-DB[*a,l*]P-8,9-diol during incubations. All values given are reported in percent of the substrate added. [b] For relative HPLC retention times (RRT_{COR}) cf. Table 1. [c] *trans*-DB[*a,l*]P-8,9:*n,m-bis*-diols possess in addition to the 8,9-diol group one further vicinal diol increment with $m = n+1$.

racemic *trans*-DB[*a,l*]P-8,9-diol are compiled in Table 5. As indicated by the amount of remaining substrate in the incubation mixtures, metabolic turnover catalyzed by human P450 1B1 was considerably higher compared to human P450 1A1 (77 vs 32%). The *trans*-8,9-diol was converted by both human enzymes to four (1A1) or five (1B1) different *trans*-8,9-diol phenols and four different *bis*-diols (Table 5). The nature of these metabolites was elucidated by their UV spectra and by mass spectra of their *O*-TMS-ethers (Table 2). The molecular ions (*m/z*) of 568 and 658 and the characteristic fragmentation pattern observed in the MS spectra are consistent with the proposed structure assignment as *trans*-DB[*a,l*]P-8,9-diol phenols and *trans*-DB[*a,l*]P-8,9:*n,m-bis*-diols (with $m = n+1$), respectively.

Discussion

Among all enzymes tested to date, P450 1A1 has been considered to play an outstanding role in toxification of carcinogenic PAH irrespective of its species origin (*2*, *4*, *49*). Its highest catalytic activity and, therefore, its particular importance for metabolic activation of a wide range of different PAH compared to other P450 enzymes have been frequently demonstrated by measurement of the total metabolic turnover (*8*−*10*, *50*−*53*), the cytotoxicity (*10*, *51*), or the level of DNA binding (*13*, *20*, *54*) using different cellular test systems that gained metabolic competence after induction or transfection of individual P450 enzymes. However, isolation and cloning of the P450 1B1 enzyme, recently discovered in rodent and human tissues (*14*−*18*, *55*), subsequently allowed generation of human P450 1B1-expressing cell lines and led to the observation that this enzyme contributes in a comparable extent to the genotoxicity of various PAH (*10*, *19*, *20*).

The present study indicates that human P450 1B1- and 1A1-expressing V79 cells show a remarkable similarity in the regioselective oxidation of the potent carcinogen DB[*a,l*]P, despite only a 40% amino acid sequence homology. Metabolism of DB[*a,l*]P by both enzymes proceeds at the 11,12-position of the parent hydrocarbon, whereas oxidation at the K-region (8,9-position) plays a minor role (Scheme 1). This is in contrast to recent studies from this laboratory with the related fjord region PAH B[*c*]Ph (*10*, *56*) which demonstrated a high preference for human P450 1B1 to oxidize the K-region (5,6-position), whereas human P450 1A1 catalyzed the oxidation of the K-region and 3,4-position of B[*c*]Ph to a similar extent. Different regioselectivities between P450s 1A1 and 1B1 were also previously noted in studies by Christou et al. (57) using 7,12-dimethylbenz[*a*]anthracene (DMBA) as substrate allowing discrimination between these individual P450 enzymes involved in PAH metabolism.

The strong genotoxicity of DB[*a,l*]P is predominantly mediated by its fjord region 11,12-diol 13,14-epoxides (*21*, *28*, *54*). In MCF-7 or genetically engineered V79 cells, DNA adduct formation occurred via stereoselective formation of (+)-*syn*- and (−)-*anti*-DB[*a,l*]PDE [cf. Scheme 1, (*20*, *22*)]. However, formation of (+)-*syn*-DB[*a,l*]PDE was observed only after exposure to high concentrations of the parent compound (>1 μM). Besides the corresponding DB[*a,l*]PDE−DNA adducts, no further adducts could be detected in human P450 1B1-expressing V79 cells and in MCF-7 cells, which constitutively express this P450 enzyme (*57*), after exposure to DB[*a,l*]P or its *trans*-11,12-diols (*20*, *22*, *58*). In contrast, HPLC patterns obtained after treatment of human P450 1A1-expressing V79 cells with DB[*a,l*]P contained in addition to the well-characterized DB[*a,l*]PDE-DNA adducts several as yet unknown DNA adducts with increased polar character (*20*). In the present study, human P450 1A1- and 1B1-expressing V79 cell lines were exposed to DB[*a,l*]P and its enantiomeric *trans*-11,12-diols in a concentration range of 0.01 nM through 10 μM in order to monitor the cytotoxicity of these compounds activated by the respective human P450 enzymes. After exposure, measurement of the Neutral Red uptake served as an indicator for cell viability and revealed in each case an increased toxicity on V79 cells expressing human P450 1A1 compared to those expressing human P450 1B1 (Figure 1). Irrespective of the cell line, the *R,R*-configurated (−)-*trans*-DB-[*a,l*]P-11,12-diol displayed the strongest cytotoxic effects with EC_{50} values of 1.4 and 15 nM in P450 1A1- and P450 1B1-expressing cells, respectively. This compound is the immediate metabolic precursor of the DNA binding (−)-*anti*-DB[*a,l*]PDE possessing *R,S,S,R*-configuration (Scheme 1). In contrast, the cytotoxicity of the (+)-*trans*-DB[*a,l*]-P-(11*S*,12*S*)-diol, the immediate metabolic precursor of (+)-*syn*-DB[*a,l*]PDE (Scheme 1), was found in both cell lines at an approximately 30−40-fold lower range compared to its enantiomer (Figure 1). Using different metabolism systems such as microsomes (*34*), MCF-7

cells (*58*), and V79 cells expressing single P450 enzymes including human P450 1A1 and 1B1 (*20*) revealed that the (+)-*trans*-DB[*a*,*l*]P-11,12-diol is only a poor substrate for P450 enzymes. Almost no metabolic turnover and no significant P450-mediated mutagenicity or DNA binding could be observed. On the other hand, DNA binding of (+)-*syn*-DB[*a*,*l*]PDE has been found to be 3-fold lower compared to its diastereomeric (−)-*anti*-DB[*a*,*l*]PDE (*59*), possibly due to an accelerated hydrolytic sequestration of this diastereomer as a consequence of the preferred adopted so-called *aligned* conformation of the partially saturated benzo ring (*35*, *60*). Thus, differences in the cytotoxic activity of the enantiomeric *trans*-DB[*a*,*l*]P-11,12-diols can be partially rationalized in terms of the extremely different DB[*a*,*l*]PDE-DNA adduct levels developing during incubation and which may affect critical target genes required for survival of the V79 cells. However, and in consideration of the comparable levels of total DNA binding catalyzed by human P450 1A1 and 1B1 during exposure to DB[*a*,*l*]P and its (−)-*trans*-11,12-diol (*20*), the 5−10-fold increase displayed by the parent PAH and each individual *trans*-11,12-diol enantiomer in P450 1A1-expressing cells (Figure 1) clearly shows that a simple explanation for the observed cytotoxicity based on the total level of DNA adducts must fail.

Measurement of the metabolites after 6 and 12 h of incubation (Table 3) and analysis of the enantiomeric composition of the *trans*-11,12-diol formed (Table 4) revealed that the human P450 1B1-expressing V79 cell clone used in this study generated considerably more (−)-*trans*-DB[*a*,*l*]P-11,12-diol and (*r*,*t*,*t*,*c*)-DB[*a*,*l*]P-11,12,13,14-tetrol compared to human P450 1A1-expressing V79 cells. These data indicate that human P450 1B1 produced higher levels of (−)- *anti*-DB[*a*,*l*]PDE, a result in line with the higher amount of (−)-*anti*-DB[*a*,*l*]PDE-DNA adducts found in the same cell line at 6 and 24 h after exposure to 1 μM DB[*a*,*l*]P or 0.05 μM (−)-*trans*-DB[*a*,*l*]P-11,12-diol (*20*). Consequently, the increased cytotoxicity of DB-[*a*,*l*]P and its *trans*-11,12-diols observed in P450 1A1-expressing V79 cells (Figure 1) must be mediated either by the highly polar adducts only observed in this cell line (*20*) or by mechanisms other than DNA adduct formation, e.g., generation of reactive oxygen species and membrane and ion channel disintegration. Further investigations are needed to clarify this question.

In order to perform metabolism studies and to avoid any considerable cell damage at the same time, the strong cytotoxicity on V79 cells expressing human P450 1A1 and 1B1 did not allow any exposure to concentrations higher than 1 μM DB[*a*,*l*]P. For this reason, increased incubation times were required to enhance enzymatic turnover and to ensure that concentrations of metabolites exceeded the detection thresholds of the analytical approaches used. On the other hand, strong DNA binding of DB[*a*,*l*]P already observed after 6 h of incubation (*20*) was known to compete with the attempt to provide a representative view of the individual metabolites formed. As shown in Table 3, total recovery of the substrate and its metabolites found after incubation decreased with prolonged incubation times possibly due to the mentioned sequestration of electrophilically reactive intermediates by cellular proteins and DNA. This view is supported by the results obtained after incubation of the (±)-*trans*-DB-[*a*,*l*]P-8,9-diol (Table 5). Although the incubation period was increased up to 48 h, the total recovery in both cell lines was found to be quantitative (100%), possibly due to an extremely low level of DNA binding determined after prolonged exposure times.[3]

The K-region *trans*-DB[*a*,*l*]P-8,9-diol (Scheme 1) was detected as a metabolite of its parent PAH only after 48 h of incubation and only in human P450 1A1-expressing V79 cells (cf. footnotes in Tables 3 and 4). It has been reported that this compound is one of the major metabolites of DB[*a*,*l*]P formed with liver microsomes from 3-methylcholanthrene-treated rats (*43*) or human P450 1A1-expressing microsomes obtained by transfection of human hepatoma G2 cells (*53*). Employment of subcellular fractions in the mentioned studies allowed incubation with extremely high concentrations of DB[*a*,*l*]P (80 μM and 20 μM, respectively) that may account for the differences in kinetics and proportions compared to this study. Recently, Nesnow et al. (*61*) have used liver microsomes from β-naphthoflavone-treated rats and a commercially available recombinant human P450 1A1-expressing system in order to determine the enantiomeric composition of the K-region *trans*-8,9-diol metabolically formed from DB[*a*,*l*]P. Based on the separation of both enantiomeric *trans*-8,9-diols by CSP HPLC and subsequent measurement of individual CD spectra from both fractions, the assignment of individual enantiomers was presented. The authors proposed that the early eluting *trans*-8,9-diol showing a positive first Cotton effect is the (+)-*trans*-8,9-diol and the later eluting *trans*-8,9-diol showing a negative Cotton effect is the (−)-*trans*-8,9-diol (*61*). Obviously, direct measurement of the specific optical rotation of the individual fractions was omitted (*61*). In the present study, both *trans*-8,9-diol enantiomers of DB-[*a*,*l*]P were prepared via HPLC separation of their individual *bis*-(−)-MAA-esters (cf. Experimental Procedures). An unambiguous assignment of the absolute configuration of the diol increment was feasible by analysis of the coupling patterns of $-OCH_AH_BCO_2-$ methylene proton resonances in the ^{1}H NMR spectra of both diastereomeric esters (Figure 3) (*44*, *45*). In accordance with the empirical rule found for the K-region *trans*-diols of dibenz[*a*,*h*]anthracene (DB[*a*,*h*]A), B[*a*]A, B[*c*]Ph, B[*a*]P, DMBA, and other PAH investigated to date (*44*, *45*), the less polar diastereomer with larger negative optical rotation and minor magnetic nonequivalence of the methylene protons has (*R*,*R*) absolute configuration. As shown in Figure 3, the methylene protons of each ester group appeared as a singlet in the ^{1}H NMR spectrum of the early eluting, less polar (−)-DB[*a*,*l*]P-8,9-*bis*-MAA-ester, whereas magnetic nonequivalence of these protons in the late eluting (+)-DB[*a*,*l*]P-8,9-*bis*-MAA-ester provided two pairs of quartets (AB-systems) shifted into each other. Thus, magnetic equivalence in the (−)-DB[*a*,*l*]P-8,9-*bis*-MAA-ester indicates (*R*,*R*) absolute configuration, whereas magnetic nonequivalence in the (+)-DB[*a*,*l*]P-8,9-*bis*-MAA-ester indicates (*S*,*S*) absolute configuration. Subsequent methanolysis of the (−)-DB[*a*,*l*]P-(8*R*,9*R*)-*bis*-MAA-ester provided the *trans*-(8*R*,9*R*)-diol with a specific optical rotation of +173° and a negative first Cotton effect in its CD spectrum, and methanolysis of the (+)-DB[*a*,*l*]P-(8*S*,9*S*)-*bis*-MAA-ester provided the *trans*-(8*S*,9*S*)-diol with a specific optical rotation of −191° and a positive first Cotton effect in its CD spectrum (Figure 3). These results are in contrast to the assignment published by Nesnow et al. (*61*). However, in the present study an unambiguous assignment of the

[3] A. Luch et al., manuscript in preparation.

absolute configuration based on ^{1}H NMR spectra analysis of the precursors was accompanied by direct measurement of the corresponding specific optical rotation and Cotton effects of each individual enantiomeric *trans*-8,9-diol released from the ester. Including the change of the sign of the optical rotation observed upon esterification with (−)-MAA-Cl and the relationship between polarity, optical rotation, and methylene proton signals of the diastereomeric *bis*-(−)-MAA-esters, the signs of optical rotation and Cotton effects of the enantiomeric K-region diols and their absolute configuration presented in this study are supported by similar findings for other PAH such as B[*a*]A, B[*c*]Ph, pyrene, B[*a*]P, and DB[*a*,*h*]*A* (*44*, *45*).

Based on this assignment, the enantiomeric composition of the *trans*-8,9-diol formed during incubation of human P450 1A1-expressing V79 cells with DB[*a*,*l*]P was determined as shown in Table 4: human P450 1A1 preferentially generated the (+)-*trans*-(8*R*,9*R*)-diol with an enantiomeric excess of ∼80% (Scheme 1). A similar enantioselectivity was also found by Nesnow et al. (*61*). However, they reported that the (−)-*trans*-8,9-diol was preferentially formed by human P450 1A1. It is of interest to note that the CD spectrum of the major *trans*-8,9-diol presented by Nesnow et al. (*61*) is identical to the spectrum of the (+)-DB[*a*,*l*]P-8,9-diol with *R*,*R*-configuration synthesized as reference material in the present study (Figure 3). Considering the data compiled in Table 4, K-region oxidation by human P450 1A1 and subsequent hydrolysis by microsomal epoxide hydrolase form similar relative proportions of the *trans*-DB[*a*,*l*]P-8,9-diol enantiomers as found for the K-region diols of B[*a*]P, DMBA, DB[*a*,*h*]A, and other PAH (*62*). In contrast, and interestingly, as the only exception among all PAH investigated to date, the fjord region PAH B[*c*]Ph was found to be converted by human P450 1A1 at its K-region preferentially to the (−)-*trans*-5,6-diol with *S*,*S*-configuration (*10*, *44*, *56*).

Secondary metabolic conversion of the K-region (±)-*trans*-DB[*a*,*l*]P-8,9-diol was catalyzed by both human P450 enzyme-expressing cell lines used in the present study. Due to the lack of any cytotoxicity, V79 cells could be exposed up to 10 μM of this compound. After 48 h of incubation and irrespective of the cell line used, several *trans*-DB[*a*,*l*]P-8,9-diol phenols and *bis*-diols (Table 5) could be detected and subsequently characterized by spectrophotometry and GC/MS-analysis after derivatization to *O*-TMS ethers (Table 2). Recently, Nesnow et al. (*61*) identified and characterized several diastereomeric *trans*-8,9:11,12- and *trans*-8,9:13,14-*bis*-diols as products from catalytic conversion by human P450 1A1. Based on these findings and DNA binding studies, an alternative activation pathway via formation of the K-region *trans*-8,9-diol and further metabolism via *bis*-diols toward DNA-reactive *bis*-diol epoxides was proposed (*61*). However, after 48 h of incubation, the total recoveries of the substrate and its metabolites were found in the present study at 100%. Thus, one may conclude that any DNA binding of the K-region *trans*-DB[*a*,*l*]P-8,9-diol catalyzed by human P450 1A1 or 1B1 is unlikely to contribute considerably to the genotoxicity of the parent compound as proposed by Nesnow et al. (*61*).

In summary, the present study indicates that the regio- and enantioselectivity observed in the metabolism of DB-[*a*,*l*]P mediated by the human P450 1B1-expressing V79 cell line resembles that exhibited by the human P450 1A1-expressing V79 cells. DB[*a*,*l*]P and its enantiomeric *trans*-11,12-diols were more toxic in V79 cells expressing human P450 1A1 than in those expressing human P450 1B1, a finding which could not be rationalized on the basis of a higher level of metabolic activation of the parent hydrocarbon to the strong genotoxic fjord region (−)-*anti*-DB[*a*,*l*]PDE. However, its metabolic precursor, the (−)-*trans*-DB[*a*,*l*]P-11,12-diol, was found to be the strongest cytotoxic metabolite of DB[*a*,*l*]P in both cell lines. Only cells expressing human P450 1A1 catalyzed metabolic turnover of DB[*a*,*l*]P to small amounts of the K-region *trans*-8,9-diol. In contrast, both human P450s 1A1 and 1B1 converted the *trans*-8,9-diol to several diol phenols and *bis*-diols. However, both the (+)- and (−)-*trans*-DB[*a*,*l*]P-8,9-diol enantiomers displayed no cytotoxicity at all either in human P450 1A1- or in 1B1-expressing V79 cells, suggesting that metabolic activation of DB[*a*,*l*]P via further oxidation of the K-region *trans*-DB[*a*,*l*]P-8,9-diol is of minor importance.

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