Published in final edited form as:

Chem Res Toxicol. 2008 November ; 21(11): 2207–2215. doi:10.1021/tx8002005.

Oxidation of PAH *trans*-Dihydrodiols by Human Aldo-Keto Reductase AKR1B10

Amy M. Quinn[†], Ronald G. Harvey[‡], and Trevor M. Penning^{†,§}

[†]Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084

[§]Center of Excellence in Environmental Toxicology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084

[‡]Ben May Department for Cancer Research, University of Chicago, Chicago, Illinois 60637

Abstract

AKR1B10 has been identified as a potential biomarker for human non-small cell lung carcinoma and as a tobacco exposure and response gene. AKR1B10 functions as an efficient retinal reductase *in vitro*, and may regulate retinoic acid homeostasis. However, the possibility that this enzyme is able to activate polycyclic aromatic hydrocarbon (PAH) *trans*-dihydrodiols to form reactive and redox-active *o*-quinones has not been investigated to date. AKR1B10 was found to oxidize a wide range of PAH *trans*-dihydrodiol substrates *in vitro* to yield PAH *o*-quinones. Reactions of AKR1B10 proceeded with improper stereochemistry, since it was specific for the minor (+)-benzo[*a*]pyrene-7*S*,8*S*-dihydrodiol diastereomer formed *in vivo*. However, AKR1B10 displayed reasonable activity in the oxidation of both the (–)-*R,R* and (+)-*S,S* stereoisomers of benzo[*g*]chrysene-11,12-dihydrodiol and oxidized the potentially relevant, albeit minor, (+)-benz[*a*]anthracene-3*S*,4*S*-dihydrodiol metabolite. We find that AKR1B10 is therefore likely to play a contributing role in the activation of PAH *trans*-dihydrodiols in human lung. AKR1B10 retinal reductase activity was confirmed *in vitro* and found to be 5- to 150-fold greater than the oxidation of PAH *trans*-dihydrodiols examined. AKR1B10 was highly expressed at the mRNA and protein levels in human lung adenocarcinoma A549 cells, and robust retinal reductase activity was measured in lysates of these cells. The much greater catalytic efficiency of retinal reduction compared to PAH *trans*-dihydrodiol metabolism suggests AKR1B10 may play a greater role in lung carcinogenesis through dysregulation of retinoic acid homeostasis than through oxidation of PAH *trans*-dihydrodiols.

Introduction

Aldo-keto reductase (AKR) 1B10 was originally cloned from small intestine and called “aldose reductase-like 1” protein due to its high sequence identity with aldose reductase (AKR1B1) (1). Although AKR1B10 has been implicated in the detoxification of cytotoxic lipid aldehydes (1,2), it is now emerging as a potential biomarker for non-small cell lung cancer (NSCLC) (3,4). AKR1B10 was one of seven genes most overexpressed in a microarray of 40,000 genes in NSCLC. There was a positive correlation ($P < 0.0001$) between AKR1B10 overexpression and smoking (3). These data were corroborated by evidence of AKR1B10 upregulation in both tumors and bronchial epithelium of smokers (5). Additionally, cigarette smoke condensate exposure amplified AKR1B10 expression in both normal human epidermal and squamous cell

* To whom correspondence should be addressed. Department of Pharmacology, University of Pennsylvania, School of Medicine, 130C John Morgan Bldg., 3620 Hamilton Walk, Philadelphia, PA 19104-6084. Phone: (215) 898-9445. Fax: (215) 573-2236. E-mail: penning@pharm.med.upenn.edu.

carcinoma cell lines (6,7). Importantly, studies on the impact of smoking cessation on global gene expression in the bronchial epithelium of chronic smokers showed that it was one of three AKR genes that was downregulated in smokers who quit (8). These observations implicate AKR1B10 as a tobacco exposure and response gene.

Polycyclic aromatic hydrocarbons (PAHs) are an important class of chemical carcinogens found in tobacco smoke. Our laboratory has characterized the role of human AKR isoforms in the metabolic activation of PAH *trans*-dihydrodiol proximate carcinogens. Thus far our studies have been limited to aldehyde reductase, AKR1A1, and members of the AKR1C dihydrodiol/hydroxysteroid dehydrogenase subfamily, AKR1C1-AKR1C4. Production of *o*-quinone metabolites by these enzymes has been shown *in vitro* and in cell lines to amplify ROS and oxidatively damage DNA bases to form the highly mutagenic lesion 8-oxo-dGuo (9-14). However, whether AKR1B10 is involved in the metabolic activation of PAHs is unknown.

AKR1B10 has been implicated in liver carcinogenesis and the regulation of retinoid metabolism (1,15). Its role in the regulation of retinoic acid homeostasis may be an alternative mechanism by which AKR1B10 contributes to carcinogenesis. Dietary retinol (vitamin A) is absorbed and oxidized to retinal by alcohol dehydrogenase and short-chain dehydrogenase enzymes. Retinal is then oxidized by aldehyde dehydrogenase isoforms to all-*trans*-retinoic acid, the major active cellular retinoid metabolite. Binding of all-*trans*-retinoic acid and its 9-*cis* stereoisomer to nuclear retinoic acid receptors (RARs) leads to activation of these ligand-induced transcription factors and transcription of genes containing a retinoic-acid response element (RARE) in their promoter region. The biological effects of retinoic acid signaling are extensive and comprise inhibition of cell growth, induction of differentiation, and induction of apoptosis (16). The reduction of retinal to retinol may also occur; AKR1B10 is the most efficient retinal reductase identified to date (17,18). Overexpression of AKR1B10 may thus deplete the pool of retinal available for metabolism to retinoic acid, resulting in promotion of cell growth and a lack of differentiation and apoptosis, events that aid the multi-step carcinogenic process. It is not clear if overexpression of AKR1B10 in lung cancer is simply an association or if a causal relationship exists where AKR1B10 contributes to the pathogenesis of this disease.

Here we examine the oxidation of a structural series of PAH *trans*-dihydrodiols by AKR1B10. We also compare the ability of AKR1B10 to reduce retinal to retinol and extend the studies to the related AKR1B1 isoform. We find that that AKR1B10 plays a peripheral role in PAH metabolism, and that it is much more efficient in utilizing retinal than PAH *trans*-dihydrodiol substrates.

Experimental Procedures

Caution

All PAHs are potentially dangerous and were handled in accordance with NIH Guidelines for the Use of Chemical Carcinogens.

Chemicals

All-*trans*-retinal and DL-glyceraldehyde were purchased from Sigma-Aldrich (St. Louis, MO). (±)-B[a]P-7,8-dihydrodiol; (+)-B[a]P-7*S*,8*S*-dihydrodiol; and (−)-B[a]P-7*R*,8*R*-dihydrodiol, and (±)-B[a]P-4,5-dihydrodiol were obtained from the NCI Chemical Carcinogen Standard Reference Repository (Midwest Research Institute, Kansas City, MO). BA-3,4-dihydrodiol, DMBA-3,4-dihydrodiol, B[g]C-11,12-dihydrodiol, and DB[a,l]P-11,12-dihydrodiol were synthesized according to published procedures (19). B[c]Ph-3,4-dihydrodiol was kindly provided by Dr. Mahesh K. Lakshman (The City College and The City University of New

York, New York, NY). 2-Carboxybenzaldehyde was purchased from Fisher Scientific Intl. (Pittsburgh, PA). *R*-Sulforaphane was obtained from LKT Laboratories (St. Paul, MN). Ponalrestat was provided courtesy of Dr. Florante Quiocho (Baylor College of Medicine, Houston, TX).

Enzymes

AKR1B10 was cloned from human mixed tissue cDNA using the primers, forward: 5'-dAGA ATT CAT ATG GCC ACG TTT GTG-3', reverse: 5'-dTCT CGA GTC AAT ATT CTG CAT CG-3'. The PCR product was purified with QIAEX II Agarose Gel Extraction Protocol (Qiagen) and digested with *Nde*I and *Xho*I restriction enzymes for insertion into a pET-16b vector (Novagen, Madison, WI) for recombinant expression as a His₁₀-fusion protein. The enzyme was purified as previously described (17). Briefly, expression of His₁₀-AKR1B10 was induced in *E. coli* with IPTG. The bacterial pellet was lysed by sonication and applied to a nickel-charged Sepharose column (Amersham Biosciences, Piscataway, NJ and Uppsala, Sweden). Protein was eluted with a linear gradient of 60 to 500 mM imidazole and then dialyzed to remove imidazole. Purity was assessed at each step by SDS-PAGE electrophoresis and by comparison of the specific activity of DL-glyceraldehyde reduction. The final specific activity was 2.11 $\mu\text{mol DL-glyceraldehyde reduced/min/mg}$ in assays containing 0.2 mM NADPH, 20 mM DL-glyceraldehyde, and 0.2% bovine serum albumin in 135 mM sodium phosphate buffer, pH 7.0, at 30 °C. The specific activity was in agreement with published values (1). Removal of the His₁₀ tag did not affect enzyme activity.

AKR1B1 in pET-23d vector was a generous gift from Dr. Mark Petrash (Washington University in St. Louis, St. Louis, MO). Expression of AKR1B1 was induced in *E. coli* with IPTG. The bacterial cell pellet was sonicated and fractionated by 50-80% ammonium sulfate precipitation. Following application to a PBE 94 (Amersham Biosciences) chromatofocusing column, protein was eluted with 1:8 Polybuffer 74, pH 5.0. The sample was then applied to a hydroxyapatite (BioRad Laboratories, Hercules, CA) column, pH 7.0, and protein was eluted with a linear gradient of 10-330 mM potassium phosphate. Purity was assessed at each step by measuring the specific activity of DL-glyceraldehyde reduction and by SDS-PAGE gel electrophoresis. AKR1B1 was purified to a final specific activity of 1.23 $\mu\text{mol DL-glyceraldehyde reduced/min/mg}$ under published assay conditions (20).

AKR7A2 was supplied in a pET-15b vector as a kind gift from Dr. John D. Hayes (University of Dundee, Dundee, Scotland) and purified as described (21) to a specific activity of 3.54 $\mu\text{mol 2-carboxybenzaldehyde (1 mM) reduced/min/mg AKR7A2}$. Recombinant AKR7A3 protein was provided by Dr. Thomas R. Sutter (University of Memphis, Memphis, TN).

Oxidation of ($\pm$)-B[a]P-7,8-Dihydrodiol by AKR1B10

To determine the ability of AKR1B isoforms to oxidize ($\pm$)-B[a]P-7,8-dihydrodiol, incubations contained 20 μM ($\pm$)-B[a]P-7,8-dihydrodiol with a final concentration of 8% DMSO and 2.3 mM NADP⁺ in 50 mM MOPS (3-morpholinopropanesulfonic acid) buffer, pH 7.4, containing 400 μM β -mercaptoethanol and 10 μg recombinant AKR1B enzyme in a final volume of 500 μL . End-point reactions were performed up to 18 h at 37 °C. The product of these reactions, B[a]P-7,8-dione, was detected by stoichiometric trapping as a thioether conjugate with β -mercaptoethanol, as previously described (22). Consumption of B[a]P-7,8-dihydrodiol was analyzed by reversed-phase HPLC (23).

Following the observation that AKR1B10 consumed only 50% of the racemic ($\pm$)-B[a]P-7,8-dihydrodiol substrate, the stereospecificity of the reaction was determined. Incubations contained 20 $\mu\text{mol/L}$ of either the (+)-7*S*,8*S* or (–)-7*R*,8*R* diastereomer of B[a]P-7,8-dihydrodiol with a final concentration of 8% DMSO and 2.3 mM NADP⁺ in 50 mM MOPS

buffer, pH 7.4, containing 400 μM β -mercaptoethanol and 10 μg recombinant AKR1B enzyme in a final volume of 500 μL . End-point reactions were performed up to 18 h at 37 $^{\circ}\text{C}$. Consumption of B[a]P-7,8-dihydrodiol was analyzed by reversed-phase HPLC (23).

Determination of Steady State Kinetic Parameters for (+)-B[a]P-7,8-Dihydrodiol Oxidation by AKR1B Isoforms

To determine the kinetic constants for the oxidation of (+)-B[a]P-7,8-dihydrodiol catalyzed by AKR1B isoforms, incubations contained 1.25 - 20 μM (+)-B[a]P-7,8-dihydrodiol in 8% DMSO and 2.3 mM NADP^+ in 50 mM MOPS buffer, pH 7.4, containing 400 μM β -mercaptoethanol and homogenous recombinant AKR enzyme (AKR1B1 6.7 μg , AKR1B10 15 μg) in a final volume of 250 μL . End-point reactions were performed in duplicate over 60 min at 37 $^{\circ}\text{C}$. AKR1B reactions were terminated and consumption of B[a]P-7,8-dihydrodiol was analyzed by reversed-phase HPLC. Velocity versus substrate concentration data were plotted linearly to obtain an estimate of k_{cat}/K_m as $v/[S] \approx V_{\text{max}}/K_m$ when $[S] \ll K_m$. Values were corrected for inhibition of AKR1B isoforms by 8% DMSO (25% remaining activity).

Oxidation of PAH *trans*-Dihydrodiols by AKR Isoforms

To determine the specific activities for the oxidation of a series of PAH *trans*-dihydrodiols catalyzed by AKR isoforms, incubations contained 20 μM PAH ($\pm$)-*trans*-dihydrodiol in 8% DMSO and 2.3 mM NADP^+ in 50 mM MOPS buffer, pH 7.4, containing 400 μM β -mercaptoethanol and AKR enzyme. End-point reactions were performed in duplicate over 60 min at 37 $^{\circ}\text{C}$. AKR reactions were terminated by addition of 250 μL ice-cold acetone and extracted twice with 500 μL ethyl acetate. B[a]P-4,5-dihydrodiol (0.5 nmol) was used as an internal standard to correct for extraction efficiency.

Combined extracts were dried under vacuum and redissolved in methanol for analysis by reversed-phase HPLC-UV. Reaction products were separated using a Waters Alliance 2695 HPLC system (Waters Corp., Milford, MA) with a Zorbax-ODS C18 column (5 μm , 4.6 $\times$ 250 mm; Agilent Technologies, Palo Alto, CA) and a flow rate of 0.5 mL/min. Reaction products were separated with a linear gradient of 68-73% methanol (v/v) over 40 min (B[a]P-7,8-dihydrodiol, t_R 31 min), 70-85% methanol (v/v) over 36 min (BA-3,4-dihydrodiol, t_R 17 min), 65-80% methanol (v/v) over 60 min (B[g]C-11,12-dihydrodiol, t_R 43 min), 60-70% methanol (v/v) over 60 min (B[c]Ph-3,4-dihydrodiol, t_R 38 min), 75-95% methanol (v/v) over 45 min (DB[a,l]P-11,12-dihydrodiol, t_R 27 min), and 70-90% methanol (v/v) over 48 min (DMBA-3,4-dihydrodiol, t_R 28 min). Elution was monitored with a Waters 2996 Photodiode Array Detector. Calculation of initial rates (nmol/min) of *trans*-dihydrodiol consumption was achieved by comparison of peak areas to standard curves constructed for each compound at the wavelength of maximum absorption (272 nm for B[a]P-4,5-dihydrodiol, 367 nm for B[a]P-7,8-dihydrodiol, 262 nm for BA-3,4-dihydrodiol, 271 nm for B[g]C-11,12-dihydrodiol, 224 nm for B[c]Ph-3,4-dihydrodiol, 304 nm for DB[a,l]P-11,12-dihydrodiol, 271 nm for DMBA-3,4-dihydrodiol). Data were normalized to the B[a]P-4,5-dihydrodiol internal standard.

Circular Dichroism Spectroscopy

AKR1B enzymes were incubated with 20 μM racemic PAH *trans*-dihydrodiol in 8% DMSO (final concentration) in the presence of 100 mM potassium phosphate buffer, pH 9.0, at 37 $^{\circ}\text{C}$. Reactions were monitored by HPLC-UV chromatography to determine the end-point of the reaction, i.e. when 50% of the racemic PAH *trans*-dihydrodiol had been consumed. The unreacted *trans*-dihydrodiol was extracted with ethyl acetate and dried *in vacuo*. The dried sample was resuspended in methanol and separated by semipreparative TLC using GF silica gel plates (Analtech, Inc., Newark, DE) and a mobile phase of 55:45 ethyl acetate/hexane. The R_f value of the unreacted *trans*-dihydrodiol was compared under UV light to an authentic

standard. The *trans*-dihydrodiol was eluted with methanol from the silica, using caution to minimize exposure to light. CD spectra were recorded on a Jasco Spectropolarimeter Model J-810 at 20 °C in a 0.1 cm path length cell. The spectra were recorded at 1 nm intervals over the wavelength range 240 - 400 nm. Results were expressed as ellipticity in units of millidegrees (mdeg).

Determination of Steady State Kinetic Parameters for Retinal Reduction

To determine the kinetic constants for the reduction of all-*trans*-retinal catalyzed by AKR1B10, incubations contained 0.1 - 50 μ M all-*trans*-retinal in 8% DMSO and 200 μ M NADPH in 100 mM sodium phosphate buffer, pH 7.5, containing 0.02% Tween 80 and AKR enzyme (AKR1B1 6.7 μ g, AKR1B10 15 μ g) in a final volume of 1 mL. Consumption of all-*trans*-retinal was measured in triplicate spectrophotometrically at 400 nm [$\epsilon_{400\text{ nm}} = 29,500\text{ M}^{-1}\text{ cm}^{-1}$, (17)] and 25 °C. Data were corrected for inhibition by 8% DMSO and 0.02% Tween 80 (20% activity remaining) and analyzed by fitting velocity versus substrate concentration data for the equation for a hyperbola, $v = V_{\text{max}}[S] / (K_m + [S])$ to generate mean $\pm$ standard error for steady state kinetic parameters.

Cell Culture

A549, H358, and HepG2 cells were obtained from the ATCC (Manassas, VA) and maintained as described (24). HBEC-KT cells were provided courtesy of Dr. John D. Minna (The University of Texas Southwestern Medical Center, Dallas, TX) and maintained as described (25).

Real-Time RT-PCR

Real-time RT-PCR was used to compare transcript levels for human AKR isoforms in human cell lines. One μ g of total RNA was reverse-transcribed using the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). Each plate contained nine full-length standards (0.025 - 2,500,000 fg) in duplicate and samples in triplicate. The amount of target cDNA was established through comparison of threshold cycle (C_T) values for each sample to a standard curve generated for each gene. Expression was normalized to high abundance and low abundance housekeeping genes, i.e. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogen deaminase (PBGD), respectively.

The primer sequences for PCR amplification of AKR1A1, AKR1C1-1C4, GAPDH, and PBGD genes were obtained from previous publications (23,26,27). The primers for AKR1B1 are, forward: 5'-dAGA ACG CAT TGC TGA GAA CTT TAA GGT C-3', reverse: 5'-dGTA ATC CTT GTG GGA GGT ACA GCT CAA C-3' giving a 130 bp product. Primers for AKR1B10 and AKR7A2 were purchased as QuantiTect Primer Assay primer sets (Qiagen, Valencia, CA). Quantitative analysis of the specific expression of each gene was performed on a DNA Engine Thermal Cycler (MJ Research, Waltham, MA) with SYBR Green detection. PCR conditions were as follows: 95 °C for 15 min followed by 40 cycles of 94 °C for 15 sec of denaturation, X °C for 30 sec for annealing, and 72 °C for 30 sec for extension (where X = 60 °C for AKR1A1, AKR1C2, AKR1C3, and AKR7A2; 57 °C for AKR1C1; 56 °C plus 4% DMSO for AKR1C4; 55 °C for AKR1B1 and AKR1B10; and 58 °C for GAPDH and PBGD). Specificity of AKR primers was validated with the 2,500 fg full-length standards of each AKR isoform.

Full-length cDNA standards of AKR1A1 and AKR1C1-1C4 were generated from the prokaryotic expression vectors pET-16b containing the respective cDNA clones (28,29) via digestion with either *Nco*I and *Bam*HI (AKR1A1) or *Xho*I and *Bgl*II (AKR1Cs). cDNA standards for AKR1B1, AKR1B10, and AKR7A2 were similarly prepared from their prokaryotic expression vectors via digestion with *Nco*I and *Hind*III (AKR1B1), *Nde*I and *Xho*I (AKR1B10), or *Xba*I and *Xho*I (AKR7A2). Standards for GAPDH and PBGD were

generated by isolating the PCR product following PCR amplification of human kidney cDNA. Digestion products were isolated by gel purification and used as standards with correction factors for GAPDH (3.30) and PBGD (7.48) due to differences in molecular weight between full-length and PCR product standards.

Immunoblotting

Cell lysates were prepared by homogenization of cells in RIPA buffer supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany), and centrifugation at 10,000 *g* to remove cell debris. Twenty micrograms of each sample were electrophoresed on a 12 % SDS polyacrylamide gel and transferred to nitrocellulose. AKR1B10 protein was detected with 1:1000 dilution of rabbit polyclonal *anti*-AKR1B10 antibody (Dr. Deliang Cao, Southern Illinois University, Springfield, IL) and visualized with horseradish peroxidase-conjugated goat *anti*-rabbit IgG. Specific bands were detected using ECL Western Blot detection reagent (Amersham, Buckinghamshire, England).

Functional Enzyme Assay

Cell lysates for AKR1B assays were prepared by scraping cells into 100 mM sodium phosphate buffer, pH 7.5, homogenization on ice with a Dounce homogenizer, and centrifugation at 10,000 *g* to remove cell debris. Cell lysates (100 µg/mL) were incubated at 37 °C in 10 mM sodium phosphate buffer, pH 6.2, with 10 mM DL-glyceraldehyde in 150 µM NADPH to determine AKR1B activity. Consumption of NADPH was monitored in triplicate spectrophotometrically at 340 nm. DL-glyceraldehyde is a standard substrate for AKR1B1 and AKR1B10, but is not specific and will also detect AKR1A1 activity. Inhibition of AKR1B activity was achieved with 150 µM ponalrestat.

Reduction of all-*trans*-retinal was measured in A549 cell lysates prepared as described above. Cell lysates (100 µg/mL) were incubated at 37 °C in 100 mM sodium phosphate buffer, pH 7.5, with 10 µM all-*trans*-retinal in 2% DMSO and 200 µM NADPH. Consumption of all-*trans*-retinal was measured in triplicate spectrophotometrically at 400 nm. Inhibition of AKR1B activity was achieved with 100 µM ponalrestat.

Results

Oxidation of (±)-B[a]P-7,8-Dihydrodiol by AKR1B Isoforms

AKR1B10 was recombinantly expressed and purified to homogeneity. It was able to catalyze the NADP⁺-dependent oxidation of (±)-B[a]P-7,8-dihydrodiol *in vitro*. The product of this reaction was trapped as a B[a]P-7,8-dione thioether conjugate with β-mercaptoethanol and identified by comparison of both retention time and UV spectra to an authentic synthetic standard (Fig. 1). AKR1B10-mediated consumption of racemic (±)-B[a]P-7,8-dihydrodiol proceeded only to 50% completion, indicating stereochemical specificity for one *trans*-dihydrodiol diastereomer. Incubations of AKR1B10 with (–)-B[a]P-7*R*,8*R*-dihydrodiol were nonproductive, while those with (+)-B[a]P-7*S*,8*S*-dihydrodiol proceeded at a specific activity of 1.3 nmol/min/mg and demonstrated that AKR1B10 is stereospecific for the (+)-7*S*,8*S* stereoisomer (data not shown). Thus, AKR1B10 and AKR1A1 show opposing stereospecificity for the oxidation of (±)-B[a]P-7,8-dihydrodiol, while the AKR1C isoforms are not stereoselective for this PAH *trans*-dihydrodiol substrate (28,29), Table 1.

The identification of AKR1B10 as a PAH-metabolizing enzyme led us to consider other human AKR isoforms in PAH *trans*-dihydrodiol oxidation. AKR1B1 (aldose reductase) was recombinantly expressed and shown to metabolize (±)-B[a]P-7,8-dihydrodiol to generate B[a]P-7,8-dione *in vitro*; however, it also failed to oxidize the major (–)-B[a]P-7*R*,8*R*-dihydrodiol isomer formed metabolically. (±)-B[a]P-7,8-dihydrodiol was not a substrate for

either recombinantly-expressed AKR7A2 or AKR7A3; accordingly, these isoforms were excluded from further consideration as PAH-metabolizing enzymes.

Determination of Steady State Kinetic Parameters for (+)-B[a]P-7S,8S-Dihydrodiol Oxidation

We next determined the kinetic constants for the oxidation of (+)-B[a]P-7S,8S-dihydrodiol catalyzed by AKR1B10 (Fig. 2A). The rate of *trans*-dihydrodiol oxidation was measured by monitoring the disappearance of substrate by reversed-phase HPLC methods. Due to limited substrate solubility, AKR1B10 could not be saturated with (+)-B[a]P-7,8-dihydrodiol and the relationship between velocity and substrate concentration was linear. The Michaelis-Menten equation, under these conditions, simplifies to $v/[S] = V_{max}/K_m$, providing a direct estimation of utilization ratio. Using the enzyme concentration of AKR1B10, the catalytic efficiency (k_{cat}/K_m) of (+)-B[a]P-7,8-dihydrodiol oxidation was calculated to be $2.36 \text{ mM}^{-1} \text{ min}^{-1}$. Catalytic efficiencies can be used to compare similar substrates for different enzymes, Table 1. Among the AKR1A1 and AKR1C isoforms, catalytic efficiencies for racemic ($\pm$)-B[a]P-7,8-dihydrodiol oxidation vary from 17 (AKR1C4) to 53 (AKR1C2) $\text{mM}^{-1} \text{ min}^{-1}$, making AKR1B10 the least efficient of the AKR isoforms examined to date in the oxidation of B[a]P-7,8-dihydrodiol.

The kinetic parameters for AKR1B1-catalyzed oxidation of (+)-B[a]P-7S,8S-dihydrodiol were also examined (Fig. 2B) and the catalytic efficiency was determined to be $k_{cat}/K_m = 10.3 \text{ mM}^{-1} \text{ min}^{-1}$. Thus, the catalytic efficiency of (+)-B[a]P-7S,8S-dihydrodiol oxidation for AKR1B1 is four times greater than that seen for AKR1B10, but inferior to the oxidation of (–)-B[a]P-7R,8R-dihydrodiol and the oxidation of ($\pm$)-B[a]P-7,8-dihydrodiol catalyzed by AKR1A1 and AKR1C isoforms, respectively, Table 1.

Oxidation of PAH *trans*-Dihydrodiols by AKR1B Isoforms

Additional racemic PAH *trans*-dihydrodiols, including methylated bay- and *fjord*-region *trans*-dihydrodiols, were evaluated as substrates for AKR1B10 *in vitro*, Scheme 1. Enhanced steric strain in the bay region renders the PAH nonplanar and more carcinogenic (30). AKR1B10 utilization of bay region ($\pm$)-B[a]P-7,8-dihydrodiol and methylated bay region ($\pm$)-DMBA-3,4-dihydrodiol substrates proceeded with V_{max}/K_m values ($\mu\text{mol}/\text{min}/\mu\text{mol}/[\text{S}]$, $\text{min}^{-1}\text{mM}^{-1}$) of 2.34 and 2.70, respectively, Table 2. *Fjord*-region ($\pm$)-B[c]Ph-3,4-dihydrodiol and ($\pm$)-DB[a,l]P-11,12-dihydrodiol were poor substrates for AKR1B10 (V_{max}/K_m values, 1.51 and $0.38 \text{ min}^{-1}\text{mM}^{-1}$). Only the bay region ($\pm$)-BA-3,4-dihydrodiol and *fjord*-region ($\pm$)-B[g]C-11,12-dihydrodiol had significant V_{max}/K_m values of 12.8 and 9.55, respectively. AKR1B10 only oxidized 50% of ($\pm$)-BA-3,4-dihydrodiol, suggesting stereospecificity for one of the BA-3,4-dihydrodiol stereoisomers. Notably, AKR1B10 did not display stereoselectivity for oxidation of ($\pm$)-DMBA-3,4-dihydrodiol and ($\pm$)-B[g]C-11,12-dihydrodiol.

Circular Dichroism Spectroscopy

The unreacted isomer remaining after AKR1B10-catalyzed oxidation of ($\pm$)-BA-3,4-dihydrodiol was analyzed by CD spectroscopy. Assignment was based on comparison to published spectra (31). The sign of the Cotton effect was negative, indicating the unreacted substrate was enriched with the (–)-3R,4R stereoisomer, and that the (+)-3S,4S stereoisomer was preferentially oxidized (Fig. 3A).

The best substrates for AKR1B10, namely ($\pm$)-BA-3,4-dihydrodiol and ($\pm$)-B[g]C-11,12-dihydrodiol, were then monitored for stereochemical preference in AKR1B1-catalyzed reactions. Similar to AKR1B10, the unreacted substrate of AKR1B1-mediated oxidation of ($\pm$)-BA-3,4-dihydrodiol yielded a negative Cotton effect and indicated stereochemical preference of AKR1B1 for the (+)-*S,S trans*-dihydrodiol stereoisomer (Fig. 3B). None of the reactions of AKR1B1 with PAH *trans*-dihydrodiols utilized the major (–)-*R,R* stereoisomer

generated metabolically. While AKR1B1 oxidized only the (+)-*S,S* isomer of B[g]C-11,12-dihydrodiol (Fig. 3C) (32), AKR1B10 was not stereoselective in the oxidation of (±)-B[g]C-11,12-dihydrodiol.

Determination of Steady State Kinetic Parameters for Retinal Reduction

We next determined the kinetic constants (k_{cat} , K_m , and k_{cat}/K_m) for the reduction of all-*trans*-retinal catalyzed by AKR1B10. The rate of retinal reduction was measured spectrophotometrically by monitoring the disappearance of substrate. AKR1B10 was found to have a k_{cat} value of 39 min^{-1} and a K_m value of $6.2 \text{ } \mu\text{mol/L}$, yielding a k_{cat}/K_m value of $6,300 \text{ mM}^{-1} \text{ min}^{-1}$ (Fig. 4). This value is comparable to previously reported values (17,18), and is several orders of magnitude greater than the value seen for the oxidation of any PAH *trans*-dihydrodiol substrate examined to date.

Expression of AKR1B Isoforms in A549 Cells

Transcript levels of AKR1A1, AKR1C, AKR1B, and AKR7A2 isoforms were measured in the A549 human lung adenocarcinoma cell line, as shown in Figure 5. AKR1B1 and particularly AKR1B10 were the most abundant AKR isoforms expressed in these cells. The AKR1B10 mRNA expression level in A549 cells was compared to those of HBEC-KT immortalized human bronchial epithelial cells, H358 human bronchoalveolar carcinoma cells, and HepG2 human hepatocellular carcinoma cells (Fig. 6A). A549 cells were found to have significantly greater AKR1B10 transcript levels than the other cells examined. High AKR1B10 protein expression in A549 cells was confirmed by immunoblot analysis (Fig. 6B).

A549 cells were analyzed for functional AKR1B expression using a standard assay for reduction of DL-glyceraldehyde. Cell lysates were determined to have significant AKR1B activity, $18.6 \pm 2.1 \text{ nmol/min/mg cell lysate}$, that was 73% inhibited with $150 \text{ } \mu\text{M}$ ponalrestat, an AKR1B inhibitor. The specific activity of all-*trans*-retinal reduction in A549 cell lysates was determined to be $9.6 \pm 0.8 \text{ nmol/min/mg cell lysates}$. This activity was 50% inhibited by $100 \text{ } \mu\text{M}$ ponalrestat. AKR1B10 mRNA, protein expression, and functional enzyme activity show that this isoform is more abundantly expressed in human lung adenocarcinoma A549 cells, and the levels are not as high in bronchoalveolar carcinoma H358 cells or immortalized bronchial epithelial HBEC-KT cells.

Discussion

AKR1B10 has been identified as a potential biomarker for NSCLC and a tobacco exposure and response gene (3,4,8,33). The functional role of this enzyme in human lung carcinogenesis is unknown. Human AKR isoforms of the 1A1 and 1C families have been shown to oxidize PAH *trans*-dihydrodiols to form active and redox-active *o*-quinones (9-14). These compounds cause oxidative DNA damage in human lung cell lines through amplification of ROS and may contribute to PAH-induced lung carcinogenesis (34). Based on the upregulation of AKR1B10 in response to cigarette smoke in both human lung tissue and cell lines (5-7), and its downregulation in past smokers (8), we sought to investigate the potential of AKR1B10 to metabolically activate PAH *trans*-dihydrodiol proximate carcinogens.

B[a]P is the most frequently studied PAH, in part due to its identification in coal tar as the first carcinogenic compound in this chemical class, as well as its presence in many environmental PAH mixtures. However, other PAHs may have greater carcinogenic potential than B[a]P. Accordingly, a series of PAH *trans*-dihydrodiols, including compounds of bay-, methylated bay-, and *ffjord*-region classifications, were investigated as substrates for AKR1B10. BA is present in mainstream tobacco smoke ($13.2\text{--}22.6 \text{ ng/cigarette}$) at levels comparable to those of B[a]P (35), and it is a major component of total PAHs in the environment (36). DMBA is the

7,12-methylated derivative of BA where enhanced steric strain in the bay region renders it non-planar and significantly more carcinogenic than the nonmethylated compound (37). Although a synthetic compound, DMBA is frequently used to study carcinogenesis (38), and both its (+)-3*S*,4*S* and (-)-3*R*,4*R* *trans*-dihydrodiol metabolites have been shown to be substrates for AKR-catalyzed oxidation (24). While benzo[*g*]chrysene (B[*g*]C) has not been routinely quantitated in tobacco smoke condensate, it has been found in coal tar, petroleum distillates, and in a standard reference sample of urban air particulate (39,40). B[*g*]C-11,12-dihydrodiol oxidation has previously been measured for human AKR1 isoforms where high catalytic efficiency for (+)-11*S*,12*S* and (-)-11*R*,12*R* stereoisomers by AKR1C4 was noted (29). The *ffjord*-region PAHs benzo[*c*]phenanthrene (B[*c*]Ph) and DB[*a,l*]P are constituents of tobacco smoke and are found in other environmental sources (41). DB[*a,l*]P is the most tumorigenic PAH tested to date in mouse skin and rat mammary gland models (42), and turnover of its *trans*-dihydrodiol metabolite has not yet been observed for any human AKR isoform.

Stereochemistry appears to be an important determinant of substrate preference for AKR-mediated PAH *trans*-dihydrodiol activation. Stereospecificity was not observed in AKR1B10-mediated oxidation of (±)-DMBA-3,4-dihydrodiol and (±)-B[*g*]C-11,12-dihydrodiol. The lack of stereoselectivity and high utilization ratio ($V_{\max}/K_m$) of (±)-B[*g*]C-11,12-dihydrodiol oxidation by AKR1B10 indicates biological relevance for this reaction.

Metabolism of (±)-BA-3,4-dihydrodiol paralleled that seen for (±)-B[*a*]P-7,8-dihydrodiol, in that oxidation by AKR1B10 proceeded with strict stereospecificity for the (+)-*S,S* stereoisomer. The (-)-*R,R* stereoisomer of BA-3,4-dihydrodiol is preferentially formed *in vivo*, although to a lesser extent than for B[*a*]P-7,8-dihydrodiol. The (+)-*S,S* stereoisomer has been shown to constitute 10-31% of BA-3,4-dihydrodiol diastereomers formed in rat liver microsomes (43,44). Thus, the minor (+)-BA-3*S*,4*S*-dihydrodiol metabolite may be formed in reasonable quantities as BA is present in both tobacco smoke and environmental sources (35, 36). AKR1B10-catalyzed conversion of (+)-BA-3*S*,4*S*-dihydrodiol to BA-3,4-dione may impact PAH-mediated lung carcinogenesis via amplification of ROS and oxidative DNA damage caused by redox cycling of this PAH *o*-quinone. Additionally, BA-3,4-dione may influence PAH metabolism through induction of P450 1A1 and 1B1 expression, similar to that seen for the known aryl hydrocarbon receptor-ligand B[*a*]P-7,8-dione (45,46). The high expression of AKR1B10 in A549 cells lends further support to a contributing role for this isoform in PAH *trans*-dihydrodiol activation.

The oxidation of PAH *trans*-dihydrodiols by the closely related (71% amino acid identity) AKR1B1 enzyme was also analyzed. This enzyme is widely expressed in the human body, including the lung. The broad substrate specificity of AKR1B1 and its upregulation under oxidative stress (47,48), a chronic condition in smokers' lungs, made it a reasonable candidate for PAH *trans*-dihydrodiol oxidation. Similar to AKR1B10, AKR1B1 was able to oxidize PAH *trans*-dihydrodiols to form *o*-quinones. However, reactions were stereospecific for the minor (+)-*S,S* diastereomers formed *in vivo* for all *trans*-dihydrodiols examined.

Formation of B[*a*]P-7,8-dione in intact A549 cells was recently measured by LC-ESI/MS following treatment with (±)-B[*a*]P-7,8-dihydrodiol (34). Based on our current observation that AKR1B10 and AKR1B1 are highly expressed in these cells, these AKR1B isoforms may contribute to the measured oxidation of the (+)-B[*a*]P-7*S*,8*S*-dihydrodiol diastereomer. However, AKR1C1-1C3 are likely to be the dominant *o*-quinone-generating enzymes, due to their high turnover and ability to oxidize both the (+)- and (-)-B[*a*]P-7,8-dihydrodiols.

AKR1B10 may also promote human lung carcinogenesis via regulation of retinoic acid homeostasis. Proposed as a retinal reductase, upregulation of AKR1B10 in the lung may disrupt signaling of the growth-inhibitory, pro-differentiation, and pro-apoptotic molecule retinoic

acid through depletion of its metabolic precursor retinal. Other AKRs have been shown to regulate ligand occupancy of nuclear receptors (49), including pre-receptor regulation of the AhR by altering levels of PAH *o*-quinone ligands (45,46). Following confirmation that AKR1B10 was a catalyst for reduction of all-*trans*-retinal *in vitro*, we examined its effect on retinoid metabolism in A549 cells. AKR1B10 was found to be highly expressed at the mRNA, protein, and functional enzyme levels in this non-small cell carcinoma cell line. Additionally, a significant rate of retinal reduction was measured in lysates of A549 cells. The catalytic efficiency of this reaction ($k_{\text{cat}}/K_m = 6,300 \text{ mM}^{-1} \text{ min}^{-1}$) was several orders of magnitude greater than that observed with oxidation of any PAH *trans*-dihydrodiol by AKR1B10.

Decreased expression of all RAR and RXR subtypes is frequently observed in tumor tissue of NSCLC patients, indicating a fundamental dysregulation of the retinoic acid-pathway in this cancer (50-52). In particular, decreased RAR β is frequently observed in both cancer cells and tissues (53), and may lead to resistance to retinoids. RAR β is inducible by retinoic acid due to the RARE in its promoter region (54,55). Treatment with all-*trans*-retinoic acid leads to growth inhibition in some human lung cancer cell lines and is accompanied by an increase in RAR β (56). In lung cancer cells lines unresponsive to all-*trans*-retinoic acid, RAR β induction was not observed. In fact, most NSCLC cell lines are resistant to all-*trans*-retinoic acid *in vitro*, including A549 cells (57-59). Indeed, we find A549 cells to be retinoid-insensitive,² as measured by the failure of all-*trans*-retinoic acid treatment to either inhibit cellular proliferation or enhance connexin 43 expression, a marker of gap-junctional intercellular communication (60,61). These data suggest that resistance to retinoids is an early event in carcinogenesis. Upregulation of AKR1B10 may also be an early event in carcinogenesis, as its expression is elevated in squamous metaplasia, precancerous lesions of small cell lung carcinoma (3). Thus, the role of AKR1B isoforms in the regulation of retinoid homeostasis may be better examined in a normal human bronchial epithelial or premalignant cell line, such as the retinoid-responsive BEAS-2B cell line (62).

The retinal reductase activity of AKR1B10 may account for the failure of β -carotene supplementation to reduce lung cancer risk (63,64). β -carotene is cleaved symmetrically to form two molecules of retinol, and dietary β -carotene is the major source of retinol in developing countries (65). High expression of AKR1B10 may deprive cells of the anticipated pro-apoptotic and pro-differentiation effects of retinoic acid signaling by shifting the equilibrium towards the reduced retinoid. While this may explain why no positive effects were seen with carotenoid supplementation, it does not account for the increased lung cancer risk observed. This may be due to increased lung capacity with β -carotene and thus greater inhalation of carcinogens, or the result of procarcinogenic carotenoid oxidation products (66, 67).

AKR1B10 may contribute to human lung carcinogenesis through metabolic activation of PAH *trans*-dihydrodiols. However, it is unlikely to dominate PAH activation via previously examined AKR1A1 and AKR1C isoforms due to its low catalytic efficiency and its selectivity for minor *trans*-dihydrodiol stereoisomers. Exceptions may exist for the *trans*-dihydrodiols of BA and B[g]C based on the level of enzyme expression observed. It is unclear if AKR1B10 disrupts retinoic acid homeostasis in human lung cells to promote transformation, although this enzyme was shown to have retinal reductase activity *in vitro* and in A549 lung adenocarcinoma cells.

Acknowledgements

This work was supported by NIH grants, R01 CA39504, R01 ES015857, P30 ES013508, and PA PDOH4100038714

²Quinn, A. M., and Penning, T. M. Unpublished data.

References

1. Cao D, Fan ST, Chung SS. Identification and characterization of a novel human aldose reductase-like gene. *J Biol Chem* 1998;273:11429–11435. [PubMed: 9565553]
2. Yan R, Zu X, Ma J, Liu Z, Adeyanju M, Cao D. Aldo-keto reductase family 1 B10 gene silencing results in growth inhibition of colorectal cancer cells: Implication for cancer intervention. *Int J Cancer* 2007;121:2301–2306. [PubMed: 17597105]
3. Fukumoto S, Yamauchi N, Moriguchi H, Hippo Y, Watanabe A, Shibahara J, Taniguchi H, Ishikawa S, Ito H, Yamamoto S, Iwanari H, Hironaka M, Ishikawa Y, Niki T, Soharu Y, Kodama T, Nishimura M, Fukayama M, Dosaka-Akita H, Aburatani H. Overexpression of the aldose-keto reductase family protein AKR1B10 is highly correlated with smokers' non-small cell lung carcinomas. *Clin Cancer Res* 2005;11:1776–1785. [PubMed: 15755999]
4. Kim B, Lee HJ, Choi HY, Shin Y, Nam S, Seo G, Son DS, Jo J, Kim J, Lee J, Kim J, Kim K, Lee S. Clinical validity of the lung cancer biomarkers identified by bioinformatics analysis of public expression data. *Cancer Res* 2007;67:7431–7438. [PubMed: 17671213]
5. Woenckhaus M, Klein-Hitpass L, Grepmeier U, Merk J, Pfeifer M, Wild P, Bettstetter M, Wuensch P, Blaszyk H, Hartmann A, Hofstaedter F, Dietmaier W. Smoking and cancer-related gene expression in bronchial epithelium and non-small-cell lung cancers. *J Pathol* 2006;210:192–204. [PubMed: 16915569]
6. Nagaraj NS, Beckers S, Mensah JK, Waigel S, Vigneswaran N, Zacharias W. Cigarette smoke condensate induces cytochromes P450 and aldose-keto reductases in oral cancer cells. *Toxicol Lett* 2006;165:182–194. [PubMed: 16713138]
7. Gumus ZH, Du B, Kacker A, Boyle JO, Bocker JM, Mukherjee P, Subbaramaiah K, Dannenberg AJ, Weinstein H. Effects of tobacco smoke on gene expression and cellular pathways in a cellular model of oral leukoplakia. *Cancer Prevention Research* 2008;1940–6207.CAPR-1908-0007
8. Zhang L, Lee JJ, Tang H, Fan YH, Xiao L, Ren H, Kurie J, Morice RC, Hong WK, Mao L. Impact of smoking cessation on global gene expression in the bronchial epithelium of chronic smokers. *Cancer Prevention Research* 2008;1940–6207.CAPR-1907-0017
9. Flowers-Geary L, Harvey RG, Penning TM. Cytotoxicity of polycyclic aromatic hydrocarbon *o*-quinones in rat and human hepatoma cells. *Chem Res Toxicol* 1993;6:252–260. [PubMed: 7686407]
10. Penning TM, Ohnishi ST, Ohnishi T, Harvey RG. Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon *trans*-dihydrodiols catalyzed by dihydrodiol dehydrogenase. *Chem Res Toxicol* 1996;9:84–92. [PubMed: 8924621]
11. Flowers L, Bleczynski WF, Burczynski ME, Harvey RG, Penning TM. Disposition and biological activity of benzo[a]pyrene-7,8-dione: a genotoxic metabolite generated by dihydrodiol dehydrogenase. *Biochemistry* 1996;35:13664–13672. [PubMed: 8885846]
12. Tang DW, Chang KW, Chi CW, Liu TY. Hydroxychavicol modulates benzo[a]pyrene-induced genotoxicity through induction of dihydrodiol dehydrogenase. *Toxicol Lett* 2004;152:235–243. [PubMed: 15331132]
13. Park JH, Troxel AB, Harvey RG, Penning TM. Polycyclic aromatic hydrocarbon (PAH) *o*-quinones produced by the aldose-keto-reductases (AKRs) generate abasic sites, oxidized pyrimidines, and 8-oxo-dGuo via reactive oxygen species. *Chem Res Toxicol* 2006;19:719–728. [PubMed: 16696575]
14. Penning TM, Drury JE. Human aldose-keto reductases: function, gene regulation, and single nucleotide polymorphisms. *Arch Biochem Biophys* 2007;464:241–250. [PubMed: 17537398]
15. Zeindl-Eberhart E, Haraida S, Liebmann S, Jungblut PR, Lamer S, Mayer D, Jager G, Chung S, Rabes HM. Detection and identification of tumor-associated protein variants in human hepatocellular carcinomas. *Hepatology* 2004;39:540–549. [PubMed: 14768008]
16. Blomhoff R, Blomhoff HK. Overview of retinoid metabolism and function. *J Neurobiol* 2006;66:606–630. [PubMed: 16688755]
17. Crosas B, Hyndman DJ, Gallego O, Martras S, Pares X, Flynn TG, Farres J. Human aldose reductase and human small intestine aldose reductase are efficient retinal reductases: consequences for retinoid metabolism. *Biochem J* 2003;373:973–979. [PubMed: 12732097]
18. Gallego O, Belyaeva OV, Porte S, Ruiz FX, Stetsenko AV, Shabrova EV, Kostereva NV, Farres J, Pares X, Kedishvili NY. Comparative functional analysis of human medium-chain dehydrogenases,

- short-chain dehydrogenases/reductases and aldo-keto reductases with retinoids. *Biochem J* 2006;399:101–109. [PubMed: 16787387]
19. Harvey RG, Dai Q, Ran C, Penning TM. Synthesis of the *o*-quinones and other oxidized metabolites of polycyclic aromatic hydrocarbons implicated in carcinogenesis. *J Org Chem* 2004;69:2024–2032. [PubMed: 15058949]
20. Tarle I, Borhani DW, Wilson DK, Quiocho FA, Petrash JM. Probing the active site of human aldose reductase. Site-directed mutagenesis of Asp-43, Tyr-48, Lys-77, and His-110. *Journal of Biological Chemistry* 1993;268:25687–25693. [PubMed: 8245005]
21. O'Connor T, Ireland LS, Harrison DJ, Hayes JD. Major differences exist in the function and tissue-specific expression of human aflatoxin B₁ aldehyde reductase and the principal human aldo-keto reductase AKR1 family members. *Biochem J* 1999;343:487–504. [PubMed: 10510318]
22. Jiang H, Shen YM, Quinn AM, Penning TM. Competing roles of cytochrome P450 1A1/1B1 and aldo-keto reductase 1A1 in the metabolic activation of (±)-7,8-dihydroxy-7,8-dihydro-benzo[*a*]pyrene in human bronchoalveolar cell extracts. *Chem Res Toxicol* 2005;18:365–374. [PubMed: 15720144]
23. Quinn AM, Penning TM. Comparisons of (±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol activation by human cytochrome P450 and aldo-keto reductase enzymes: effect of redox state and expression levels. *Chem Res Toxicol*. 2008
24. Palackal NT, Lee SH, Harvey RG, Blair IA, Penning TM. Activation of polycyclic aromatic hydrocarbon *trans*-dihydrodiol proximate carcinogens by human aldo-keto reductase (AKR1C) enzymes and their functional overexpression in human lung carcinoma (A549) cells. *J Biol Chem* 2002;277:24799–24808. [PubMed: 11978787]
25. Ramirez RD, Sheridan S, Girard L, Sato M, Kim Y, Pollack J, Peyton M, Zou Y, Kurie JM, DiMaio JM, Milchgrub S, Smith AL, Souza RF, Gilbey L, Zhang X, Gandia K, Vaughan MB, Wright WE, Gazdar AF, Shay JW, Minna JD. Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. *Cancer Res* 2004;64:9027–9034. [PubMed: 15604268]
26. Berge G, Mollerup S, Ovrebø S, Hewer A, Phillips DH, Eilertsen E, Haugen A. Role of estrogen receptor in regulation of polycyclic aromatic hydrocarbon metabolic activation in lung. *Lung Cancer* 2004;45:289–297. [PubMed: 15301869]
27. Bauman DR, Steckelbroeck S, Peehl DM, Penning TM. Transcript profiling of the androgen signal in normal prostate, benign prostatic hyperplasia, and prostate cancer. *Endocrinology* 2006;147:5806–5816. [PubMed: 16959841]
28. Burczynski ME, Harvey RG, Penning TM. Expression and characterization of four recombinant human dihydrodiol dehydrogenase isoforms: oxidation of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene to the activated *o*-quinone metabolite benzo[*a*]pyrene-7,8-dione. *Biochemistry* 1998;37:6781–6790. [PubMed: 9578563]
29. Palackal NT, Burczynski ME, Harvey RG, Penning TM. The ubiquitous aldehyde reductase (AKR1A1) oxidizes proximate carcinogen *trans*-dihydrodiols to *o*-quinones: potential role in polycyclic aromatic hydrocarbon activation. *Biochemistry* 2001;40:10901–10910. [PubMed: 11535067]
30. Ittah Y, Thakker DR, Levin W, Croisy-Delcey M, Ryan DE, Thomas PE, Conney AH, Jerina DM. Metabolism of benzo[*c*]phenanthrene by rat liver microsomes and by a purified monooxygenase system reconstituted with different isozymes of cytochrome P-450. *Chem Biol Interact* 1983;45:15–28. [PubMed: 6872097]
31. Yang SK, Fu PP. Stereoselective metabolism of 7-methylbenz[*a*]anthracene: absolute configuration of five dihydrodiol metabolites and the effect of dihydrodiol conformation on circular dichroism spectra. *Chem Biol Interact* 1984;49:71–88. [PubMed: 6722941]
32. Bushman DR, Grossman SJ, Jerina DM, Lehr RE. Synthesis of optically active fjord-region 11,12-diol 13,14-epoxides and the K-region 9,10-oxide of the carcinogen benzo[*g*]chrysene. *J Org Chem* 1989;54:3533–3544.
33. Penning TM, Lerman C. Genomics of smoking exposure and cessation: lessons for cancer prevention and treatment. *Cancer Prevention Research* 2008;1940–6207.CAPR-1908-0047

34. Park JH, Mangal D, Tacka KA, Quinn AM, Harvey RG, Blair IA, Penning TM. Evidence for the aldo-keto reductase pathway of polycyclic aromatic *trans*-dihydrodiol activation in human lung A549 cells. *Proc Natl Acad Sci U S A* 2008;105:6846–6851. [PubMed: 18474869]
35. Ding YS, Trommel JS, Yan XJ, Ashley D, Watson CH. Determination of 14 polycyclic aromatic hydrocarbons in mainstream smoke from domestic cigarettes. *Environ Sci Technol* 2005;39:471–478. [PubMed: 15707046]
36. IARC. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Polynuclear Aromatic Compounds, Part I, Chemical, Environmental, and Experimental Data. Vol. 32. IARC; Lyon: 1983.
37. Wislocki PG, Fiorentini KM, Fu PP, Yang SK, Lu AY. Tumor-initiating ability of the twelve monomethylbenz[*a*]anthracenes. *Carcinogenesis* 1982;3:215–217. [PubMed: 7067048]
38. DiGiovanni J. Multistage carcinogenesis in mouse skin. *Pharmacol Ther* 1992;54:63–128. [PubMed: 1528955]
39. Levit DI. Polyaromatic hydrocarbons in high-boiling petroleum distillates: isolation by gel permeation chromatography and identification by fluorescence spectrometry. *Anal Chem* 1973;45:1050–1055.
40. Wise SA, Benner BA, Chesler SN, Hilpert LR, Vogt CR, May WE. Characterization of the polycyclic aromatic hydrocarbons from two standard reference material air particulate samples. *Anal Chem* 1986;58:3067–3077.
41. Stevenson JL, Von Haam E. Carcinogenicity of benzo(*a*)anthracene and benz(*c*)phenanthrene derivatives. *Am Ind Hyg Assoc J* 1965;26:475–478. [PubMed: 5865317]
42. Cavalieri EL, Higginbotham S, RamaKrishna NV, Devanesan PD, Todorovic R, Rogan EG, Salmasi S. Comparative dose-response tumorigenicity studies of dibenzo[*a,l*]pyrene versus 7,12-dimethylbenz[*a*]anthracene, benzo[*a*]pyrene and two dibenzo[*a,l*]pyrene dihydrodiols in mouse skin and rat mammary gland. *Carcinogenesis* 1991;12:1939–1944. [PubMed: 1934274]
43. Thakker DR, Levin W, Yagi H, Turujman S, Kapadia D, Conney AH, Jerina DM. Absolute stereochemistry of the *trans*-dihydrodiols formed from benzo[*a*]anthracene by liver microsomes. *Chem Biol Interact* 1979;27:145–161. [PubMed: 498352]
44. Yang, SK.; Mushtaq, M.; Chiu, PL. Stereoselective metabolism and activation of polycyclic aromatic hydrocarbons. In: Harvey, RG., editor. *Polycyclic Hydrocarbons and Carcinogenesis*. 1985. p. 19–34. ACS Symposium Series 283
45. Burczynski ME, Penning TM. Genotoxic polycyclic aromatic hydrocarbon *ortho*-quinones generated by aldo-keto reductases induce CYP1A1 via nuclear translocation of the aryl hydrocarbon receptor. *Cancer Res* 2000;60:908–915. [PubMed: 10706104]
46. Jiang H, Vudathala DK, Blair IA, Penning TM. Competing roles of aldo-keto reductase 1A1 and cytochrome P4501B1 in benzo[*a*]pyrene-7,8-diol activation in human bronchoalveolar H358 cells: role of AKRs in P4501B1 induction. *Chem Res Toxicol* 2006;19:68–78. [PubMed: 16411658]
47. Spycher SE, Tabataba-Vakili S, O'Donnell VB, Palomba L, Azzi A. Aldose reductase induction: a novel response to oxidative stress of smooth muscle cells. *Faseb J* 1997;11:181–188. [PubMed: 9039961]
48. Nakamura N, Obayashi H, Fujii M, Fukui M, Yoshimori K, Ogata M, Hasegawa G, Shigeta H, Kitagawa Y, Yoshikawa T, Kondo M, Ohta M, Nishimura M, Nishinaka T, Nishimura CY. Induction of aldose reductase in cultured human microvascular endothelial cells by advanced glycation end products. *Free Radic Biol Med* 2000;29:17–25. [PubMed: 10962201]
49. Penning TM, Jin Y, Rizner TL, Bauman DR. Pre-receptor regulation of the androgen receptor. *Mol Cell Endocrinol* 2008;281:1–8. [PubMed: 18060684]
50. Xu XC, Sozzi G, Lee JS, Lee JJ, Pastorino U, Pilotti S, Kurie JM, Hong WK, Lotan R. Suppression of retinoic acid receptor β in non-small-cell lung cancer *in vivo*: implications for lung cancer development. *J Natl Cancer Inst* 1997;89:624–629. [PubMed: 9150186]
51. Picard E, Seguin C, Monhoven N, Rochette-Egly C, Siat J, Borrelly J, Martinet Y, Martinet N, Vignaud JM. Expression of retinoid receptor genes and proteins in non-small-cell lung cancer. *J Natl Cancer Inst* 1999;91:1059–1066. [PubMed: 10379969]
52. Brabender J, Metzger R, Salonga D, Danenberg KD, Danenberg PV, Holscher AH, Schneider PM. Comprehensive expression analysis of retinoic acid receptors and retinoid X receptors in non-small

- cell lung cancer: implications for tumor development and prognosis. *Carcinogenesis* 2005;26:525–530. [PubMed: 15618235]
53. Lippman SM, Lotan R. Advances in the Development of Retinoids as Chemopreventive Agents. *J Nutr* 2000;130:479.
 54. de The H, Marchio A, Tiollais P, Dejean A. Differential expression and ligand regulation of the retinoic acid receptor α and β genes. *Embo J* 1989;8:429–433. [PubMed: 2542014]
 55. de The H, Vivanco-Ruiz MM, Tiollais P, Stunnenberg H, Dejean A. Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature* 1990;343:177–180. [PubMed: 2153268]
 56. Shah VO, Scavini M, Nikolic J, Sun Y, Vai S, Griffith JK, Dorin RI, Stidley C, Yacoub M, Vander Jagt DL, Eaton RP, Zager PG. Z-2 microsatellite allele is linked to increased expression of the aldose reductase gene in diabetic nephropathy. *J Clin Endocrinol Metab* 1998;83:2886–2891. [PubMed: 9709964]
 57. van der Leede BM, van den Brink CE, van der Saag PT. Retinoic acid receptor and retinoid X receptor expression in retinoic acid-resistant human tumor cell lines. *Mol Carcinog* 1993;8:112–122. [PubMed: 7691069]
 58. Kim YH, Dohi DF, Han GR, Zou CP, Oridate N, Walsh GL, Nesbitt JC, Xu XC, Hong WK, Lotan R, et al. Retinoid refractoriness occurs during lung carcinogenesis despite functional retinoid receptors. *Cancer Res* 1995;55:5603–5610. [PubMed: 7585641]
 59. Sun SY, Yue P, Dawson MI, Shroot B, Michel S, Lamph WW, Heyman RA, Teng M, Chandraratna RAS, Shudo K, Hong WK, Lotan R. Differential Effects of Synthetic Nuclear Retinoid Receptor-selective Retinoids on the Growth of Human Non-Small Cell Lung Carcinoma Cells. *Cancer Res* 1997;57:4931–4939. [PubMed: 9354460]
 60. Zhang ZQ, Zhang W, Wang NQ, Bani-Yaghoub M, Lin ZX, Naus CC. Suppression of tumorigenicity of human lung carcinoma cells after transfection with connexin43. *Carcinogenesis* 1998;19:1889–1894. [PubMed: 9854998]
 61. Avanzo JL, Menecier G, Mesnil M, Hernandez-Blazquez FJ, Fukumasu H, da Silva TC, Rao KV, Dagli ML. Deletion of a single allele of Cx43 is associated with a reduction in the gap junctional intercellular communication and increased cell proliferation of mouse lung pneumocytes type II. *Cell Prolif* 2007;40:411–421. [PubMed: 17531084]
 62. Petty WJ, Li N, Biddle A, Bounds R, Nitkin C, Ma Y, Dragnev KH, Freemantle SJ, Dmitrovsky E. A novel retinoic acid receptor β isoform and retinoid resistance in lung carcinogenesis. *J Natl Cancer Inst* 2005;97:1645–1651. [PubMed: 16288117]
 63. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med* 1994;330:1029–1035. [PubMed: 8127329]
 64. Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Hammar S. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med* 1996;334:1150–1155. [PubMed: 8602180]
 65. Bendich A, Olson JA. Biological actions of carotenoids. *Faseb J* 1989;3:1927–1932. [PubMed: 2656356]
 66. Gradelet S, Leclerc J, Siess MH, Astorg PO. β -Apo-8'-carotenal, but not β -carotene, is a strong inducer of liver cytochromes P450 1A1 and 1A2 in rat. *Xenobiotica* 1996;26:909–919. [PubMed: 8893038]
 67. Wang XD, Russell RM. Procarcinogenic and anticarcinogenic effects of beta-carotene. *Nutr Rev* 1999;57:263–272. [PubMed: 10568335]

Abbreviations

AKR

aldo-keto reductase

ARE

antioxidant response element

BA	benz[<i>a</i>]anthracene
BA-3,4-dihydrodiol	(±)- <i>trans</i> -3,4-dihydroxy-3,4-dihydro-BA
B[<i>g</i>]C	benzo[<i>g</i>]chrysene
B[<i>g</i>]C-11,12-dihydrodiol	(±)- <i>trans</i> -11,12-dihydroxy-11,12-dihydro-B[<i>g</i>]C
B[<i>a</i>]P	benzo[<i>a</i>]pyrene
B[<i>a</i>]P-7,8-dihydrodiol	(±)- <i>trans</i> -7,8-dihydroxy-7,8-dihydro-B[<i>a</i>]P
(±)-<i>anti</i>-B[<i>a</i>]PDE	(±)- <i>trans</i> -7,8-dihydroxy-7,8-dihydro-9 α ,10 α -epoxy-7,8,9,10-tetrahydro-B[<i>a</i>]P
B[<i>c</i>]Ph	benzo[<i>c</i>]phenanthrene
B[<i>c</i>]Ph-3,4-dihydrodiol	(±)- <i>trans</i> -3,4-dihydroxy-3,4-dihydro-B[<i>c</i>]Ph
Cx43	connexin 43
DB[<i>a,l</i>]P	dibenzo[<i>a,l</i>]pyrene
DB[<i>a,l</i>]P-11,12-dihydrodiol	(±)- <i>trans</i> -11,12-dihydroxy-11,12-dihydro-DB[<i>a,l</i>]P
DMBA	7,12-dimethylbenz[<i>a</i>]anthracene
DMBA-3,4-dihydrodiol	(±)- <i>trans</i> -3,4-dihydroxy-3,4-dihydro-DMBA
EH	epoxide hydrolase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GJIC	gap junctional intercellular communication
8-oxo-dGuo	8-oxo-2'-deoxyguanosine
MC	3-methylcholanthrene
NSCLC	non-small cell lung cancer

P450	cytochrome P450
PAHs	polycyclic aromatic hydrocarbons
PB	phenobarbitol
PBGD	porphobilinogen deaminase
ROS	reactive oxygen species

Figure 1.

AKR1B10 oxidizes B[a]P-7,8-dihydrodiol to form B[a]P-7,8-dione *in vitro*. 20 μ M of ($\pm$)-B[a]P-7,8-dihydrodiol was incubated with AKR1B10 in the presence of 400 μ M β -mercaptoethanol for 0 min (A) and 120 min (B). Reactions were analyzed by RP-HPLC with UV detection at 254 nm. ($\pm$)-B[a]P-7,8-dihydrodiol substrate (C) and the thioether conjugate of the B[a]P-7,8-dione product (D) were identified by comparison of retention times and UV spectra to authentic standards.

Figure 2.

Velocity versus substrate concentration plots for the oxidation of (+)-B[a]P-7S,8S-dihydrodiol catalyzed by 6.7 μg homogenous AKR1B10 (A) and 15 μg AKR1B1 (B). End-point assays were performed in duplicate over 1 h and (+)-B[a]P-7S,8S-dihydrodiol consumption was measured by RP-HPLC. Plots of pmol/min (+)-B[a]P-7S,8S-dihydrodiol consumed versus substrate concentration are shown.

Figure 3.

AKR1B10 (A) and AKR1B1 (B) stereospecifically oxidize the (+)-*S,S* isomer of BA-3,4-dihydrodiol, and AKR1B1 stereospecifically oxidizes the (+)-*S,S* isomer of B[g]C-11,12-dihydrodiol (C). Reactions (25 mL) contained 20 μ M racemic ($\pm$)-BA-3,4-dihydrodiol plus either AKR1B10 (3.5 mg) or AKR1B1 (1.5 mg), or 20 μ M racemic ($\pm$)-B[g]C-11,12-dihydrodiol plus AKR1B1 (1.5 mg) in the presence of 2.3 mM NADP⁺. Reactions were run to completion and the unreacted stereoisomer was purified by TLC. Racemic and unreacted diols were dissolved in methanol and spectra were recorded in 1 nm increments from 240 to 400 nm. CD spectroscopy of all diols yielded a negative Cotton effect, indicating that the unreacted (–)-*R,R* stereoisomer remains and that AKR1B isoforms are stereospecific for the (+)-*S,S* *trans*-dihydrodiol substrate in these reactions. Racemic diols are shown in black, and unreacted diols of AKR1B-catalyzed reactions are shown in gray.

Figure 4.

Determination of steady-state kinetic parameters for reduction of all-*trans*-retinal catalyzed by AKR1B10. Consumption of retinal was measured spectrophotometrically in incubations of 0.1 – 50 μM substrate with AKR1B10 in the presence of NADPH. A plot of nmol/min all-*trans*-retinal consumed versus substrate consumed is shown.

Figure 5.

Expression of AKR transcripts in the A549 human lung adenocarcinoma cell line. mRNA transcripts were evaluated in triplicate using real-time RT-PCR and normalized to the GAPDH housekeeping gene. Data are shown as the mean and standard deviation for each AKR transcript.

Figure 6.

AKR1B10 expression in human cell lines by real-time RT-PCR (A), immunoblot analysis (B), and enzyme activity (C). (A) mRNA transcripts were evaluated in triplicate using real-time RT-PCR methods and expressed as log (fg per fg GAPDH $\times 10^4$). Data are shown as the mean and standard deviation for each AKR transcript. (B) Lysates of A549 (lane 2), HBEC-KT (lane 3), H358 (lane 4), and HepG2 (lane 5) cells containing 20 μ g total protein were loaded in each lane. Specific bands were detected with 1:1000 polyclonal rabbit *anti*-AKR1B10 and goat *anti*-rabbit IgG-HRP conjugate. Purified recombinant AKR1B10 (250 ng) was loaded for comparison of protein expression (lane 1). (C) AKR1B10 activity was measured in cell lysates in triplicate by monitoring the reduction of 10 mM DL-glyceraldehyde. The mean and standard deviation of specific activities (nmol/min/mg total protein) are shown.

Bay Region PAH *trans*-Dihydrodiols

Benzo[a]pyrene-7,8-dihydrodiol

Benz[a]anthracene-3,4-dihydrodiol

Methylated Bay Region PAH *trans*-Dihydrodiols

7,12-Dimethylbenz[a]anthracene-3,4-dihydrodiol

Fjord Region PAH *trans*-Dihydrodiols

Benzo[g]chrysene-11,12-dihydrodiol

Benzo[c]phenanthrene-3,4-dihydrodiol

Dihydrobenzo[a,l]pyrene-11,12-dihydrodiol

Scheme 1.

Structures of PAH *trans*-dihydrodiol substrates examined for AKR1B10. Absolute stereochemistry is shown for the major stereoisomer of each *trans*-dihydrodiol formed metabolically.

Table 1
Steady-State Kinetic Parameters for Oxidation of B[a]P-7,8-Dihydrodiol Catalyzed by Human AKR Isoforms

	B[a]P-7,8-Diol	k_{cat}/K_m , mM ⁻¹ min ⁻¹
AKR1A1	(-)	30.8 ^a
AKR1C1	(±)	22.6 ^a
AKR1C2	(±)	53.3 ^a
AKR1C3	(±)	24.7 ^a
AKR1C4	(±)	16.7 ^a
AKR1B10	(+)	2.36
AKR1B10	(-)	N.D. ^b
AKR1B1	(+)	10.3
AKR1B1	(-)	N.D.

^aValues taken from (23).

^bN.D., not detected.

Table 2
Comparisons of PAH *trans*-Dihydrodiol Substrate Specificity for AKR1B10

PAH <i>trans</i> -dihydrodiols	$V_{\max}/K_m^a$	Stereospecificity
Bay Region		
Benzo[a]pyrene-7,8-diol	2.34	(+)- <i>S,S</i>
Benz[a]anthracene-3,4-diol	12.8	(+)- <i>S,S</i>
Methylated Bay Region		
7,12-Dimethylbenz[a]anthracene-3,4-diol	2.70	(-)- <i>R,R</i> , (+)- <i>S,S</i>
Fjord Region		
Benzo[g]chrysene-11,12-diol	9.55	(-)- <i>R,R</i> , (+)- <i>S,S</i>
Benzo[c]phenanthrene-3,4-diol	1.51	N/D ^b
Dihydrobenzo[a,l]pyrene-11,12-diol	0.38	N/D

^a $V_{\max}/K_m = \mu\text{mol}/\text{min}/\mu\text{mol}/[\text{S}]$ where $v/[\text{S}] = V_{\max}/K_m$ when $K_m \gg [\text{S}]$, to yield units of $\text{min}^{-1}\text{mM}^{-1}$.

^b N.D., not determined.