Chemical Research in Toxicology

Review

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Human Aldo-Keto Reductases and the Metabolic Activation of Polycyclic Aromatic Hydrocarbons

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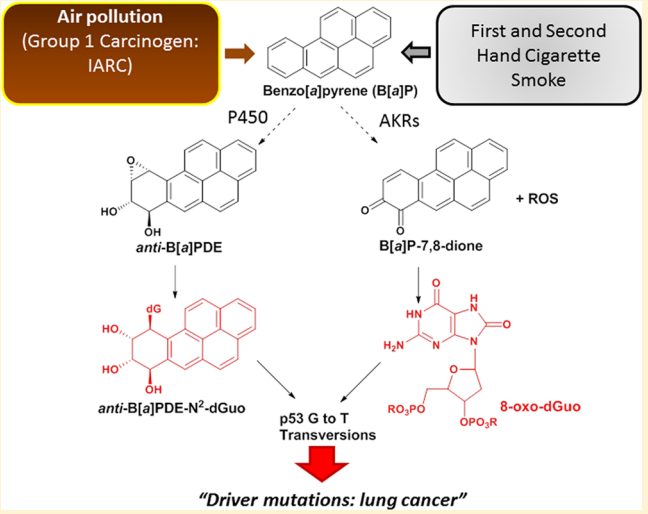
ABSTRACT: Aldo-keto reductases (AKRs) are promiscuous NAD(P)(H) dependent oxidoreductases implicated in the metabolic activation of polycyclic aromatic hydrocarbons (PAH). These enzymes catalyze the oxidation of non-K-region *trans*-dihydrodiols to the corresponding *o*-quinones with the concomitant production of reactive oxygen species (ROS). The PAH *o*-quinones are Michael acceptors and can form adducts but are also redox-active and enter into futile redox cycles to amplify ROS formation. Evidence exists to support this metabolic pathway in humans. The human recombinant AKR1A1 and AKR1C1−AKR1C4 enzymes all catalyze the oxidation of PAH *trans*-dihydrodiols to PAH *o*-quinones. Many human AKRs also catalyze the NADPH-dependent reduction of the *o*-quinone products to air-sensitive catechols, exacerbating ROS formation. Moreover, this pathway of PAH activation occurs in a panel of human lung cell lines, resulting in the production of ROS and oxidative DNA damage in the form of 8-oxo-2′-deoxyguanosine. Using stable-isotope dilution liquid chromatography tandem mass spectrometry, this pathway of benzo[*a*]pyrene (B[*a*]P) metabolism was found to contribute equally with the diol-epoxide pathway to the activation of this human carcinogen in human lung cells. Evaluation of the mutagenicity of *anti*-B[*a*]P-diol epoxide with B[*a*]P-7,8-dione on p53 showed that the *o*-quinone produced by AKRs was the more potent mutagen, provided that it was permitted to redox cycle, and that the mutations observed were G to T transversions, reminiscent of those observed in human lung cancer. It is concluded that there is sufficient evidence to support the role of human AKRs in the metabolic activation of PAH in human lung cell lines and that they may contribute to the causation of human lung cancer.



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INTRODUCTION

The last comprehensive review of the role of aldo-keto reductases (AKRs) in the metabolic activation of polycyclic aromatic hydrocarbons (PAH) appeared in this journal in 1999.[1] At that time, the AKRs involved were referred to as dihydrodiol dehydrogenases; however, these promiscuous enzymes are now recognized as being members of the AKR superfamily and will be discussed as such. The focus of this

Received: July 17, 2014
Published: October 3, 2014

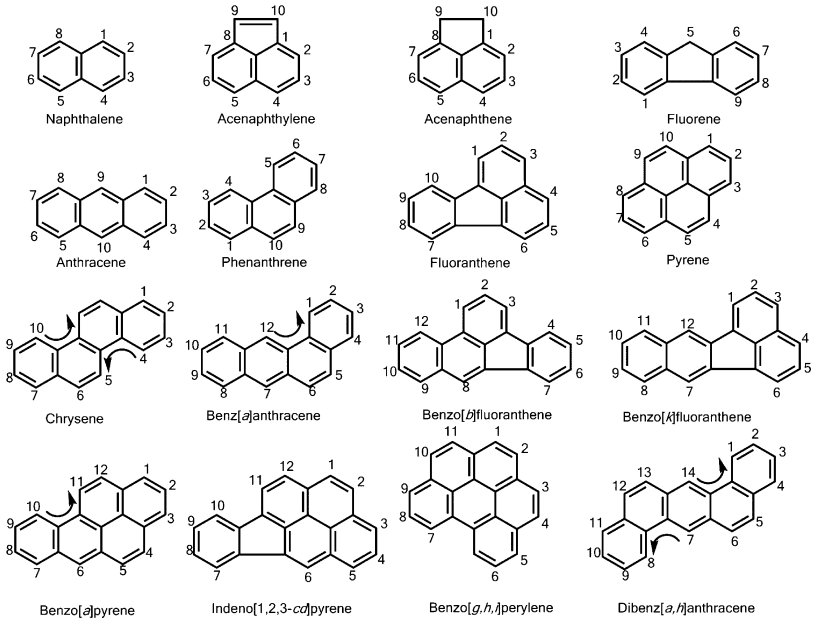

Figure 1. EPA priority PAH pollutants. Carcinogenic PAH have four or more fused benzene rings and contain a bay region denoted by the curly arrow.

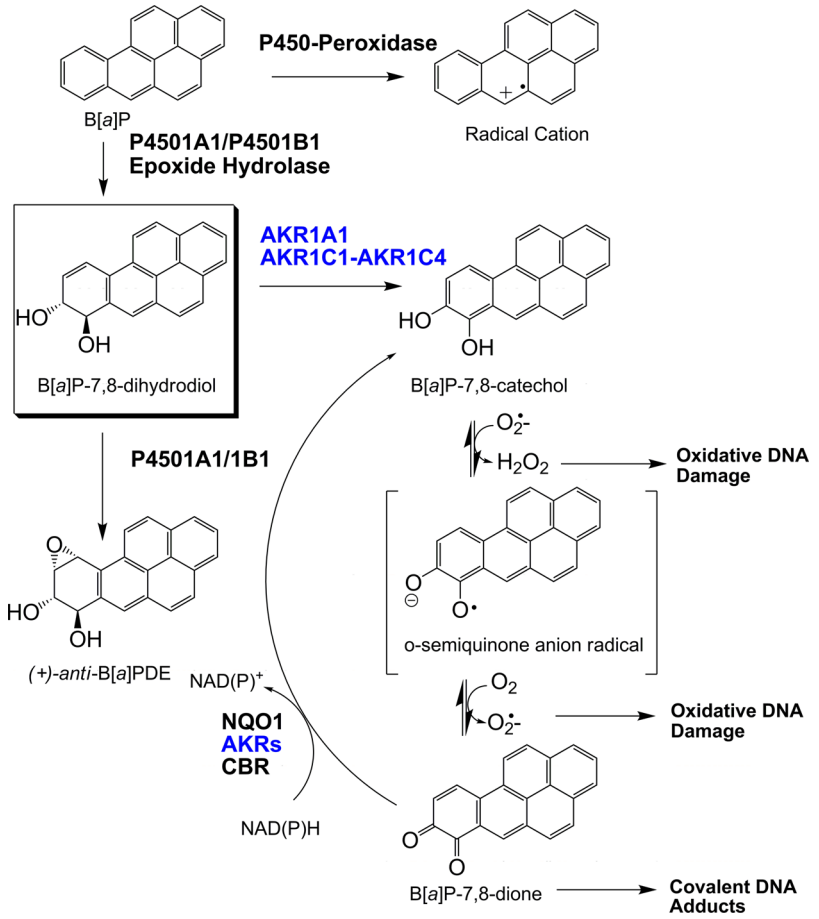

Figure 2. Pathways of PAH activation. Three pathways are shown: the radical-cation pathway catalyzed by P450 peroxidase activity, the diol-epoxide pathway that gives rise to *anti*-B[*a*]PDE, and the *o*-quinone pathway that gives rise to B[*a*]P-7,8-dione.

review will be to describe (a) the human AKRs and their ability to act as dihydrodiol dehydrogenases, (b) their role in the metabolism of PAH in human lung cells, (c) the genotoxicity of their PAH *o*-quinone products, (d) AKR genomics (regulation of gene expression, splice variants, and polymorphisms), and (e) future directions. Before discussing these topics, a brief description of the position of AKRs in PAH metabolism/activation is warranted to orient the reader.

PAH are ubiquitous environmental pollutants and are products of incomplete combustion of fossil fuels and other organic matter; they are prevalent in the air we breathe and can be found in diesel exhaust, air-borne fine particulate matter ($PM_{2.5}$), and mainstream and second-hand tobacco smoke.[2−6] PAH can also enter the food supply and can be introduced by either food preparation or food processing, e.g., barbecuing and smoking of food, etc. or by the growth of leafy plants and vegetables in PAH-contaminated soil.[7,8] The most thoroughly studied PAH is benzo[*a*]pyrene (B[*a*]P), which has five fused benzene rings and is now listed as a Group 1 or known human carcinogen by the International Agency for the Research on Cancer (IARC).[5] The United States Environmental Protection Agency (EPA) PAH priority air pollutants and their relationship to B[*a*]P is shown in Figure 1.

B[*a*]P is innocuous by itself and must be metabolically activated to mediate its deleterious effects. Three major pathways of metabolic activation have been proposed in the literature, and these pathways are generally applicable to other PAH as well, Figure 2. A fourth activation pathway, the benzylic sulfonation pathway has been reported for alkylated PAH in mouse studies.[9,10] There is also one report indicating that this may occur with nonalkylated PAH, such as B[*a*]P in animals.[11] However, this pathway is not discussed since we have yet to compare its involvement in PAH activation versus that observed with AKRs.

In the first pathway of B[*a*]P activation, B[*a*]P is used as a coreductant for complex I formed in the peroxidase cycle of P450 or other peroxidases. Complex I is the equivalent of perferryl-oxygen (FeV^{+}═O), which must be reduced back to the resting ferric state. By removing electrons from C6 of B[*a*]P, a reactive radical-cation is formed that can form depurinating DNA adducts. This pathway is known as the radical-cation pathway.[12,13] Evidence for this pathway includes detection of depurinating DNA adducts from dibenzo[*a,l*]-pyrene in mouse skin, where this PAH is one of the most potent PAH tumorigens known.[14] This pathway has not been widely accepted due to the short half-life of the radical-cation and the need for this reactive intermediate to transit from the microsomes into the nucleus to attack unfolded DNA. Importantly, this pathway does not have a requirement in the PAH structure for a bay region; yet, the presence of the bay region is a critical determinant of PAH carcinogenicity.[15]

In the second pathway, B[*a*]P undergoes monooxygenation on the terminal benzo-ring catalyzed by cytochrome P4501A1 (hepatic) and P4501B1 (extrahepatic) enzymes[16] to the corresponding 7*S*,8*S*-arene oxide.[17,18] Ring opening catalyzed by epoxide hydrolase leads to the formation of the non-K-region dihydrodiol to form the stereochemically preferred (−)7*R*,8*R*-*trans*-dihydrodiol-7,8-dihydroxy-B[*a*]P (B[*a*]P-7,8-*trans*-dihydrodiol). If the dihydrodiol is not intercepted, then it can undergo a subsequent round of monooxygenation to form the 7α,8β-dihydroxy-9α,10α-oxo-7,8,9,10-tetrahydro-B[*a*]P ((+)-*anti*-diol-B[*a*]P-epoxide, (+)-*anti*-B[*a*]PDE).[19,20] (+)-*Anti*-B[*a*]PDE forms stable (+)-*anti*-B[a]PDE-N^2-dGuo adducts that have been detected *in vitro* and *in vivo*.[21−23] Compelling evidence exists that (+)-*anti*-B[*a*]PDE is the most mutagenic B[*a*]P metabolite in the Ames test[24] and the most tumorigenic metabolite in the new born mouse model of lung carcinogenesis.[25] This pathway is known as the *anti*-diol epoxide pathway and is shared by all bay region containing PAH, e.g., 5-methyl-chrysene,[26] benz[*a*]anthracene,[27] benzo[*c*]phenanthrene,[28] dibenz[*a,h*]anthracene,[29] and dibenzo[*a,l*]-pyrene.[30]

In the third pathway, the intermediate B[*a*]P-7,8-*trans*-dihydrodiol is oxidized by dihydrodiol dehydrogenase using $NAD(P)^+$ as cofactor to form the corresponding ketol. The ketol tautomerizes to form the air-sensitive catechol, 7,8-dihydroxy-B[*a*]P. This catechol undergoes one-electron oxidation in air to form an *o*-seminquinone anion radical and a subsequent one-electron oxidation in air to form the fully oxidized B[*a*]P-7,8-dione (*o*-quinone) (eqs 1−3).[31,32] The two-electron oxidation of the catechol to the *o*-quinone consumes molecular oxygen and produces reactive oxygen species (ROS; superoxide anion radical and hydrogen peroxide), eq 3.[33]

$$QH_2 + O_2^{\bullet -} = Q^{\bullet} + H_2O_2 \quad (1)$$

$$Q + O_2 = O_2^{\bullet -} + Q \quad (2)$$

$$QH_2 + O_2 = Q + H_2O_2 \text{ (net sum of equations 1 and 2)} \quad (3)$$

$$NADPH + H^+ + Q = NADP^+ + QH_2 \quad (4)$$

where QH_2, catechol; Q, quinone.

Once formed, the *o*-quinone is a highly reactive Michael acceptor and can form conjugates with glutathione and covalent adducts with DNA.[34−36] However, the *o*-quinone is also redox-active, and in the presence of reducing equivalents, e.g., NADPH, it is reduced back to the catechol (eq 4). If the catechol is not intercepted by catechol-*O*-methyltransferase (COMT), sulfotransferases (SULTs), or urdine glucuronsyl transferases (UGTs), then it will be reoxidized to the *o*-quinone to establish a futile redox cycle. In this cycle, NADPH and molecular oxygen are consumed and ROS are generated, establishing a ROS amplification system, Figure 2.[37] The link between B[*a*]P metabolism and ROS generation is compelling since the generation of a prooxidant state has been linked to tumor promotion.[38] This pathway of PAH activation is known as the *o*-quinone or AKR pathway of B[*a*]P activation and resembles the estrogen *o*-quinone pathway, suggesting a common mechanism in PAH and estrogen carcinogenesis involving catechols and their corresponding *o*-quinones.[39,40] Components of the AKR pathways were first demonstrated with the rat liver dihydrodiol dehydrogenase (AKR1C9), where it was shown that it was generally applicable to other carcinogenic PAH (e.g., chrysene, 5-methyl-chrysene, benz[*a*]-anthracene, 7-methylbenz[*a*]anthracene, 12-methylbenz[*a*]-anthracene, and 7,12-dmethylbenz[*a*]anthracene (DMBA)).[31,32,41] The oxidation of PAH *trans*-dihydrodiols by AKR1C9 leads to the formation of ROS, as measured by spin-trapping and EPR.[33] Both the diol-epoxide and *o*-quinone pathways have a requirement for the non-K-region *trans*-dihydrodiol intermediate and therefore the presence of a bay region within the PAH structure is required.[31] K-region *trans*-dihydrodiols were not substrates for AKR1C9.[31]

HUMAN ALDO-KETO REDUCTASES AND PAH *TRANS*-DIHYDRODIOL OXIDATION

There are five major AKR enzymes involved in the metabolic activation of PAH *trans*-dihydrodiols in humans. These are AKR1A1 (aldehyde reductase)[42] and AKR1C1−AKR1C4 (hydroxysteroid dehydrogenases).[43] AKR1A1 is an enzyme of intermediary metabolism that converts D,L-glyceraldehyde to glycerol for triglyceride biosynthesis and also converts

dx.doi.org/10.1021/tx500298n | *Chem. Res. Toxicol.* 2014, 27, 1901−1917

Table 1. Properties of Human AKRs that Oxidize PAH *trans*-Dihydrodiols

AKR1A1	AKR1C1−AKR1C4
Aldehyde Reductase	Human hydroxysteroid dehydrogenases (>86% sequence identity)
Enzyme of intermediary metabolism involved in triglyceride biosynthesis	Involved in steroid hormone metabolism
Constitutively expressed in tissues where P4501A1/1B1 and epoxide hydrolase are coexpressed	AKR1C1−AKR1C2 are highly overexpressed in nonsmall cell lung carcinoma
	AKR1C4 is liver-specific
	AKR1C1−AKR1C3 are upregulated by an ARE
Recombinant enzyme has highest k_{cat}/K_m for B[*a*]P-7,8-*trans*-dihydrodiol oxidation	Recombinant enzymes prefer bay-region substituted *trans*-dihydrodiols
Stereospecific for (−)-B[*a*]P-7,8-*trans*-dihydrodiol	No stereospecificity
Reaction product B[*a*]P-7,8-dione	Reaction products PAH *o*-quinones

Table 2. Specific Activities for B[*a*]P-7,8-*trans*-Dihydrodiol Oxidation and B[*a*]P-7,8-Dione Reduction Catalyzed by Human AKRs

AKR	B[*a*]P-7,8-*trans*-dihydrodiol isomer	B[*a*]P-7,8-*trans*-dihydrodiol oxidation (nmol/min/mg)	B[*a*]P-7,8-dione reduction (nmol/min/mg)
AKR1A1	(−)	16.0	350
AKR1B1	(+)	0.13	250
AKR1B10	(+)	0.15	250
AKR1C1	(+/−)	3.6	64
AKR1C2	(+/−)	4.7	350
AKR1C3	(+/−)	0.44	130
AKR1C4	(+/−)	2.0	130
AKR7A2	(+/−)	ND[a]	1270
AKR7A3	(+/−)	ND	1170

[a]ND = not detected; values taken from ref 54.

melvadate (aldehyde precursor of the primary alcohol in mevalonic acid) to mevaloinc acid for cholesterol biosynthesis.[44] AKR1A1 is ubiquitously expressed in human tissues and can therefore be coexpressed with induced P4501A1/1B1 and epoxide hydrolase. Recombinant AKR1A1 has the highest catalytic efficiency (k_{cat}/K_m) for the oxidation of B[*a*]P-7,8-*trans*-dihydrodiol and it is stereospecific for (−)-7*R*,8*R*-*trans*-dihydrodiol, the major stereoisomer formed metabolically.[42] The reaction product B[*a*]P-78-dione can be trapped as a thio-ether conjugate with 2-mercaptoethanol *in vitro*[45] and can be detected in H358 (human broncholaveolar) cells stably transfected with AKR1A1.[46] The highly related AKR1B1 (aldose reductase) and AKR1B10 (retinal reductase) were found to preferentially oxidize the (+)-B[*a*]P-7*S*,8*S*-*trans*-dihydrodiol, which is the minor isomer formed metabolically.[47] Thus, AKR1B enzymes do not play a major role in B[*a*]P activation, Table 1.

The human AKR1C1−AKR1C4 enzymes play the predominant role in PAH activation via their dihydrodiol dehydrogenase activity.[48] These enzymes display 3-keto-, 17-keto-, and 20-keto-steroid reductase activities.[49] Of these, AKR1C4 appears to be liver-specific. By contrast, AKR1C1−AKR1C2 are among the most highly expressed genes in non-small cell lung carcinoma (NSCLC).[50] Importantly, AKR1C1−AKR1C3 are among the most highly regulated genes controlled by the antioxidant response element (ARE).[51] This raises the prospect that ROS produced in the redox cycling of *o*-quinones can lead to further induction of AKR1C enzymes to amplify the metabolic activation of PAH via a positive feed-back loop. The AKR1C enzymes have a preference for bay region-substituted *trans*-dihydrodiols, which are proximate carcinogens derived from the highly tumorigenic bay region-methylated PAH. Bay region-methylated PAH, e.g., 5-methyl-chrysene and DMBA, are significantly more tumorigenic than either chrysene or benz[*a*]anthracene, respectively.[52] Unlike AKR1A1, AKR1C isoforms do not display stereochemical preference but instead oxidize both the (−)*R*,*R*- and the (+)*S*,*S*-dihydrodiols of a variety of PAH. The reactions products are again the PAH *o*-quinones, which can be trapped as their thio-ether conjugates.[48]

HUMAN ALDO-KETO REDUCTASES AND PAH *O*-QUINONE REDUCTION

The ability of AKRs to oxidize non-K-region PAH *trans*-dihydrodiols is unusual since these enzymes prefer to act as carbonyl reductases with a significant preference for NADPH over NAD^+. The enzymes display nanomolar affinity for the former and high micromolar affinity for the latter cofactor.[53] These affinities are close to the prevailing intracellular cofactor concentrations. However, the thermodynamic driving force for *trans*-dihydrodiol oxidation appears to be the formation of the fully aromatic catechol. Because of their preference for NADPH and carbonyl substrates, the ability of human AKRs to reduce PAH *o*-quinones was also examined.

Using a panel of human recombinant AKRs, it was found that these enzymes favored the reduction of B[*a*]P-7,8-dione (the AKR product) by several orders of magnitude over *trans*-dihydrodiol oxidation, Table 2.[54] In these reactions, all of the excess NADPH was consumed concomitant with the disappearance of molecular oxygen and the formation of superoxide anion and hydrogen peroxide. Interestingly, those AKR isoforms with the highest specific activities for the reduction of B[*a*]P-7,8-dione were not the same isoforms that have the highest specific activity for B[*a*]P-7,8-*trans*-dihydrodiol oxidation. Importantly, the ability of the AKR7A enzymes to reduce B[*a*]P-7,8-dione was similar to that observed for NADPH:quinone oxidoreductase (NQO1) and greater than that observed with the carbonyl reductases CBR1 and CBR3. Thus, once B[*a*]P-7,8-*trans*-dihdyrodiol is oxidized to B[*a*]P-7,8-dione, rapid enzymatic redox cycling will ensue, Table 3.

Table 3. Specific Activities for the Conjugation of B[*a*]P-7,8-Catechol and Reduction of B[*a*]P-7,8-Dione

human recombinant enzyme	specific activity with 10 μM B[*a*]P-7,8-dione (nmol/min/mg)
NQO1	1070[a]
AKR7A2	1270[a]
AKR1C1	64[a]
sCOMT	55[b]
SULT1A1	0.8[b]
UGT1A3	>1.0[c]

[a]Six replicates with SD < 10% [b]From Michaelis–Menten plot. [c]Commercial Supersomes.

MAJOR PATHWAYS OF PAH ACTIVATION IN HUMAN LUNG CELLS

With three pathways potentially contributing to PAH activation (radical-cation, diol-epoxide, and *o*-quinone pathways), it becomes critically important to identify the major pathway(s) in human lung since PAH are inhaled carcinogens. Such knowledge will lead to biomarkers of PAH exposure, biomarkers of PAH effect, e.g., PAH–DNA adducs, and the identification of candidate pathway genes for expression profiling and SNP variants that may determine individual genetic susceptibility to lung cancer.

To identify the major pathways of PAH activation in human lung cells, signature metabolites and signature DNA adducts of the three pathways need to be measured, Figure 3. For the

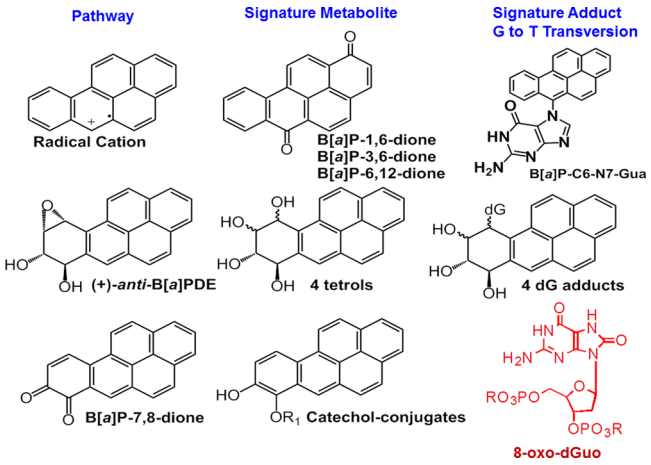

Figure 3. Signature metabolites and DNA adducts of three pathways of PAH activation.

radical-cation pathway, the signature metabolites are the extended diones B[*a*]P-1,6-dione, B[*a*]P-3,6-dione, and B[*a*]*P*-6,12-dione, and a representative DNA adduct would be the B[*a*]P–C6-N7-Gua adduct. For the diol-epoxide pathway, the signature metabolites would be the B[*a*]P-tetrols that result from hydrolysis of the (+)-*anti*-B[*a*]PDE, and a representative DNA adduct would be (+)-*anti*-B[*a*]PDE–N^2-dGuo. For the *o*-quinone pathway, the signature metabolites would be either B[*a*]P-7,8-dione or B[*a*]P-7,8-catechol, and the DNA adduct of interest would be 8-oxo-2′-deoxyguanosine (8-oxo-dGuo).

To measure these analytes, stable-isotope dilution liquid chromatographic tandem mass spectrometric (SID-LC-MS/MS) methods were developed and applied to studies of B[*a*]P metabolism and DNA adduct formation in three different human cell lines: H358 cells (human bronchoalveolar cells type 2 cells), which have low but inducible P4501B1 and AKR1C enzymes, A549 cells (human adenocarcinoma cells derived from non-small cell lung carcinoma, NSCLC), which have low P4501B1 expression but high constitutive expression of AKR1C isozymes, and HBEC-tk cells (immortalized human bronchial epithelial cells), which contain inducible *CYP1B1* and *AKR1C* expression. Signature metabolites for the radical-cation pathway, the diol-epoxide pathway, and the *o*-quinone pathway were best separated and detected using LC-atmospheric pressure chemical ionization/multiple reaction monitoring/MS/MS. Isotopically labeled [$^{13}C_4$]-B[*a*]P metabolites were synthesized as internal standards for each pathway using total synthesis employing palladium-catalyzed cross-coupling chemistry, Figure 4.[55] The limit-of-detection on column for each of the metabolites was in the range of 1.6–6 fmoles. H358 cells were then incubated with either DMSO or 10 nM of the aryl hydrocarbon receptor (AhR) inducer, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), for 12 h and then incubated with 4.0 μM B[*a*]P. It was found that B[*a*]P was more rapidly metabolized in cells treated with TCDD since analytes appeared earlier in the time course. Importantly, the levels of B[*a*]P-1,6-dione plus B[*a*]P-3,6-dione, B[*a*]P-tetrol-1, and B[*a*]P-7,8-dione formed were almost identical.[56] These data showed that in this human lung epithelial cell line all three pathways make a significant and equal contribution to B[*a*]P activation, Figure 5.

We applied similar methods to measure the two dominant DNA adducts of interest, (+)-*anti*-B[*a*]PDE–N^2-dGuo and 8-oxo-dGuo. H358 cells were pretreated with DMSO or TCDD and then challenged with 2 μM B[*a*]P-7,8-*trans*-dihydrodiol, the common precursor of the diol-epoxide and *o*-quinone pathways. It was found that (+)-*anti*-B[*a*]PDE–N^2-dGuo adducts were detected at 3 adducts per 10^7 normal bases but that the levels of adducts were reduced in the TCDD-treated cells.[57,58] This paradoxical result was explained by TCDD induction of GST and the subsequent scavenging of the diol-epoxide as a glutathione (GSH) conjugate.[59] The SID-LC-MS/MS method for measuring 8-oxo-dGuo included the use of a [$^{15}N_5$]-8-oxo-dGuo as an internal standard and the rigorous use of antioxidants to prevent the adventitious oxidation of dGuo in the workup.[60] A concentration-dependent increase in 8-oxo-dGuo levels was also observed when cells were treated with B[*a*]P-7,8-*trans*-dihydrodiol, and levels reached 16 oxo-dGuo/10^7-dGuo, Figure 6. Thus, more 8-oxo-dGuo adducts were observed than stable (+)-*anti*-B[*a*]PDE adducts when H358 cells were treated similarly with B[*a*]P-7,8-*trans*-dihydrodiol.

The metabolic activation of B[*a*]P-7,8-*trans*-dihydrodiol was also examined in A549 cells. Early transcriptomic studies showed that 317/381 NSCLC patients overexpressed AKR1C1 by differential display.[50] Using tissue microarrays, we found *AKR1C* genes to be highly overexpressed in A549 cells, which was confirmed by immunoblot analysis and enzyme assay.[48] The overexpression of *AKR1C* genes in NSCLC and A549 cells is due to a mutation in Keap-1, which leads to high constitutive expression of Nrf2 and high levels of AKR1C enzymes.[61,62] We next exposed A459 cell lysates to B[*a*]P-7,8-*trans*-dihydrodiol and were able to trap the B[*a*]P-7,8-dione as a B[*a*]P-7,8-dione-thioether conjugate and authenticate its structure by LC-MS. When intact A549 cells were exposed to B[*a*]P-7,8-*trans*-dihydrodiol, there was a concomitant disappearance of this AKR1C substrate and disappearance of B[*a*]P-7,8-dione, the AKR product.[63] Concurrently, the formation of ROS was detected by measuring increases in dichlorofluorescein diacetate (DCFH-DA) fluoresence in cells treated with both

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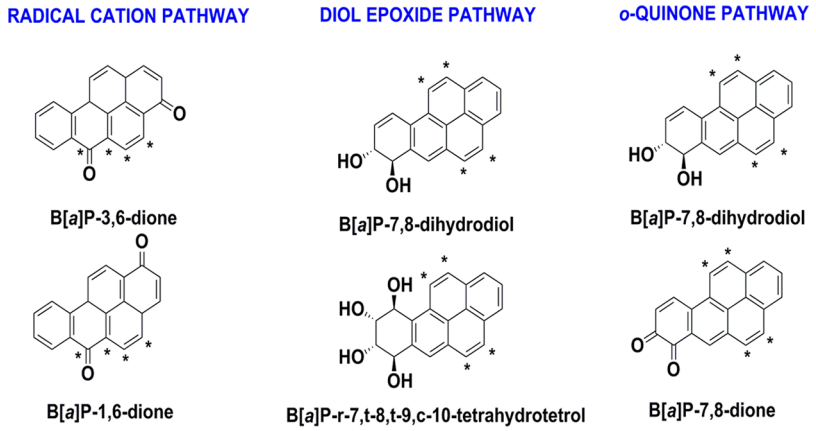

Figure 4. [$^{13}C_4$]-Internal standards for measuring signature metabolites of pathways of PAH activation. Asterisks (*) denote the position of [^{13}C] incorporation.

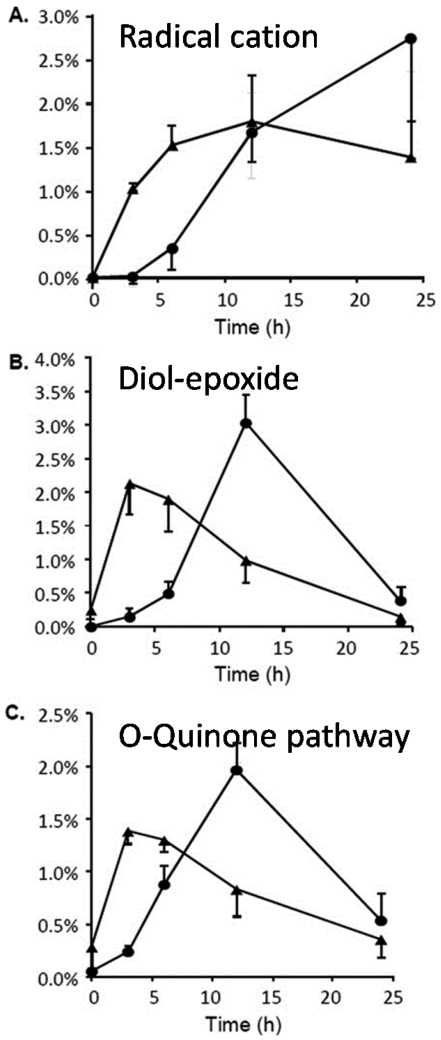

Figure 5. Equal contribution of the radical-cation, diol-epoxide, and *o*-quinone pathways in the metabolic activation of B[*a*]P in human bronchoalveolar type 2 (H358) cells. The radical-cation pathway was measured as B[*a*]P-1,6-dione and B[*a*]*P*-6,3-dione (A), the diol-epoxide pathway was measured as B[*a*]P-tetrol 1 (B), and the *o*-quinone pathway was measured as B[*a*]P-7,8-dione (C). All metabolites were measured by SID-LC-MS/MS. Reprinted from ref 56. Copyright 2012 American Chemical Society.

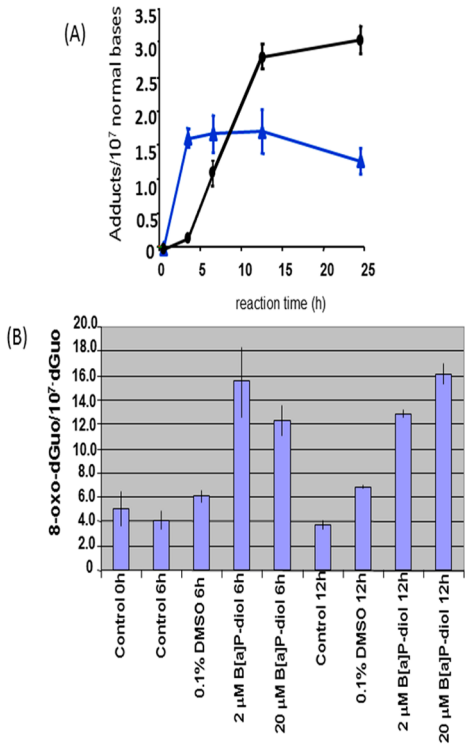

Figure 6. Formation of *anti*-B[*a*]PDE and 8-oxo-dGuo measured by SID-LC-MS/MS in human broncholaveolar type 2 (H358) cells. *anti*-B[*a*]PDE–N^2-dGuo adducts formed after incubation with 2 μM B[*a*]P-7,8-*trans*-dihydrodiol, solvent control (black), and pretreatment with 10 nM TCDD (blue) (A); 8-oxo-dGuo formation in the presence of either DMSO or increasing concentrations of B[*a*]P-7,8-*trans*-dihydrodiol as indicated (B). Adducts were measured by stable-isotope dilution liquid chromatography tandem mass spectrometry.

the AKR substrate and AKR product. However, no increase in DCFH-DA fluorescence was observed with (±)-*anti*-B[*a*]PDE (diol-epoxide) or the regioisomer B[*a*]*P*-4,5-*trans*-dihydrodiol (a non-AKR substrate). The DCFH-DA fluorescence was attenuated with ROS scavengers. These results demonstrated that ROS formation was AKR-dependent. In the same experiments, B[*a*]P-7,8-*trans*-dihydrodiol increased DNA strand breaks, as measured by the COMET assay. The strand breaks increased significantly in the presence of human oxoguanine glycosylase (hOGG1), the base excision repair enzyme

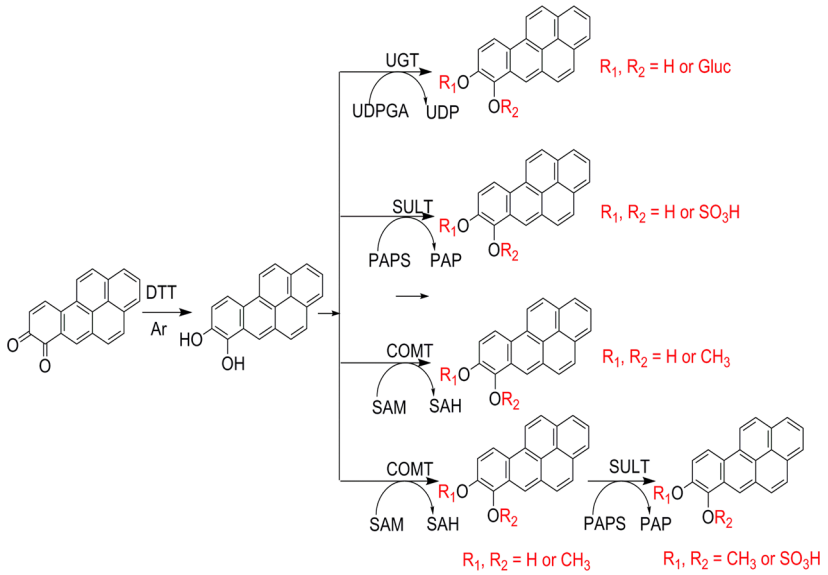

Figure 7. Synthesis of PAH-catechol standards. B[*a*]P-7,8-dione (2 μM) is reduced to the catechol in the presence of DTT in the absence of air. The formed catechol is coupled with the conjugating enzyme indicated in the presence of the corresponding cofactor at 37 °C for 1 h. For the formation of the *bis*-conjugate, the O-methylated catechol is formed first. COMT, catechol-O-methyl transferase; PAP, phosphoadenosine phosphate; PAPS, phosphoadenosine phosphosulfate; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine; SULT, sulfotransferase; UGT, uridine glucuronsyl transferase; UDPGA, uridine diphosphoglucuronic acid; and UDP, uridine diphosphate.

that removes 8-oxo-dGuo to generate a DNA strand break. B[*a*]P-7,8-*trans*-dihydrodiol-dependent DNA strand breaks were enhanced by a COMT inhibitor. SID-LC-MS/MS was also used to measure the formation of 8-oxo-dGuo directly after treating the cells with B[*a*]P-7,8-*trans*-dihydrodiol. Similar to the results with DNA strand breaks, 8-oxo-dGuo levels were significantly increased with B[*a*]P-7,8-*trans*-dihydrodiol treatment but were enhanced with a COMT inhibitor.[63] These data showed that ROS and oxidative DNA damage observed following B[*a*]P-7,8-*trans*-dihydrodiol oxidation were dependent on the interconversion of B[*a*]P-7,8-catechol and B[*a*]P-7,8-dione and their resultant redox cycling since the affects were excaerbated by a COMT inhibitor. These studies also show that the entire AKR pathway is functional in A549 cells and that this leads to ROS production, oxidative DNA damage, and the formation of 8-oxo-dGuo.[63]

METABOLISM OF PAH *O*-QUINONES

Our studies in A549 cells demonstrated that the redox cycling of PAH *o*-quinones could be intercepted by COMT. To conduct a systematic study of the enzymatic conjugation of PAH-catechols, anaerobic reduction of the PAH *o*-quinones was performed in the presence of the relevant conjugating enzyme and respective cofactor.[64] This approach was used to generate steady-state kinetic parameters for the conjugating enzymes and to generate authentic standards for product identification by LC-MS/MS, Figure 7. These authentic standards were then used to identify these metabolites in human lung cells.

We found that human recombinant COMT formed two regioisomeric B[*a*]P-7,8-catechol metabolites.[65] The dominant isomer corresponded to the 8-*O*-monomethylated-B[*a*]P-7,8-catechol and was also identified as the major COMT metabolite in A549 cells.[66] Subsequently, the major sulfotransferases (SULTs) and uridine glucuronsyl transferases (UGTs) that were expressed in A549, H358, and HBEC-tk cells were profiled by RT-PCR so that only those isoforms expressed were examined for the formation of conjugates *in vitro*.[67,68]

SULT1A1 was found to be the dominant enzyme expressed in lung cells. Incubation of human recombinant SULT1A1 led to the formation of 7-*O*-monosulfonated-B[*a*]P-7,8-catechol, which was also the product formed in A549 cells.[67] RT-PCR showed that UGTs were expressed only in A549 cells and the isoforms detected were UGT1A1, 1A3, and 2B7. These recombinant UGTs were examined for their kinetic constants and product profile using B[*a*]P-7,8-catechol as a substrate.[68] On the basis of expression level and product profile, it was concluded that UGT1A3 was the isoform responsible for the formation of 8-*O*-monoglucuronsyl-B[*a*]P-7,8-dione.[68] Examination of the specific activities of COMT, SULT1A1, and UGT1A3 to conjugate B[*a*]P-7,8-catechol versus the ability of either NQO1 or AR7A2 to reduce B[*a*]P-7,8-dione showed that quinone reduction reactions had specific activities that exceeded those for the conjugating enzymes by 20−1000-fold, Table 3. Thus, the ability of conjugating enzymes to intercept the catechol could be overwhelmed by the propensity of B[*a*]P-7,8-dione to be enzymatically redox-cycled. Thus, B[*a*]P-7,8-dione has the capability of causing significant redox stress even in the presence of conjugating enzymes, and this is supported by the data obtained in A549 cells (see earlier). Further evidence that AKRs divert PAH *trans*-dihydrodiols to PAH *o*-quinones was demonstrated in H358 cells transfected with AKR1A1. These cells were protected from the cytotoxic effects of diol-epoxides, but the ensuing ROS caused an increase in expression of hemeoxygenase.[69]

The metabolic fate of [^{3}H]-B[*a*]P-7,8-dione was further examined in A549, H358, and HBEC-tk cells.[66] It was found that [^{3}H]-B[*a*]P-7,8-dione was rapidly consumed and that radioactivity was distributed between the organic and aqueous phases of the cell media and was also found in the cell lysate pellets. Using an identical amount of unlabeled B[*a*]P-7,8-dione, GSH, and *N*-acetyl-L-cysteine (NAC), B[*a*]P-7,8-dione conjugates were identified. 8-*O*-Monomethylated-catechol, 8-*O*-

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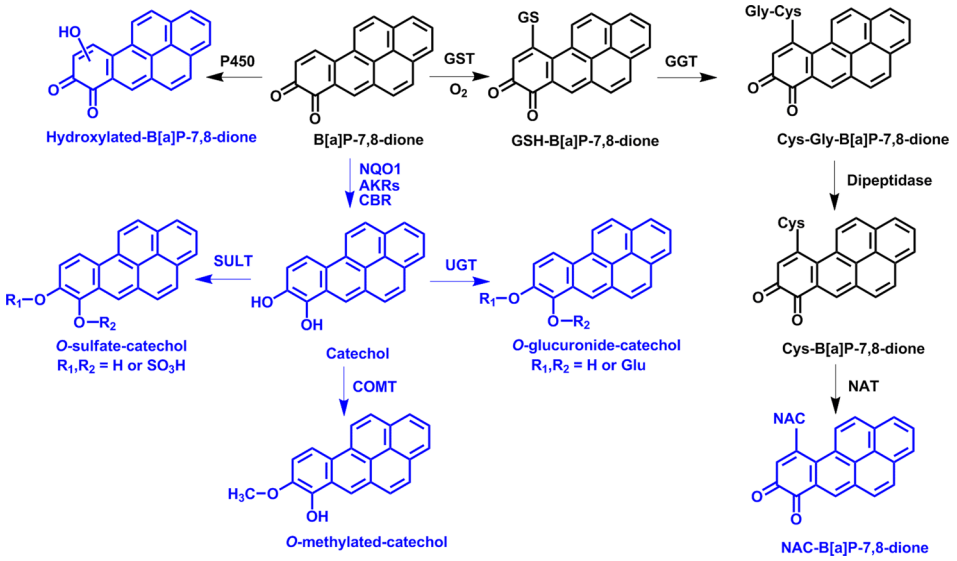

Figure 8. Metabolism of B[*a*]P-7,8-dione in human lung cells. B[*a*]P-7,8-dione was incubated with H358, A549, and HBEC-tk cells. The metabolites were subsequently identified by ion-trap mass spectrometry using authentic synthetic standards. COMT, catechol-*O*-methyl transferase; GST, glutathione-S-transferase; GGT, gamma-glutamyl transpeptidase; and NAT, cysteine-S-conjugate-*N*-acetyl transferase. Reprinted from ref 66. Copyright 2012 American Chemical Society.

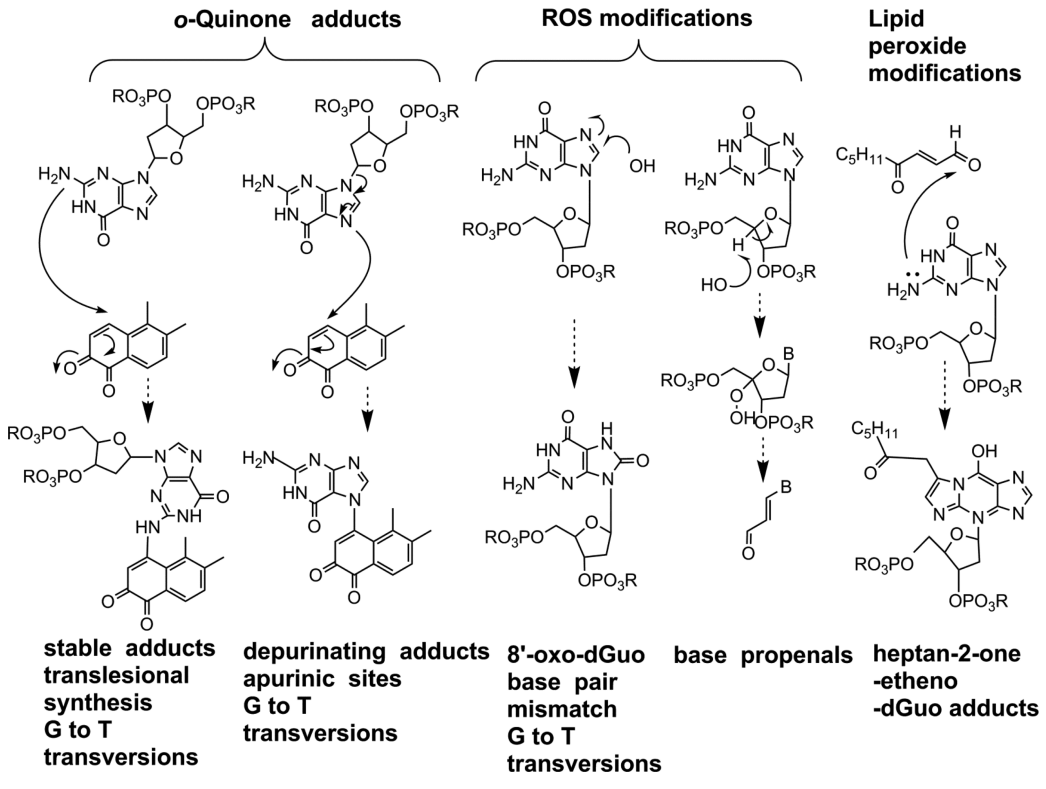

Figure 9. PAH *o*-quinone adducts and pathways to G to T transversions. Covalent B[*a*]P-7,8-dione adducts (stable and depurinating adducts) are shown on the left; adducts that can arise form reactive oxygen species (ROS) are shown on the right and would be derived from the redox cycling of PAH *o*-quinones. Reprinted from ref 1. Copyright 1999 American Chemical Society.

monoglucuronsyl-, and monohydroxylated-B[*a*]P-7,8-dione were also detected in all three cell lines. These studies showed for the first time that B[*a*]P-7,8-dione could undergo monohydroxylation. Evidence for the formation of an adenine adduct of B[*a*]P-7,8-dione was also obtained, but under the experimental conditions it was not possible to determine whether this adduct resulted from base depurination in DNA or whether it was derived from the NTP pool. The majority of these conjugates were identified by comparison to the authentic standards synthesized enzymatically *in vitro*. The GSH and NAC conjugates were shown to result from conjugation at the C10 position of B[*a*]P-7,8-dione, Figure 8.[66] The detection of B[*a*]P-7,8-dione-GSH conjugates suggests that these could arise from nonenzymatic conjugation or enzymatically from GSTs. The specificity of the human GST isoforms to conjugate PAH *o*-quinones remains to be elucidated.

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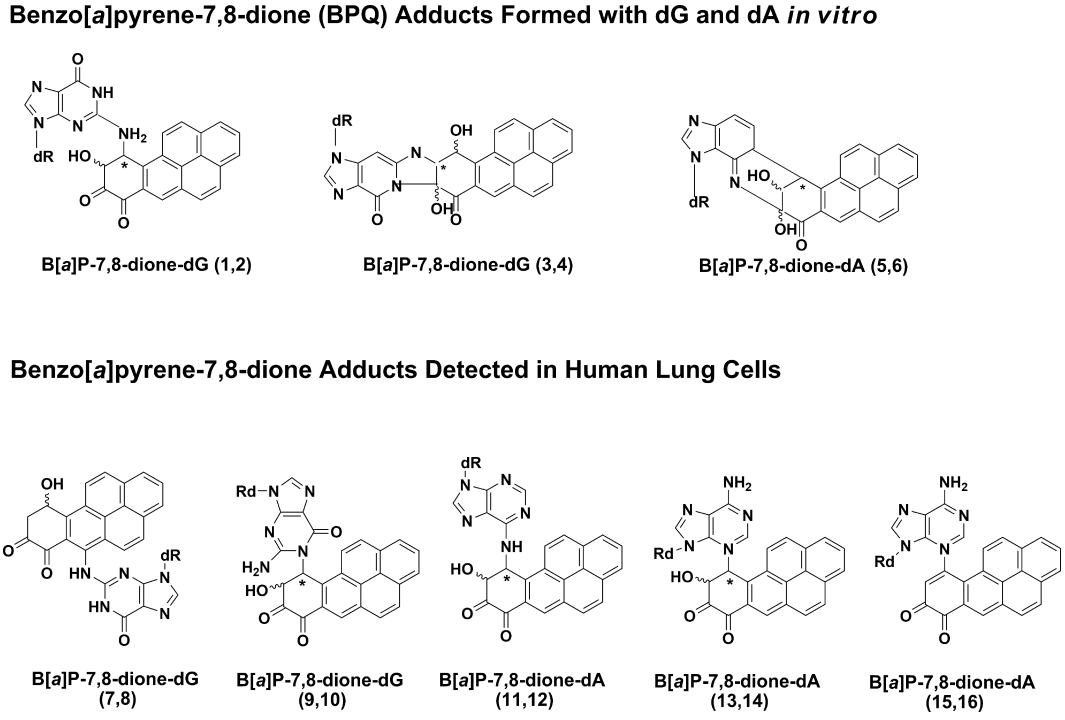

Figure 10. Stable covalent bulky DNA adducts derived from B[*a*]P-7,8-dione. B[*a*]P-7.8-dione−dA and −dG adducts formed *in vitro* used reaction conditions of 20% DMF at 55 °C for 6 h. B[*a*]P-7,8-dione adducts observed in human lung cells were simpler in structure. A total of 10 possible structures exist in cells. Asterisks (*) denote the presence of a stereochemical center.

■ PAH *O*-QUINONE−DNA ADDUCTS

G to T transversions are the most common mutations in *K-ras*[70,71] and *p53*[70,72,73] observed in human lung cancer. Any molecular mechanism by which PAH cause lung cancer must account for these mutations. Thus, interest has evolved in how radical-cations, diol-epoxides, and *o*-quinones derived from PAH may cause DNA adducts that may account for these mutations. Interestingly, PAH *o*-quinones can give rise to a spectrum of DNA adducts, and several of these may cause these mutations. These adducts include stable bulky DNA adducts, depurinating DNA adducts, the formation of abasic sites, 8-oxo-dGuo, base propenals, and those that may arise as a result of reactions of DNA bases with lipid peroxidation byproducts, Figures 9 and 10.

Stable Bulky DNA Adducts. B[*a*]P-7,8-Dione is a Michael acceptor and can undergo both 1,4- and 1,6-Michael addition to form stable bulky DNA adducts. Stable bulky DNA adducts, if not repaired by nucleotide excision repair, can lead to mutation due to translesional synthesis by error-prone translesional bypass DNA polymerases. Because these enzymes have low processivity, they often stall after reading through the lesion, and DNA polymerase II is then recruited to continue with DNA synthesis. Thus, the route to G to T transversions is not straightforward, but it can occur.

[^{3}H]-B[*a*]P-7,8-Dione forms a stable bulky DNA adduct with calf thymus DNA and plasmid DNA.[36] Upon digestion of the DNA to the constituent deoxyribonucleosides, a single labeled adduct was identified as coeluting with a similar adduct formed by reaction of B[*a*]P-7,8-dione with poly dG. Thus, the adduct was tentatively assigned as a B[*a*]P-7,8-dione-N^2-dGuo adduct without structural validation. Subsequently, reaction of B[*a*]P-7,8-dione with deoxyribonucleosides *in vitro* identified the formation of unusual cyclic and hydrated Michael addition products.[74] For example, the reaction of B[*a*]P-7,8-dione with dG afforded four characteristic Michael addition products: two diastereomers of 10-(N^2deoxyguanosyl)-9,10-dihydro-9-hydroxy-B[*a*]P-7,8-dione (adducts **1**, **2**) and two diastereomers of 8-N1,9-N^2-deoxyguanosyl-8,10-dihydroxy-9,10-dihydro-B[*a*]P-7(8H)-one (adducts **3**, **4**). The formation of adducts **1**, **2** suggests a 1,4-Michael addition reaction of dG at C10, an oxidation of the hydroquinone to the *o*-quinone, and a subsequent 1,6-Michael addition of water. The formation of adducts **3**, **4** suggests a 1,6-Michael addition reaction of dG at C9, oxidation of the hydroquinone to the *o*-quinone, a subsequent 1,4-Michael addition of water, followed by an internal cyclization. The reaction of B[*a*]P-7,8-dione with dA produced only one diastereomeric pair of adducts, identified as 8-N^6,10-N1-deoxyadenosyl-8,9-dihydroxy-9,10-dihydro-B[*a*]P-7(8*H*)-one, **5**, **6**. The formation of adducts **5**, **6** suggests a 1,4-Michael addition reaction of dA, an oxidation of the hydroquinone to the *o*-quinone, a subsequent 1,6-Michael addition of water, and an internal cyclization, Figure 10.

The mononucleotide derivatives of several of these deoxyribouncleoside adducts were then synthesized as standards for [^{32}P]-postlabeling studies using calf thymus DNA. [^{32}P]-Postlabeling revealed the formation of 8 major and at least 10 minor DNA adducts. Of these, one B[*a*]P-7,8-dione-dGMP adduct, two B[*a*]P-7,8-dione−dAMP adducts, and three B[*a*]P-7,8-dione−dCMP adducts were identified.[75] However, the vast majority remained unidentified.

Attempts were made to detect stable covalent B[*a*]P-7,8-dione DNA adducts in A/J mouse lung by injecting i.p. B[*a*]P-7,8-*trans*-dihydrodiol (AKR substrate) and B[*a*]P-7,8-dione (AKR product) using the [^{32}P]-postlabeling technique, but the only adducts detected were those that came from (+)-*anti*-B[*a*]PDE.[76] Subsequently, unique stable covalent B[*a*]P-7,8-dione-dGuo and B[*a*]P-7,8-dione-dAdo adducts were detected in A549 and HBEC-KT cells. In A549 cells, the structures of

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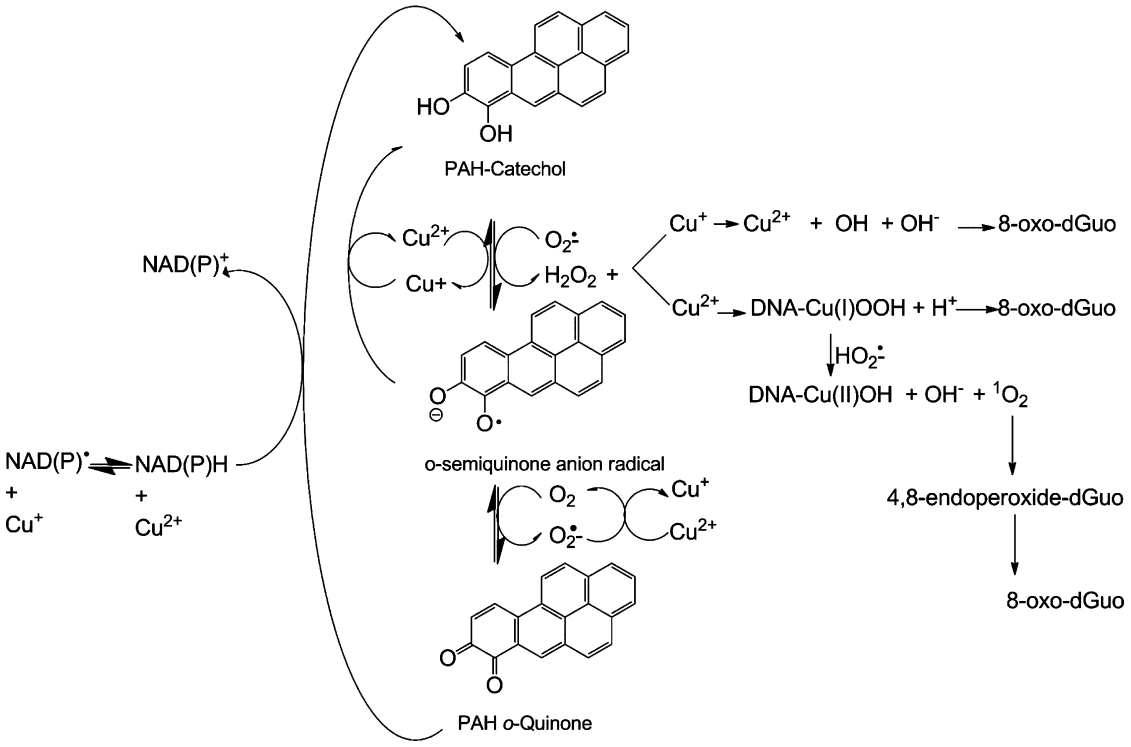

Figure 11. Mechanisms of oxidative DNA damage mediated by B[*a*]P-7,8-dione. Formation of 8-oxo-dGuo is highest in the presence of a complete redox cycling system involving NADPH, $CuCl_2$, and PAH *o*-quinone. This system gives rise to Cu(I)OOH, which is the source of singlet oxygen to form 4,8-endoperoxide-dGuo as the precursor lesion for 8-oxo-dGuo. Reprinted from ref 82. Copyright 2005 American Chemical Society.

stable B[*a*]P-7,8-dione−DNA adducts were identified as hydrated-B[*a*]P-7,8-dione−N^2-2′-dGuo, adducts **7**, **8** and hydrated-B[*a*]P-7,8-dione−N1-2′-dGuo, adducts **9**, **10**.[77] In HBEC-KT cells, the structures of stable B[*a*]P-7,8-dione−DNA adducts were identified as hydrated-B[*a*]P-7,8-dione−N^6-2′-dAdo, hydrated-B[*a*]P-7,8-dione−N1- or N3-2′-dAdo, and B[*a*]P-7,8-dione−N1- or N3-2′-dAdo, adducts **11**−**16**. In each case, adduct structures were characterized by MS^n spectra. Adduct structures were also compared to those synthesized from reactions of B[*a*]P-7,8-dione with either deoxyribonucleosides or salmon testis DNA *in vitro* but were found to be different. Collectively, these studies show that stable B[*a*]P-7,8-dione−DNA adducts do form in human lung cells, but their mutational properties and contribution, if any, to lung cancer tumorigenesis is unknown and remains to be determined.

PAH *o*-Quinone Depurinating Adducts. B[*a*]P-7,8-dione has the capacity to react with the N7 endocyclic nitrogen of dGuo and dAdo, leading to depurinating DNA adducts.[78] Depurinating adducts will leave behind abasic sites. Abasic sites can give rise to G to T transversions since translesional bypass DNA polymerases often insert an A opposite the abasic site, the so-called A-rule.[79] The B[*a*]P-7,8-dione−N7-Gua adduct can be formed synthetically by conducting Michael addition reactions with dGuo under acidic conditions to ensure hydrolysis of the N7 glycosidic bond, Figure 9. However, this adduct has yet to be seen in reactions with calf thymus DNA or in cell culture. Abasic sites that result from depurination when formed can undergo ring opening of the deoxyribose to form an aldehydic site. Using the aldehyde reactive probe, only a modest number of aldehydic sites were detected in salmon testis DNA following reaction with B[*a*]P-7,8-dione.[80] It is concluded that B[*a*]P-7,8-dione depurinating adducts do not contribute to the PAH *o*-quinone adduct profile to any significant degree.

Oxidative DNA Lesions. The most common DNA adduct formed as a result of ROS is 8-oxo-dGuo. If this is unrepaired by hOOG1, then it is mis-paired with A during replication, providing a straightforward route to G to T transversions.[81] 8-Oxo-dGuo has been detected in salmon testis DNA as a result of PAH *o*-quinone cycling, where the rank order of 8-oxo-dGuo formation was naphthalene-1,2-dione > benz[*a*]anthracene-3,4-dione > DMBA-3,4-dione > B[*a*]P-7,8-dione. The amount of 8-oxo-dGuo formed was exacerbated by the presence of NADPH and $CuCl_2$.[82] A detailed investigation of the mechanism indicated that Cu(I)OOH was likely the final oxidant and that this formed a guanine-4,8-endoperoxide, which was the precursor of 8-oxo-dGuo as described by Cadet.[83] Subsequently, 8-oxo-dGuo formation was observed in H358 and A549 cells as a direct result of PAH *o*-quinone formation. Taken together, the adduct literature would suggest that 8-oxo-dGuo may be the most important DNA lesion that arises from PAH *o*-quinones.

Further examination of the mechanism by which oxidative lesions are formed showed that the mechanism was metal ion-dependent. With Cu(II), the oxidant appeared to be Cu(I)-OOH, which in turn gave rise to $O_2{}^1$, Figure 11. By contrast, when Fe(II) was added to the system, the oxidant appeared to be $OH^•$. Hydroxyl radical produced by redox cycling can also attack deoxyribose and via a Criegee rearrangement can lead to the formation of a base propenal and malondialdehyde.[84] Using the aldehyde-reactive probe, it was found that reactive aldehydes formed in salmon testis DNA under PAH *o*-quinone redox cycling conditions increased after treatment with hOOG1, suggesting that the major culprit was excision of 8-oxo-dGuo.[80,82]

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Lipid Peroxidation DNA Lesions. Thus far, no experiments have been performed to determine whether PAH *o*-quinone redox cycling will lead to an increase in lipid peroxidation byproducts such as 4-hydroxy-2-nonenal or 4-oxo-2-nonenal (4-ONE), which can then react with DNA. For example, 4-ONE can give rise to heptan-2-one-etheno-dGuo and heptan-2-one-etheno-dAdo adducts.[85,86] However, the ability of AKRs to give rise to these adducts when challenged with PAH-*trans*-dihydrodiol substrates has not been established and could be confounded by the observation that 4-hydroxy-2-nonenal is a good substrate for the AKR1C enzymes.[87]

MUTAGENIC PROPERTIES OF PAH *O*-QUINONES

The mutagenicities of PAH *o*-quinones has been examined in the Ames test and a model yeast p53-based mutagenicity assay.[88,89] Although the former test showed modest mutagenic potency, the latter was quite revealing. In the yeast assay, the cDNA for p53 is treated with the mutagen plus/minus an activation system and then used to transform a yeast strain containing a p53 Ade reporter gene. In the presence of wild-type p53 cDNA, the Ade reporter is expressed so that yeast colonies turn white when grown on limiting adenine. By contrast, in the presence of mutated p53 cDNA, the Ade reporter gene is not expressed, and, in an attempt to make adenine, yeast colonies turn red when grown on limiting Ade. The assay thus distinguishes between wild-type and mutant p53 cDNA based on white/red colony detection, and since it is a functional assay, it selects for p53 mutants in which transcriptional competency has been lost. The p53 plasmids can be rescued from the yeast strain and sequenced so that mutational patterns and the mutational spectra can be ascertained, Figure 12.[89]

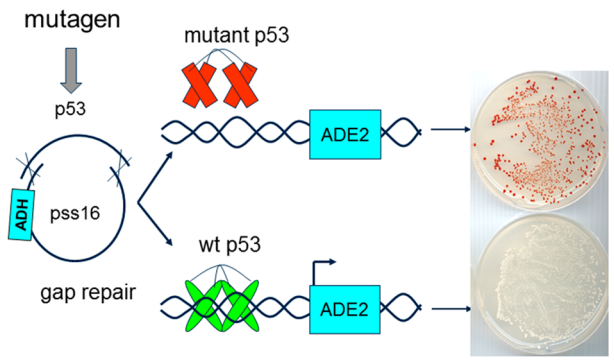

Figure 12. Yeast p53 reporter gene assay for PAH mutagenicity. In this assay, p53 cDNA is treated with a mutagen and undergoes homologous recombination in yeast with a gap repair plasmid. Wild-type and mutant p53 are then transcribed using a yeast-specific ADH promotor, and the recombinant proteins are used to drive a p53 Ade reporter gene, which results in white colonies (wild-type p53) and red colonies (mutant p53). ADH, alcohol dehydrogenase; ADE2, adenine reporter gene. Reprinted from ref 89. Copyright 2002 American Chemical Society.

Using this assay, it was found that B[*a*]P-7,8-dione was mutagenic only when a complete redox cycling system was present (NADPH and $CuCl_2$).[89] B[*a*]P-7,8-Dione was not mutagenic alone, suggesting that, if formed, stable covalent and depurinating adducts were not mutagenic in this assay. The mutation frequency was concentration-dependent, and B[*a*]P-7,8-dione was found to be 80-fold more potent than that of (±)-*anti*-B[*a*]PDE. The pattern of mutations observed with B[*a*]P-7,8-dione was predominantly G to T transversions, and the pattern was remarkably similar to the mutational pattern observed in lung cancer patients. Similar results were observed with benz[*a*]anthracene-3,4-dione and DMBA-3,4-dione. By contrast, the pattern of mutations observed with (±)-*anti*-B[*a*]PDE were G to C transversions. The inability to observe G to T transversions with (±)-*anti*-B[*a*]PDE may be due to the expression of yeast strain-specific translesional-bypass DNA polymerases.[90] p53 cDNA subjected to treatment with either B[*a*]P-7,8-dione under redox cycling conditions or *anti*-B[*a*]PDE alone was analyzed to detect either 8-oxo-dGuo by electrochemical-HPLC or stable (+)-*anti*-B[*a*]PDE−N^2-dGuo adducts by using SID-LC-MS/MS, Figure 13.[91] In both instances, a linear correlation was seen between adduct formation and mutation frequency, establishing that the mutations observed were due to 8-oxo-dGuo for B[*a*]P-7,8-dione and (+)-*anti*-B[*a*]PDE−N^2-dGuo for (±)-*anti*-B[*a*]PDE, respectively. Interestingly, a lower level of adducts was observed with B[*a*]P-7,8-dione than with (±)-*anti*-B[*a*]PDE, even though the former compound was more mutagenic under redox cycling conditions, indicating that 8-oxo-dGuo is the more mutagenic lesion.

The mutational spectra observed with B[*a*]P-7,8-dione and *anti*-B[*a*]PDE showed point mutations that were randomly distributed throughout the p53 cDNA. Whether sequence context determines adduct formation in specific codons in p53, that would account for the mutational spectra seen in human lung cancer, or whether this mutational spectra results from biological selection of those mutations that provide a growth advantage, remains controversial.[72,92] To address this issue, p53 mutations observed with B[*a*]P-7,8-dione were selected for dominance. The dominant mutations were found to cluster in DNA binding regions of p53, and many mutations were located in hot spots observed in p53 in lung cancer patients. By contrast, the recessive mutations were randomly distributed throughout p53 and did not reside in the DNA binding domain.[91] Our data suggest that mutation of p53 by 8-oxo-dGuo formed by PAH *o*-quinones is predominantly random, whereas biological selection gives a spectrum that accounts for the one observed in human lung cancer.

In subsequent experiments, we tested whether radical-cations could account for p53 mutation. It was found that the mutagenic potency of B[*a*]P radical-cations generated *in situ* was 200-fold less than that with B[*a*]P-7,8-dione derived from the AKR pathway. We conclude that radical-cations were weak mutagens in this yeast p53 assay.[93] In this assay, the following rank order of mutagenic potency was observed: B[*a*]P-7,8-dione plus redox cycling > *anti*-B[*a*]PDE > B[*a*]P-1,6-dione and B[*a*]P-3,6-dione.

TUMORIGENICITY ASSAYS WITH B[*A*]P-7,8-DIONE

We established the A/J mouse lung model of B[*a*]P tumorigenicity and then used B[*a*]P-7,8-catechol diacetate as a bioavailable precursor of B[*a*]P-7,8-dione to test its tumorigenic potential. In short-term assays, mouse urine was collected, and an increase in urinary 8-oxo-dGuo was observed as a biomarker of oxidative stress. It should be remembered that 8-oxo-dGuo is released from DNA as 8-oxo-Gua by base excision repair and that the urinary levels of 8-oxo-dGuo are likely derived from the NTP pool. In chronic studies, an increase in tumor incidence and multiplicity was observed that was modest by comparison to that observed with B[*a*]P.

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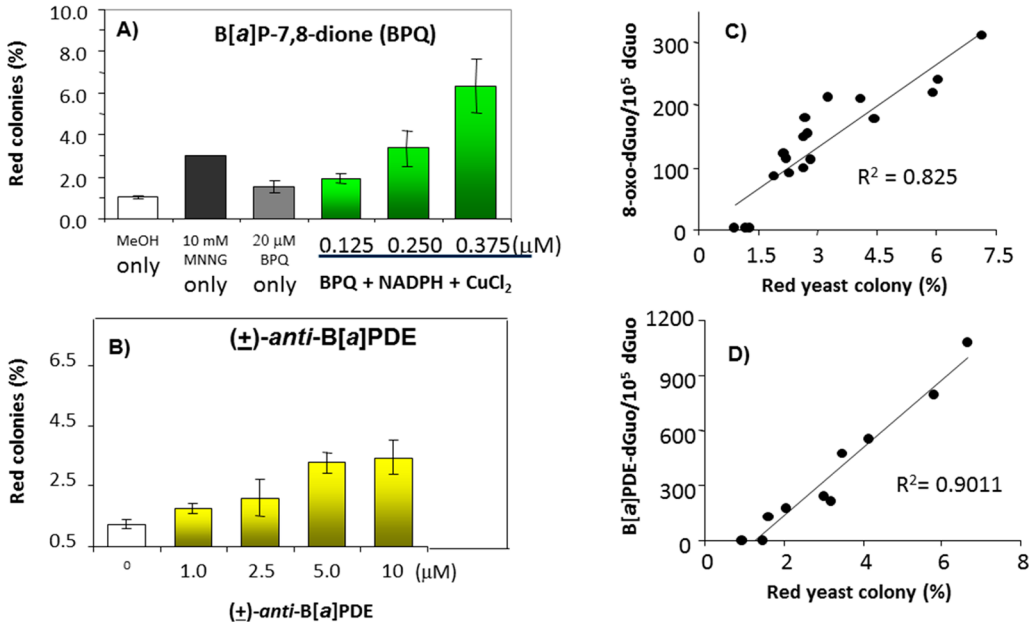

Figure 13. Linear correlation between p53 mutation frequency and adduct formation. Mutation frequency with B[*a*]P-7,8-dione in the presence of NADPH and $CuCl_2$ (A); the mutation frequency with *anti*-B[*a*]PDE is dose-dependent (B); linear relationship between 8-oxo-dGuo formation in p53 cDNA treated with B[*a*]P-7,8-dione, NADPH, and $CuCl_2$ and mutation frequency (C); and linear relationship between *anti*-B[a]PDE–N^2-dGuo adducts and mutation frequency (D). MNNG, *N*′-methyl-*N*′-nitro-*N*′-nitrosoguanidine.

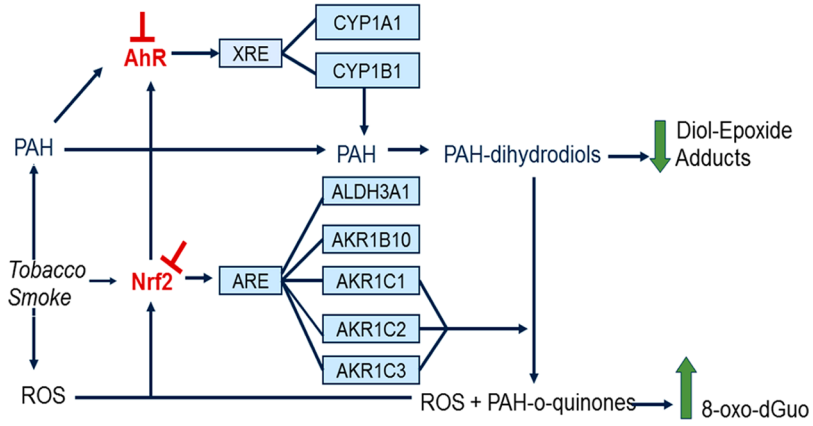

Figure 14. Consequences of upregulation of the smoking gene battery. PAH in tobacco smoke induces gene transcription by the AhR and metals, and quinones in tobacco smoke induce gene transcription by the NRf2 system. AhR activation induces *CYP1A1* and *CYP1B1* to increase the formation of PAH-*trans*-dihydrodiols. Nrf2 activation induces *AKR1C1*–*AKR1C3* to convert PAH-*trans*-dihydrodiols to PAH *o*-quinones and ROS. ROS will increase 8-oxo-dGuo formation and act to induce the Nrf2 system still further forming a positive feed-back loop. Reprinted with permission from ref 99. Copyright 2008 American Association for Cancer Research.

However, evidence for chronic inflammation was observed in the lung, suggesting that in this model B[*a*]P-7,8-dione may be acting as a tumor promoter (Melpo-Christofidou Solomidou and Penning, unpublished).

■ TRANSCRIPTOMICS AND GENETIC VARIANTS

AKRs and the Smoking Gene Battery. A large number of reports have appeared to address the expression of *AKR* genes in human lung cancer and their response to cigarette smoke. Fukumoto et al. showed that *AKR1C1* and *AKR1B10* were 2 out of the 7 most overexpressed genes by Affymetrix microarray in NSCLC.[94] Subsequently, it was found that *CYP1A1*, *CYP1B1*, *AKR1C1*, and *AKR1B10* gene expression was increased 15–30-fold in oral squamous carcinoma and induced by cigarette smoke condensate in oral dysplastic cells.[95] Woenckhaus et al. demonstrated that *AKR1C1*, *AKR1B10*, and *ALDH3A1* were 3 out of the 10 genes most overexpressed in tobacco-exposed bronchial epithelial cells.[96] Gumus et al. showed that *CYP1A1*, *CYP1B1*, *AKR1C1*, *NQO1*, and *ALDH3A1* were part of a gene battery that was upregulated in buccal oral specimens of smokers.[97] Importantly, Zhang et al. demonstrated that these same genes were most upregulated in bronchial epithelial cell brushes of smokers and downregulated in smokers that quit.[98] These findings led to the concept that *AKR* genes are part of a "smoking-gene battery" and could be used as a biomarker of cigarette smoke exposure and were likely upregulated as a stress response. They also raise the possibility that this smoking-gene battery may also contribute to the pathogenesis of smoking-related lung disease, including lung cancer.

dx.doi.org/10.1021/tx500298n | *Chem. Res. Toxicol.* 2014, 27, 1901–1917

We proposed the following scenario:[99] PAH in tobacco smoke bind to the AhR, which induces xenobiotic response element genes, e.g., *CYP1A1/CYP1B1*, leading to the formation of PAH *trans*-dihydrodiols. Simultaneously, other components of tobacco smoke, e.g., heavy metals, hydroquinones, and ROS, activate Nrf2, leading to induction of an ARE gene battery including *AKR1C1*−*AKR1C3* and *AKR1B10*. These genes are then primed so that their protein products can divert PAH-*trans*-dihydrodiols away from diol-epoxides to increase the formation of PAH *o*-quinones and exacerbate ROS. Not only will this increase oxidative DNA lesions that could lead to mutation but also increased ROS could result in the further activation of Nrf2-regulated genes. Additionally, induction of *AKR1B10* will increase retinal reductase activity of the cells and prevent the formation of retinoic acid, which would be pro-proliferative, Figure 14.[94,99,100] These hypotheses remain to be tested using functional genomic approaches.

Splice Variants. With the emergence of RNA-seq technology, splice variants of human AKR transcripts involved in PAH activation are likely to emerge.[101] Among the AKR1C enzymes, a number of splice variants have already been identified. AKR1C2 is predicted to have three transcripts and two protein isoforms in the Ensembl database. Transcripts AKR1C2-001 and AKR1C2-201 encode what is classically known as a 323 amino acid residue-long AKR1C2 protein (type 3 3α-hydroxysteroid dehydrogenase, dihydrodiol dehydrogenase/bile-acid binding protein). On the other hand, transcript AKR1C2-203 encodes a protein that utilizes an alternative exon 4, which results in a significantly truncated protein (139 aa) with a different C-terminus. This protein contains the conserved catalytic tetrad found in AKRs, but its binding of cofactor is likely to be compromised, and with over half of the protein missing, it is predicted to be inactive, if expressed.

Single Nucleotide Polymorphisms. Human AKRs are highly polymorphic according to the NCBI database: AKR1A1 has >44 non-synomynous missense SNPs, but only 19 have been validated, and their minor allelic frequency is <2%; AKR1C1 has >40 non-synomynous SNPs (4 are nonsense mutations and the rest are missense mutations), of which only 29 have been validated, and they have a minor allelic frequency of <1%; AKR1C2 has >39 non-synomynous missense SNPs, of which only 22 have been validated, and one of these, $Phe_{48}Tyr$, has a minor allelic frequency of greater than 5%; and AKR1C3 has >62 non-synomynous SNPs, of which the majority are missense mutations, 3 are nonsense mutations, and 38 have been validated. Of these, five had a minor allelic frequency >1%. With the crystal structures of each of these enzymes available, these SNPs can also be mapped to the structures to determine whether they will cause a change-in-function on the basis of *in silico* analysis. It is noteworthy that a subset of SNPs occur in evolutionarily conserved positions where the amino acid in the wild-type protein is invariant in the AKR1 family. SNPs in evolutionarily conserved amino acids in protein superfamilies have been proposed to be deleterious to function.[102−104] Loss of function in AKRs involved in PAH activation could be protective against PAH exposure and lung cancer.

■ CONCLUSIONS AND FUTURE DIRECTIONS

Sufficient evidence exists to show that the AKR pathway of PAH activation occurs in humans. The conversion of PAH *trans*-dihydrodiols to PAH *o*-quinones is catalyzed by human recombinant AKR1A1 and AKR1C isoforms. This pathway of activation is observed in human lung cells and results in the subsequent formation of 8-oxo-dGuo. The demonstration that 8-oxo-dGuo can account for p53 mutation observed in human lung cancer also supports a role for human AKRs in lung cancer causation. Interestingly, 8-oxo-dGuo is an intermediate lesion that can be further converted by one-electron oxidation to form guanidinohydantoin and spiroiminodihydantoin lesions, and this will need to be considered when examining the mutagenic potential of the 8-oxo-dGuo lesion.[105]

Much work still needs to be done to examine the role of GSTs in the conjugation of PAH *o*-quinones to identify the structures of covalent PAH *o*-quinone-DNA adducts observed in human lung cells and to examine their mutagenic properties. Little is known about the tumorigencity of PAH *o*-quinones. One issue is the shortage of good animal models. The A/J mouse lung model is often used, but its use to study of the role of AKRs in B[*a*]P activation is made difficult since the murine AKR1C enzymes are not orthologues of the human enzymes.[106] Studies with B[*a*]P-7,8-dione are prohibitive due to lack of bioavailability, which can be circumvented by the use of B[*a*]P-7,8-catechol diacetate.

It is still uncertain whether the PAH *o*-quinones will act as tumor initiators, promoters, or both. These experiments need to be performed. Initiation/promotion protocols could use urethane as initiator and B[*a*]P-7,8-dione as a promoter to examine this issue.

Importantly, the AKRs appear to be part of a smoking gene battery that is turned on in smokers. Functional genomics needs to be performed in human bronchial epithelial cells exposed to cigarette smoke to determine the phenotypic underpinnings of these AKR transcript changes. These experiments could be conducted in the immortalized HBEC-tk cells where we have shown that treatment of the cells with TCDD leads to induction of *CYP1A1/CYP1B1* but not *AKR1C* genes. By contrast, *R*-sulforaphane leads to the induction of *AKR1C* genes but not *CYP1A1/CYP1B1* in these cells. Treatment of the cells with cigarette smoke condensate leads to the induction of *CYP1A1/CYP1B1* and the *AKR1C* genes (Penning and Duan, unpublished), showing that the expression of the smoking gene battery can be recapitulated in these cells. In addition, our SID-LC-MS/MS assay can provide the required sensitivity to measure B[*a*]P metabolism in as few as 10 000 cells. This will enable B[*a*]P metabolism to be studied not only in immortalized HBEC-tk cells but also in cells from human donors (smokers and non-smokers) obtained at flexible bronchoscopy to observe differences in B[*a*]P metabolism.

AKR genes are highly polymorphic, and little is still known about the effects of SNPs in *AKR* genes and whether these would affect susceptibility to human lung cancer. An association between AKR1C3 Gln5His SNP with lung cancer incidence has been observed, but functional genomics on this variant has not been performed.[107] With the large number of SNPs now detected, it will not be possible to conduct functional genomics on each one unless a high-throughput screen is established.

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Funding

This work was supported by grants 1R01-CA39504 and P30-ES013508 awarded by the National Institutes of Health to T.M.P.

Notes

The author declares no competing financial interest.

■ ABBREVIATIONS

AhR, aryl hydrocarbon receptor; AKR, aldo-keto reductase; ARE, antioxidant response element; B[*a*]P, benzo[*a*]pyrene; COMT, catechol-*O*-methyl transferase; DCFH-DA, dichlorofluorescein diacetate; DMBA, 7,12-dimethylbenz[*a*]anthracene; EPA, United States Environmental Protection Agency; GSH, reduced glutathione; GST, glutathione-S-transferase; hOOG1, human oxo-guanine glycosylase; HSD, hydroxysteroid dehydrogenase; IARC, International Agency for Research on Cancer; LOD, limit of detection; MNNG, *N*′-methyl-*N*′-nitro-*N*′-nitroso-guanidine; NAC, *N*-acetyl-L-cysteine; NAT, cysteine-S-conjugate-*N*-acetyl transferase; NTP, nucleoside triphosphate; NQO1, NADPH-quinone oxidoreductase 1; NSCLC, non-small cell lung carcinoma; 8-oxo-dGuo, 8-oxo-2′-deoxyguanosine; 4-ONE, 4-oxo-2-nonenal; PAP, phosphoadenosine phosphate; PAPS, phosphodenosine phosphosulfate; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine; SID/LC/MS/MS, stable isotope dilution liquid chromatography mass spectrometry; SULT, sulfotransferase; nsSNP, nonsynonymous single nucleotide polymorphisms; ROS, reactive oxygen species; TCDD, 2,3,7,8-tetrachlorodibenzodioxin; UDPG, uridine diphosphoglucuronic acid; UGT, uridine glucuronsyl transferase

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