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The mode of action of organic carcinogens on cellular structures

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Abstract. Most genotoxic organic carcinogens require metabolic activation to exert their detrimental effects. The present review summarizes the mechanisms of how organic carcinogens are bioactivated into DNA-reactive descendants. Beginning with the history of discovery of some important human organic carcinogens, the text guides through the development of the knowledge on their molecular mode of action that has grown over the past decades. Some of the most important molecular mechanisms in chemical carcinogenesis, the role of the enzymes involved in bioactivation, the target gene structures of some ultimate carcinogenic metabolites, and implications for human cancer risk assessment are discussed.

Key words: Aromatic amines, carcinogen-DNA adducts, cytochrome P450, metabolism, mutation profiles, phase II enzyme activation, polycyclic aromatic hydrocarbons.

Introduction

Epidemiological evidence based on geographic and temporal variations in cancer incidences and studies of migrant populations suggest that 'environmental exposures' have a substantial impact on the causation of human cancer [1]. These studies led to the conclusion that the majority of cancer deaths in Western industrial countries are attributable to exogenous factors such as tobacco, diet, infections, and occupational exposures. The notion that the environment has the principal role in the causation of sporadic cancer is also supported by analyses of cancer cases in cohorts of twins [2] and by analyses of family-cancer databases [3]. In either case there is strong evidence that the influence of 'nonshared environmental factors' predominates.

There is no doubt that a wide range of organic chemicals (as pure compounds or present in mixtures) are carcinogenic in humans [4]. Examples from the list of known or suspected human chemical carcinogens are aromatic and heterocyclic amines or amides (e.g., 2-naphthylamine, 2-NA; 2-acetylaminofluorene, 2-AAF; 2-amino-3-methylimidazo[4,5-*f*]quinoline, IQ), halogenated and unsubstituted olefines (e.g., tetra- or perchloroethylene, PER), halogenated paraffins (e.g., 1,2-dibromoethane, 1,2-DBE), *N*-nitroso compounds (e.g., *N*-nitrosodimethylamine, NDMA; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNK), benzene and polycyclic aromatic compounds (e.g.,

benzo[*a*]pyrene, B[*a*]P; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD), estrogen-receptor agonists or antagonists with residual agonistic effects (e.g., diethylstilbestrol, DES; tamoxifen), wood dust (e.g., from oak and beech), natural compounds (e.g., aflatoxins, ochratoxin A, OTA; pyrrolizidine alkaloids), and so forth. Some selected structures are shown in Figure 1.

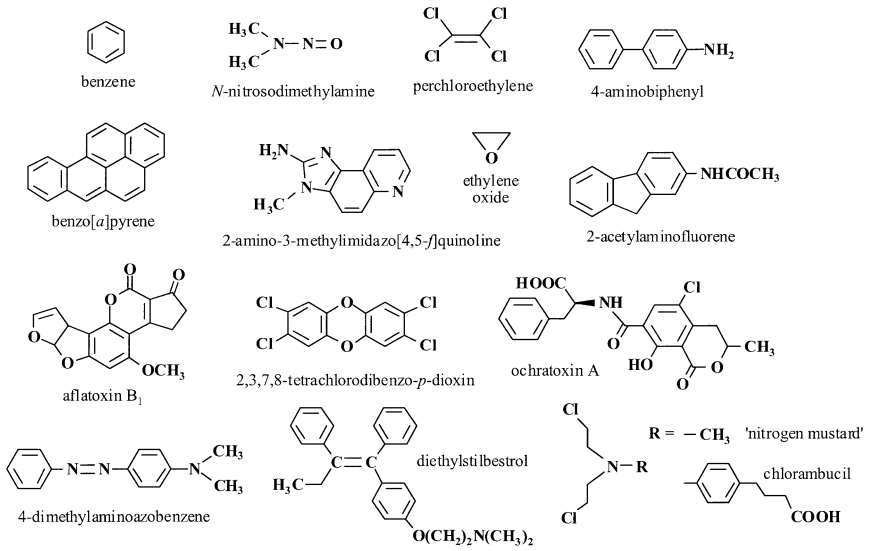


Figure 1. Some examples of organic chemicals that are known or reasonably anticipated to be carcinogenic in humans.

Discovery of organic carcinogens

In the 18th century, the two English physicians John Hill (1716–1775) and Percivall Pott (1714–1788) described the occurrence of cancer in the nose and at the skin of the scrotum, and associated it with local long-term exposure to snuff [5] and local contamination by soot at a young age [6], respectively. The interest of the latter observation lay in the first clear relationship between an occupation (chimney sweeping) and one particular form of squamous cell carcinoma; and in the possibility to prevent this disease by personal hygiene.

In the following years it became evident that certain exposures to pure organic chemicals or mixtures are associated with human carcinogenesis. Richard von Volkmann (1830–1889) and Joseph Bell (1837–1911) confirmed the early observation from Pott by describing several cases of skin affections and scrotal skin tumors among workers in the paraffin industry [7, 8]. In 1895, the surgeon Ludwig Wilhelm Carl Rehn (1849–1930) had recognized the appearance of tumors of the bladder of men employed in the German aniline dyestuff industry to produce red magenta (‘Fuchsin’) [9]. It was then noticed

that this particular occupation was associated with increased rates of cancer in the urinary bladder, subsequently referred to as 'aniline cancers'. In 1898, the German internist Otto Michael Ludwig Leichtenstern (1845–1900) considered 2-NA most likely to be involved in human bladder tumorigenesis [10].

Until the dawn of the 20th century, physicians were only able to collect the unexpected outcome of an undesigned and undesirable grand scale (occupational exposure) experiment based on the rise of industrialization. By the year 1907 it was officially recognized by the 'Workmen's Compensation Act' of Great Britain that epidermal cancer can be caused by pitch, tar or tarry compounds [11]. The imperative next step was that of experimental reproduction of cancer. After many failures to reproduce the known human outcome in laboratory animals, the Japanese pathologist Katsusaburo Yamagiwa (1863–1930) and his assistant Ichikawa successfully produced malignant tumors through application of a chemical mixture (coal tar) to the ear of rabbits [12]. While epithelial proliferation could be chemically induced already some years earlier [13, 14], this experiment produced undoubted malignant epithelial cancer for the first time. It was then experimentally and epidemiologically confirmed that tar and soot is carcinogenic in the skin of mice and in humans exposed at work place, respectively [15–17]. After chemical synthesis routes for pure higher molecular polycyclic aromatic hydrocarbons (PAHs) had been first described, Sir Ernest Laurence Kennaway (1881–1958) and his colleagues at the Royal Cancer Hospital in London successfully proved that single PAHs such as 1,2:5,6-dibenzanthracene (dibenz[*a,h*]anthracene, DB[*a,h*]A) and others are tumorigenic [18, 19]. As an indicator assay, they applied the mouse skin bioassay, which had been introduced to chemical cancer research some years earlier [20]. In 1933, Cook, Hewett and Hieger from the Cancer Hospital were successful in isolating the 'carcinogenic principle' out of coal tar pitch [21]. It turned out to be another PAH, the pentacyclic 3,4-benzpyrene (B[*a*]P), which nowadays is one of the most investigated carcinogens ever and a standard compound used in many cancer experiments as a 'positive control' (Fig. 1).

Studies with aromatic amines, aminoazo dyes and related compounds supplemented the large volume of experimental data on the carcinogenicity of industrial chemicals that had been released in high amounts during this time. The incriminated aniline itself failed to produce tumors in the urinary bladder of rabbits, as did other aromatic amines such as 2-NA [22]. Later, bladder papillomas and carcinomas were successfully induced in dogs by gavage or dermal application of 2-NA [23] – an experiment that supported the early prediction from Leichtenstern. In the meantime aminoazo dyes, such as *o*-aminoazotoluene (reduction product of 'Scarlet Red') [24] and 4-dimethylaminoazobenzene (DAB, 'butter yellow'; Fig. 1), were shown to be tumorigenic in rat liver [25]. Wilson et al. [26] reported on the tumorigenicity of 2-AAF in bladder, liver, and various other organs of rats. 2-AAF is an arylamide which was intended to be used as a pesticide, but has been later introduced as a model compound in experimental liver cancer research (Fig. 1).

With the beginning of the 1940s, a huge amount of experimental data on the bioactivity of pure and structurally defined organic compounds present in the industrial environment had been collected.

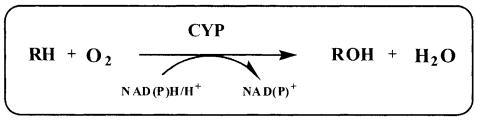
Metabolic activation of organic carcinogens – Achilles' heel of defense?

Crucial hint: DNA adducts

Based on the assumption that cancer derives from clonal expansion of a single cell [27], it seems axiomatic that chemical carcinogens must induce tumorigenesis through interaction with subcellular components that are intimately involved in mediating the underlying heritable loss of growth control. In principle, these interactions may be noncovalent and reversible or covalent, but reversible only in case repair mechanisms would restore the naïve structure. In the 'pre-Watson and Crick era', binding of carcinogens such as (brightly colored) DAB [28] or (fluorescent) B[*a*]P [29] to proteins at target tissue sites *in vivo* had been reported, and considered to be associated with the initiation of the disease [30]. Later, when the sensitivity was sufficiently high, due to the availability of radioactively labeled chemicals, binding of carcinogens, such as *N*-methyl-bis(2-chloroethyl)amine ('nitrogen mustard') [31], bis(β-chloroethyl)sulfide ('mustard gas') [32], *N*-nitrosamines (e.g., NDMA) [33], PAHs (e.g., B[*a*]P) [34], aromatic amines/amides (e.g., 2-AAF) [35], and aminoazo dyes (e.g., DAB) [36], to DNA *in vivo* was discovered (see Fig. 1). By the end of the 1960s, accumulating evidence for a correlation between the level of DNA binding of a particular carcinogen and its biological potency was overwhelming. In addition, all of these compounds were soon regarded as being mutagens as well [37]. To react with cellular macromolecules, however, most carcinogens require enzymatic activation. The parent compounds are, therefore, considered as precarcinogens or indirect carcinogens to be bioactivated into their ultimate carcinogenic forms.

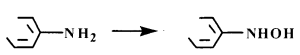
The role of biotransformation

First evidence for metabolic conversion of organic carcinogens *in vivo* had been obtained through detection of hydroxylated derivatives and their conjugates in the urine of animals treated with pure compounds [38–40]. In 1948, James A. Miller (1915–2000) and co-workers were the first to demonstrate the oxidative metabolism of DAB by rat liver microsomes [41]. Later, the activating enzymes present in microsomes were characterized as mixed-function oxidases [42], cytochrome P450 had been discovered [43], and cytochrome P450-dependent monooxygenases (CYPs) were shown to work in concert with an NADPH-dependent reductase [44] (Fig. 2).



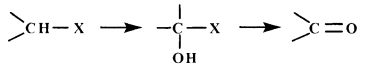
Examples (CYP enzyme/substrate)

N-Hydroxylation:

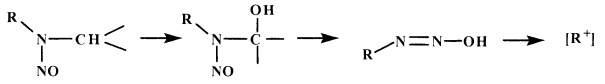


CYP1A1: IQ and other HCAs
CYP1A2: 2-NA, 4-ABP, 2-AAF, DAB, IQ
CYP1B1: IQ and other HCAs

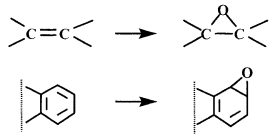
C-Hydroxylation:



CYP1A1: alkylated aromatic compounds
CYP2A6: *N*-nitroso compounds (e.g. NDMA, NNK)
CYP2E1: carbon tetrachloride, *N*-nitroso compounds



Epoxidation:



CYP1A1: PAHs
CYP3A4: AFB_1, PAH dihydrodiols
CYP1B1: PAHs
CYP2E1: VC, PER, benzene, 1,3-butadiene

Figure 2. Cytochrome P450-dependent biotransformation (monooxygenation) of organic chemicals (cf. Fig. 1) [46, 47]. The principal reaction is shown at the top (scheme). 2-AAF, 2-acetylaminofluorene; 4-ABP, 4-aminobiphenyl; AFB_1, aflatoxin B_1; CYP, cytochrome P450-dependent monooxygenase; DAB, 4-dimethylaminoazobenzene; HCAs, heterocyclic amines, also known as 'cooked food mutagens' due to their generation from amino acids during cooking processes; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; 2-NA, 2-naphthylamine; NDMA, *N*-nitrosodimethylamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PAH, polycyclic aromatic hydrocarbons; R = alkyl residue, e.g., methyl, ethyl; PER, tetra- or perchloroethylene; VC, vinyl chloride. See text for further explanations.

In principle, enzymatic biotransformation of xenobiotic compounds is aimed at ensuring detoxification and subsequent elimination via excretion pathways rather than leading to descendants of enhanced biological activity. Conversion of organic and mostly aromatic compounds into hydrophilic and excretable derivatives is catalyzed not only by CYP enzymes, from which at present 57 genes are identified in humans [45]. Rather, a great variety of additional 'xenobiotic metabolizing enzymes' (XMEs) contribute to this process which, in general, proceeds through activated intermediates that are capable of

undergoing subsequent conjugation reactions with hydrophilic functional groups or molecules. Formation of activated intermediates via CYP-mediated monooxygenation is considered as 'phase-I' in biotransformation (Fig. 2). Modification catalyzed by hydrolases, dehydrogenases, peroxidases, and reductases may also contribute to this phase. The resulting derivatives then may or may not enter 'phase-II', in which transferases catalyze conjugation to polar molecules, such as glucuronic acid (UDP-glucuronosyltransferases, UGTs) and glutathione (glutathione *S*-transferases, GSTs), or to small residues such as sulfate (sulfotransferases, SULTs) or acetic acid (*N*-acetyltransferases, NATs). On the other hand, bioactivation towards electrophilic intermediates may also lead to covalent interaction with cellular proteins or DNA. Therefore, this approach poses an inherent risk to the physiological integrity of living cells, and may thus be regarded as the Achilles' heel in the cellular defense against biohazards.

Monooxygenation of xenobiotics in mammalian species including humans is mainly catalyzed by members of the CYP families 1–3 (i.e., CYP1A1, 1A2, 1B1, 2A6, 2E1, 3A4) [46, 47]. These forms are considered as the mainstay in biotransformation of a large number of carcinogenic chemicals. For instance, CYP1A1 is the major form in human lung, CYP1A2, 2A6, 3A4, or 2E1 are mainly expressed in liver, while CYP1B1 is the main extrahepatic and extrapulmonary form with highest levels in prostate and uterus [48]. Figure 2 summarizes the major CYP-mediated bioactivation reactions of organic carcinogens.

Under certain circumstances, phase-II enzymes may also contribute to the activation of precarcinogens *in vivo*. SULT and NAT enzymes catalyze sulfonation and acetylation of nucleophilic metabolites such as *N*-hydroxylamines, phenols, benzylic alcohols and others. *N*-Hydroxylation as a bioactivation step was discovered in studies on liver carcinogenesis induced by 2-AAF [49] (Figs 2 and 3). Since *N*-hydroxy-2-AAF was found to be a more potent carcinogen than 2-AAF itself [50], it is regarded as the proximate carcinogenic metabolite. With the beginning of the 1970s, SULT-catalyzed *O*-sulfonation of *N*-hydroxy-2-AAF had been discovered [51, 52]. With time, it was then established that *O*-acetylation (by NATs, cofactor: acetyl-CoA) as well as *O*-sulfonation (by SULTs, cofactor: 5'-phosphoadenosine-3'-phosphosulfate) of *N*-hydroxy derivatives of arylamines (e.g., 2-NA), arylamides (e.g., 2-AAF), aminoazo dyes (e.g., DAB), or heterocyclic amines (HCAs, e.g., 'cooked food mutagens' such as IQ) yield highly reactive ester intermediates *in vivo* that bind to C8 or exocyclic amino groups in purine bases via intermediate release of arylnitrenium ions (Fig. 3) [53, 54].

The discovery of mercapturic acids (*N*-acetylcysteinyl conjugates) dates back to the 19th century, yet GST-mediated transfer of electrophilic metabolites onto glutathione (GSH) was not described before 1961 [55], shortly after the relationship between GSH conjugates and their excretable follow-up products had been uncovered [56]. GST enzymes interact with electrophiles via the reactive cysteinyl residue in their cofactor (Fig. 4). While GST-mediated

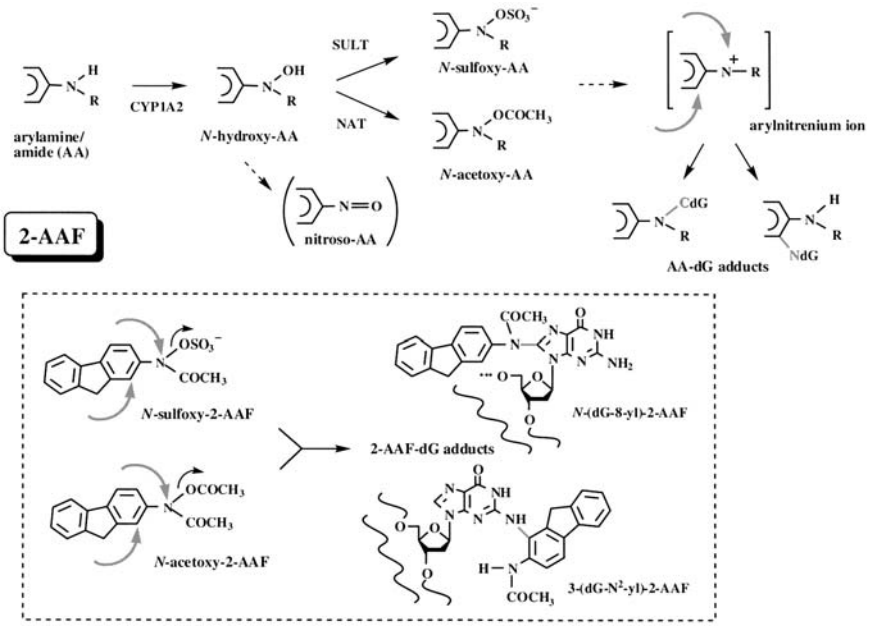


Figure 3. Bioactivation of aromatic amines or amides (AA) towards ultimate DNA-reactive sulfate or acetoxy esters. The ultimate electrophiles and major DNA binding products are exemplified in the case of 2-AAF. 2-AAF, 2-acetylaminofluorene; CYP, cytochrome P450-dependent monooxygenase; dG, 2'-deoxyguanosine; SULT, sulfotransferase; NAT, *N*-acetyltransferase; The arylnitrenium ion is a putative reactive intermediate. The grey arrows point to the position of a nucleophile attack of DNA, protein, or GSH. See text and Figure 2 for further explanations.

detoxification of activated metabolites such as epoxides or dihydrodiol epoxides (e.g., from PAHs) has been widely acknowledged since then, more recent evidence points to an additional but detrimental role of GST enzymes in the activation of certain industrial chemicals from the classes of haloalkanes (e.g., 1,2-DBE) and haloalkenes (e.g., PER; see Fig. 1). These compounds are likely to be human carcinogens due to similarities between susceptible animals and humans in bioactivation [4]. In the case of PER, there is also some limited evidence from cohort studies of laundry and dry-cleaning workers, among whom a higher than normal occurrence of non-Hodgkin's lymphoma, esophageal and cervical cancer was found [4]. Both groups of organic carcinogens are bioactivated into genotoxic GSH conjugates. While dihaloalkanes may undergo GST-catalyzed conversion into DNA-binding 'GSH half mustard' and GSH episulfonium electrophiles (Fig. 4) [57], haloalkene-GSH conjugates can be further converted via a kidney-specific cysteine conjugate β-lyase-dependent pathway. After release of the terminal amino acids γ-Glu and Gly, lysis of the β-bond in the remaining Cys adduct may eventually lead to the generation of electrophilic and toxic thioketenes, which are capable of binding to macromolecules in this tissue [58].

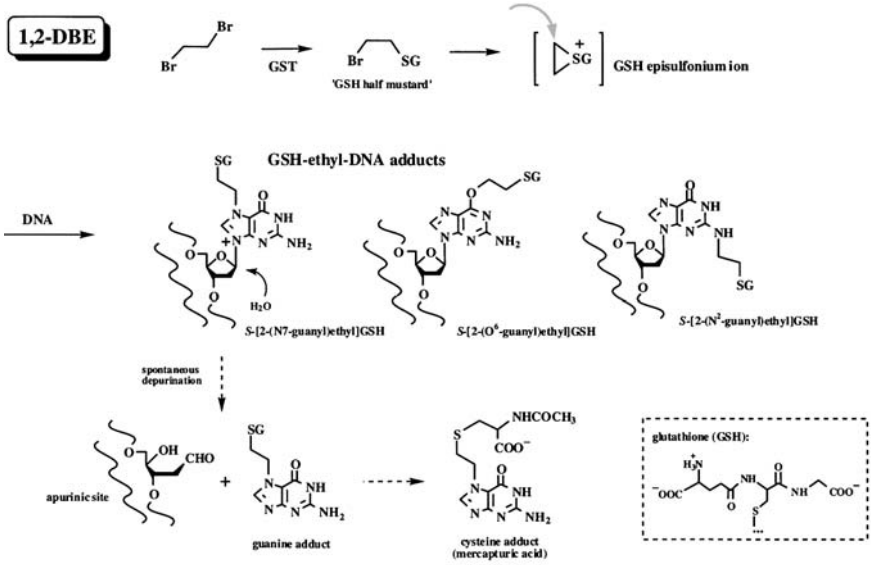


Figure 4. Glutathione *S*-transferase (GST)-dependent bioactivation of 1,2-dibromoethane (1,2-DBE) towards the DNA-reactive 'half mustard' GSH conjugate and GSH episulfonium ion. The three major DNA adducts resulting from GST-dependent activation of 1,2-DBE are depicted. The N7 adduct spontaneously depurinates and creates an apurinic site within DNA. The GSH episulfonium ion is a putative reactive intermediate. The grey arrow points to the position of the nucleophile attack. In the N7 guanine-GSH adduct the terminal amino acids are subsequently cleaved off by γ-glutamyltransferase and cysteinylglycine dipeptidase activity to give rise to a cysteine adduct that is further *N*-acetylated (→ mercapturic acid). Inset: structure of glutathione (GSH).

Genotoxic *versus* nongenotoxic mechanisms, or both?

Although most organic carcinogens require metabolic activation towards DNA-reactive intermediates, some compounds do not bind to DNA yet they are still capable of inducing tumors through nongenotoxic pathways (Fig. 5).

The arylhydrocarbon receptor

The nongenotoxic 'dioxin' TCDD, a by-product of the manufacture of polychlorinated phenols (e.g., the biocides pentachlorophenol and 2,4,5-trichlorophenoxyacetic acid, 2,4,5-T) and an inadvertently generated environmental contaminant (e.g., during waste incineration) is classified as a known human carcinogen [4]. This judgment is based on both epidemiological evidence and mechanistic information on the mode of action, which indicate a causative relationship between accidental exposures of humans and cancer at multiple sites [59]. In animals, TCDD is carcinogenic in liver, thyroid,

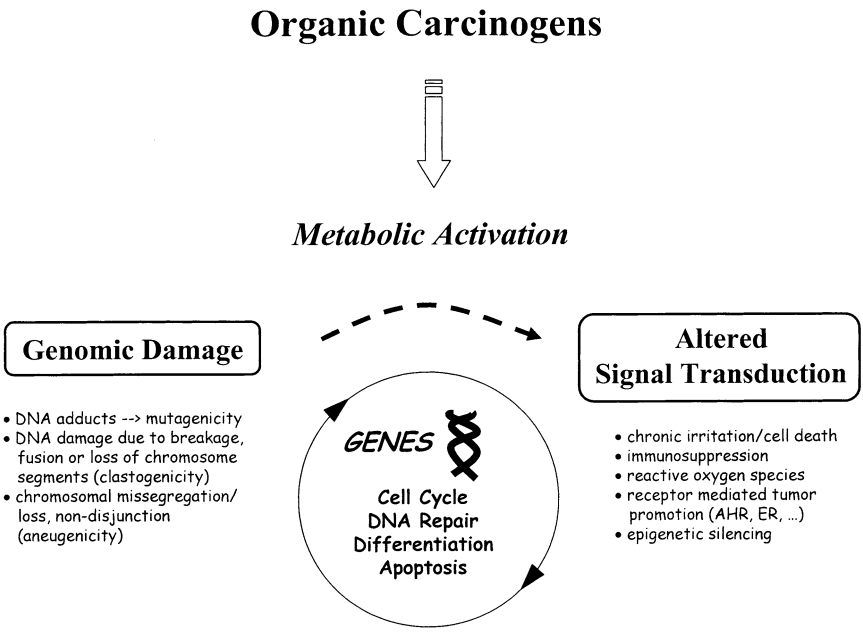


Figure 5. Genotoxic and nongenotoxic modes of action of organic carcinogens. Chemical carcinogens directly or indirectly affect the regulation and expression of genes involved in cell cycle control, DNA repair, cell differentiation, or cell death. DNA damage- or receptor-induced alterations in cellular signal transduction processes may lead to the loss of growth control and to genome instability. AHR, arylhydrocarbon receptor (agonists: TCDD, PAHs, PCBs); ER, estrogen hormone receptor (agonists: estrogen, diethylstilbestrol; ER antagonist tamoxifen with residual agonistic effects). See text for further explanations.

lung, adrenal cortex, skin, lymph nodes etc. [60]. In addition, this compound is also known as a potent tumor promotor in liver and skin in the two-stage initiation-promotion models for tumorigenesis (see below). TCDD is the strongest agonist of the arylhydrocarbon receptor (AHR) [61], an ubiquitous cytosolic protein originally discovered in connection to the inducibility of the microsomal enzyme activity designated as 'aryl hydrocarbon hydroxylase' ('AHH'). The AHH activity (now identified as being identical to certain alleles of CYP1A1 and CYP1B1) could be induced *in vitro* and *in vivo* by planar PAHs such as B[*a*]P and others [62, 63]. This cellular response is mediated by AHR [64], a member protein of the bHLH-PAS family of transcription factors characterized by an N-terminal basic helix-loop-helix DNA binding domain and a homology region originally described in the transcription factors PER, ARNT, and SIM (PAS domain) [65]. Upon binding to one of its ligands, the complex translocates into the nuclear compartment, heterodimerizes with the AHR nuclear translocator (ARNT), and then binds to specific arylhydrocarbon- or 'xenobiotic-responsive elements' (XRE = 5'-TNGCGTG-3'). XRE sequences are enhancer elements upstream of genomic target genes encoding

a diverse set of genes including enzymes involved in metabolism of PAHs and other xenobiotics (e.g., CYP1A1) [66].

TCDD induces a great diversity of toxic effects *in vivo* (strong acute toxicity, immunosuppression, teratogenicity, tumorigenicity). Since this compound does not require metabolic activation to exert its toxicity, induction of XMEs is likely to be etiologically unrelated to these biological effects. On the other hand, homozygous deletion of the *Ah* gene locus that encodes AHR renders mice resistant to the entire spectrum of TCDD-mediated toxicity [67, 68]. Conversely, a constitutively active AHR protein expressed in transgenic mice is capable of inducing tumors in the stomach [69] and promoting liver carcinogenesis [70]. Most recently, it became clear that TCDD changes the expression levels of numerous proteins involved in cell and tissue homeostasis, i.e., cellular growth, proliferation, differentiation, and apoptosis [71]. Using human hepatoma HepG2 cells, cDNA microarray analyses revealed that the levels of at least 112 [72] or 310 [73] mRNAs significantly changed in either direction due to exposure to TCDD (CYP1A1: 12- to 16-fold up). This primary response includes proteins involved in cellular proliferation (e.g., Ser/Thr kinases such as COT and NEK-2, protooncogen KRAS2, guanine nucleotide exchange factors, phospholipase A2), cell cycle regulation and apoptosis (e.g., cyclin B2, TNF receptor, HSP40), or extracellular matrix turnover, signaling and cell adhesion (e.g., human enhancer of filamentation 1, metallothioneines, plasminogen activator inhibitors, integrins beta 1 or 3) [72, 73]. In addition to their transactivation activity AHR-ligand complexes may also affect cellular signaling networks such as those triggered by hormones (e.g., estrogen receptor α, ERα), hypoxia (hypoxia inducible factor-1α, HIF-1α), nuclear factor-κB (NF-κB), retinoblastoma protein (RB), or by protein kinases, phosphatases and their coactivators through either 'molecular crosstalk' at DNA binding sites or direct protein-protein interactions, or both [74]. An example of the latter mechanism is the functional attachment of the cytosolic tyrosine kinase SRC to AHR, and the initiation of phosphorylation signaling cascades upon binding of TCDD [75]. At present, the individual roles of most of these newly discovered AHR-mediated effects in TCDD-induced carcinogenesis remain elusive. However, all alterations together contribute to an even more complex network of additional gene-gene interactions that may result in the broad-ranging interactive and tissue-specific biological outcomes observed [76].

Monooxygenation pathway and AHR-ligand activities

In 1935, Eric Boyland (1905–2002) suggested that PAHs might either be converted into more toxic substances or detoxicated [77]. About 20–25 years later, the same investigator demonstrated that arene oxides (epoxides) were shown to be crucial intermediates [78]. Since the bioactivated B[*a*]P-7,8-dihydrodiol had been found to bind to a greater extent to DNA than the parent compound,

the B[*a*]P-7,8-dihydrodiol 9,10-epoxide (B[*a*]PDE) was proposed to be the ultimate DNA-reactive intermediate of this PAH [79] (Fig. 6). Subsequent work confirmed the central role of vicinal dihydrodiol epoxide metabolites in mediating the DNA binding of B[*a*]P and other carcinogenic PAHs [80]. These compounds would not be carcinogenic if they were not metabolized by CYP enzymes and microsomal epoxide hydrolase (mEH) through subsequent steps of epoxidation and hydrolysis ('monooxygenation pathway'; Fig. 6). Deletion of the genes encoding the enzymes involved in this activation route (e.g., CYP1B1, mEH), or of the gene encoding the AHR protein involved in the induction of these enzymes (cf. above), renders mice resistant to the biological effects of potent PAHs such as B[*a*]P, 7,12-dimethylbenz[*a*]anthracene (DMBA), dibenzo[*a,l*]pyrene (DB[*a,l*]P; Fig. 6) and others [80–83].

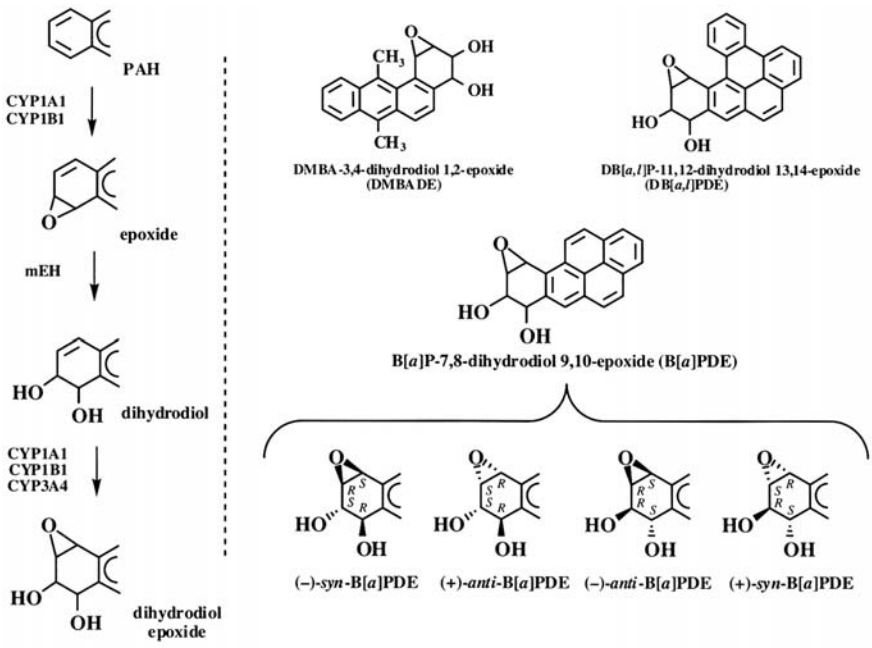


Figure 6. Bioactivation of polycyclic aromatic hydrocarbons (PAHs) towards ultimate DNA-reactive dihydrodiol epoxides. Activation of PAHs requires the activity of cytochrome P450-dependent monooxygenases (CYP). Initial conversion into epoxides is mainly catalyzed by CYP1A1 or 1B1. Subsequently, epoxides are hydrolyzed into *trans*-dihydrodiols by microsomal epoxide hydrolase (mEH). Further CYP-catalyzed epoxidation at the vicinal double bond generates the ultimate genotoxic dihydrodiol epoxides of PAHs. In addition to CYP1A1 and 1B1, CYP3A4 may also contribute to this final activation step. The ultimate genotoxic dihydrodiol epoxides from three strong carcinogenic PAHs, B[*a*]P, DMBA, and DB[*a,l*]P, are depicted. Activation towards vicinal dihydrodiol epoxides is highly stereoselective. Altogether, four different stereoisomers can be produced in one molecule region. This is exemplified in the case of the diastereomeric 7,8-dihydrodiol 9,10-epoxides of B[*a*]P (B[*a*]PDE). See text and Figure 7 for further explanations.

In the two-stage mouse skin bioassay, which had been established in 1947 [84], strong carcinogenic PAHs act as 'complete carcinogens' when repeatedly applied over time [85]. Such compounds are capable of inducing both somatic mutations in critical target genes through DNA binding ('initiation phase') and subsequent outgrowth of cells that are irreversibly transformed ('promotion phase'). This 'two-stage concept' goes back to Friedewald and Rous [86] who were the first to distinguish between the initiating and promoting effects in chemical carcinogenesis. In the early 1980s, induction of activating mutations in cellular *H-Ras* upon single application of carcinogenic PAHs was proven to be an early event in tumor initiation [87]. In this experiment, genomic DNA from skin carcinomas of mice, induced by single application of DMBA and subsequent treatment with a chemical promotor of carcinogenesis (12-*O*-tetradecanoylphorbol-13-acetate, TPA), carried an activated *H-Ras* oncogene. Transfection experiments with this DNA led to morphological transformation of fibroblasts in culture. As known today, oncogenic RAS increases cellular proliferation through multiple pathways, e.g., elevated cyclin D1 expression [88] or mitogen-activated protein kinase (MAPK) pathways (e.g., JNK, ERKs) [89]. Nevertheless, repeated application of carcinogenic PAHs is mandatory to obtain maximal tumor yield in mouse skin. This finding along with the requirement of a functional AHR protein supports the notion that both the initiating and promotional activity of carcinogenic PAHs in skin depends on AHR-mediated gene expression. This would be in agreement with the tumor promoting activity of TCDD in this organ.

Apoptotic resistance

Carcinogenic aromatic amines or amides such as 4-aminobiphenyl (4-ABP) or 2-AAF primarily induce bladder tumors in dogs, and tumors in the liver, lung or mammary gland of rodents [90] (Figs 1 and 3). Despite some new insights on the main CYP isoform involved in activation *in vivo* [91], *N*-hydroxylation and subsequent reactive ester formation have been well characterized and sufficiently explain the genotoxicity of these compounds [90, 92] (Fig. 3). However, additional tumor-promoting activities have been observed and investigated, particularly in the case of the model compound 2-AAF, a 'complete carcinogen' in rodent liver [93]. Chronic exposure of rats to 2-AAF was found to trigger adaptive responses in mitochondria permeability transition pores and BCL-2 expression levels of hepatocytes that resulted in an increased resistance to apoptosis [94]. There is evidence that this effect is an early tissue response to the presence of 'reactive oxygen species' (e.g., hydroxy or superoxide anion radicals) generated via redox-cycling of 2-AAF metabolites (i.e., 2-nitrosofluorene, cf. Fig. 3). Since mitochondrial resistance is established in the tissue before the clonal outgrowth of preneoplastic cells, this nongenotoxic effect contributes to the selection of resistant cells and hence to the tumor-promoting activity of 2-AAF in its target organ liver.

DNA methylation status

Based on their potency to induce a DNA damage response via upregulation of TP53 protein levels and subsequent transactivation of genes involved in cell cycle checkpoint control and apoptosis, gene expression profiling enables discrimination between genotoxic and nongenotoxic carcinogens at early time points and high treatment doses *in vitro* [95]. From the foregoing section, however, it can be concluded that long-term exposure to rather low doses of genotoxic carcinogens may also contribute to nongenotoxic alterations in target tissues *in vivo*, thereby promoting the outgrowth of transformed cell clones. Perturbation of balanced tissue homeostasis through interaction with factors that enhance proliferation or inhibit differentiation and cell demise would interfere with DNA damage response pathways and may result in the accumulation of DNA lesions and genomic instability that ultimately contribute to malignant progression [96] (cf. Fig. 5). Although sometimes considered as 'epigenetic', in early stages of tumorigenesis, these nongenotoxic effects are rather reversible and require continuous presence of the inducing compound. On the other hand, heritable alterations in gene expression patterns epigenetically triggered through carcinogen-induced changes in the DNA methylation status of cells have been as yet rarely described. It may be interesting to note that ions of the metals nickel (Ni) and cadmium (Cd), or of the metalloid arsenic (As) have been shown to induce epigenetic alterations in cells *in vitro* [97]. Another example is the developmental stage-specific carcinogen DES, a synthetic nonsteroidal estrogen which was taken until the early 1970s by women during pregnancy to prevent miscarriage [98] (Fig. 1). In 1971, DES exposure *in utero* was associated with vaginal clear cell adenocarcinoma in adolescent daughters of women who had taken the drug [99]. This compound was also linked to additional structural, functional, and cellular abnormalities in both females and males, following prenatal exposure [98, 100]. Neonatal exposure of mice to DES revealed that estrogen-responsive genes such as *lactoferrin* and the protooncogenes *c-Fos*, *c-Jun*, and *c-Myc* are upregulated until adulthood even when the drug had been withdrawn again [101]. For instance, mice treated with DES 1–5 days after birth expressed *lactoferrin* and *c-Fos* persistently from days 5 to 60, and subsequently developed epithelial cancers in the uterus at 18 months of age [102, 103]. In the uterine tissue, these alterations were accompanied by hypomethylation of specific CpG sites within the *lactoferrin* and *c-Fos* promotor regions. In contrast, DES-induced downregulation of a group of developmental genes *sensu strictu*, i.e., *Hox-A10* or *A11* [104], could not be associated with alterations in the methylation pattern of their gene promotor regions [105]. Therefore, the molecular mechanisms underlying persistent changes in gene expression patterns and the role of alterations in the DNA methylation status induced by neonatal (or prenatal) DES exposure remain elusive.

Molecular specificity of genotoxic carcinogens?

Carcinogen-induced DNA damage and mutations disrupt the expression of genes involved in the surveillance of cellular growth, proliferation, and death (Fig. 5). What is known about the molecular specificity in organic carcinogens' modes of action?

The impact of DNA binding level

In their landmark paper from 1964, Brookes and Lawley worked on the tissue binding levels of six PAHs, i.e., naphthalene, dibenz[*a,c*]anthracene, DB[*a,h*]A, B[*a*]P, 3-methylcholanthrene, and DMBA [34]. Based on their experiments, the authors concluded that there is a 'significant positive correlation' between the binding to DNA and the carcinogenic potency of these compounds according to Iball's index (that is, ratio between tumor incidence and average tumor latency period [106]). In contrast, this correlation was not found for the binding to proteins or RNA [34]. In more recent years it became clear that vicinal dihydrodiol epoxides of PAHs are the actual DNA-binding metabolites that mediate the biological effects associated with their parent structures (cf. above; and see Fig. 6). By direct application of these reactive descendants it could be shown that their mutagenic potency correlates nicely to the level of PAH-DNA adduct formation [107]. Given these data, it therefore seems plausible that the quantity of DNA damage rather than the individual (qualitative) adduct structure is the main factor determining the mutagenic activity of a particular genotoxic species. In addition, the tumor-inducing potencies of different PAHs in the lungs of strain A/J mice, another experimental tumor model, were found to correlate to the time-integrated DNA adduct levels (TIDAL) calculated as area under the curves of total dihydrodiol epoxide-DNA adduct levels during a time course of 30 days after injection [108]. This parameter represents the total effective molecular dose delivered to target lung DNA, and it linearly correlated to the PAH doses administered. The intimate relationships between DNA binding level and mutagenicity and between the TIDAL and carcinogenicity observed in mice *in vivo* support the notion that the DNA binding level can serve as an important (bio)indicator of the tumor threat that may result from certain exposures of humans to carcinogenic PAHs [109], and other genotoxic carcinogens as well [110].

The impact of stereochemistry

The DNA adduct level itself at a given time point is an integrated product of compound's (structure-related) toxicokinetic and toxicodynamic behavior, including metabolic activation and detoxification prior to covalent binding, as well as the effectiveness of the repair of those lesions that have been formed.

XMEs usually operate with high regio- and stereoselectivity. For instance, CYP-mediated toxification of aflatoxin B_1 (AFB_1), a natural carcinogen produced by *Aspergillus* mould and linked to human liver carcinogenesis [111], occurs at its 8,9-position (Fig. 7). Based on the isolation of the main DNA adduct (at N7 in guanine bases), formation of an 8,9-epoxide intermediate had been proposed and subsequently confirmed [112]. CYP3A4, the most important enzyme involved in AFB_1 activation in human liver, exclusively forms the *exo* isomer (Fig. 7). In contrast, another CYP form, CYP1A2, may add some small amounts of the diastereomeric *endo* epoxide [113]. However, the predominant AFB_1 *exo*-8,9-oxide is about 1000-fold more genotoxic than its *endo* diastereomer due to the spatial configuration of the epoxide moiety within the AFB_1 *exo*-8,9-oxide-DNA intercalation complex. Intercalation of the furanocoumarine residue between DNA bases directs the *exo* epoxide ring in a favorable position for an S_N2 attack by the N7 atom of guanine [114, 115]. Follow-up products of the main N7-DNA adduct of AFB_1 then result from depurination or ring opening of the purine base. These products, along with the detoxification products produced by GST- or mEH-mediated conversions, are depicted in Figure 7.

Similarly, metabolic activation of PAHs is highly selective [80]. As demonstrated for a wide range of carcinogenic PAHs the initial epoxidation → hydrolysis sequence produces a dihydrodiol with *R,R*-configuration in high enantiomeric excess (cf. above). Subsequent (diastereo)selective epoxidation at the vicinal double bond then predominantly generates the *R*,*S*-dihydrodiol *S*,*R*-epoxide with the epoxide moiety *trans* to the benzylic hydroxy group. In the case of B[*a*]P, all four possible stereoisomeric 7,8-dihydrodiol 9,10-epoxides are depicted in Figure 6, with (+)-*anti*-B[*a*]PDE as the major species formed during bioactivation. Depending on the activation system, some small amounts of the other isomers, the (–)-*syn*-(*R,S,R,S*)-, (+)-*syn*-(*S,R,S,R*) and (–)-*anti*-(*S,R,R,S*) dihydrodiol epoxides, may also be generated – the latter two through monooxygenation of the *S,S*-dihydrodiol (Fig. 6). However, B[*a*]P-induced DNA damage *in vitro* or *in vivo* predominantly results from covalent interaction of (+)-*anti*-B[*a*]PDE, most of which is trapped by 2'-deoxyguanosine (dG) residues via *trans* opening of the epoxide moiety [80, 116] (Fig. 7). If not repaired properly, the product of this reaction is likely to cause nucleotide misincorporation at the opposite DNA strand during the next round of DNA replication, and, therefore, has the potential of inducing mutations (i.e., dG base substitutions or frame shifts). DNA lesions such as the (+)-*trans*-*anti*-B[*a*]PDE-N^2-dG adduct are subject to nucleotide excision repair (NER). Induction of NER activity requires both the disruption of normal base pairing and the presence of a chemical modification ('bipartite damage recognition') [117]. It could be demonstrated that (+)-*anti*-B[*a*]PDE induces a considerably different degree of NER activity in a certain DNA base context depending on the way of epoxide ring opening during adduct formation [118]. While the *cis*-opened adduct, i.e., the (+)-*cis*-*anti*-B[*a*]PDE-N^2-dG, adopts an intercalative, internal adduct conformation with the benzo[*a*]pyrenyl moiety

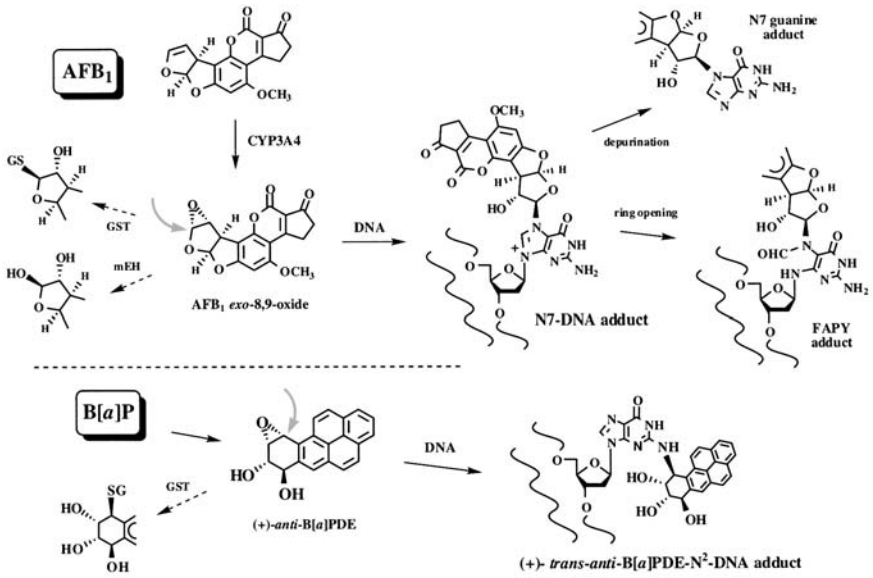


Figure 7. Stereoselective activation of aflatoxin B_1 (AFB_1) and benzo[*a*]pyrene (B[*a*]P) and their main reaction products with DNA. CYP3A4, the main CYP enzyme involved in AFB_1 activation, stereoselectively produces AFB_1 *exo*-8,9-epoxide. The *endo*-diastereomer is not formed by CYP3A4, yet it may be formed in small amounts by CYP1A2. B[*a*]P is stereoselectively activated to (+)-*anti*-B[*a*]PDE possessing *R,S,S,R*-configuration (cf. Fig. 6). The grey arrows point to the position of the nucleophile (DNA, protein, GSH) attack. GSH conjugates of AFB_1 *exo*-8,9-oxide and (+)-*anti*-B[*a*]PDE are detoxification products. AFB_1 *exo*-8,9-oxide forms primarily DNA adducts at position N7 in purine bases. The N7 adduct is then subject to further modification. Due to the positive charge in the purine ring system, the purine adduct may be released from DNA to produce an apurinic site (cf. Fig. 4). Ring opening leads to another secondary product, the formamidopyrimidine (FAPY) adduct. The major DNA binding product of B[*a*]P, the (+)-*trans*-*anti*-B[*a*]PDE-N^2-dG, derives from *trans* opening of the epoxide moiety. See text for further explanations. GST, gluthathione *S*-transferase; mEH, microsomal epoxide hydrolase.

inserted into the double helix and concomitant displacement of the modified base, the (+)-*trans*-*anti*-B[*a*]PDE-N^2-dG displays an external conformation with the aromatic ring system accommodated in the minor DNA groove [119]. Hence, the local DNA distortion induced by the *cis* product is much more severe as compared to the *trans* product, thereby resulting in a 10-fold faster removal through NER. Moreover, the poor enzymatic repair of the predominant (+)-*trans*-*anti*-B[*a*]PDE-N^2-dG adduct is preceded by an insufficiently activated DNA damage checkpoint [120]. At non-toxic doses of *anti*-B[*a*]PDE, a significant number of synchronized cells *in vitro* were found to enter S phase, with little increase of those in G_1. The failure to induce a proper DNA damage arrest in G_1 (so-called 'stealth property') along with insufficient enzymatic repair increases the likelihood of transforming mutations because DNA replication continues on a damaged template via engagement of error-prone Y-family polymerases (pol η, κ, ι, ζ, Rev1) during translesional synthesis [121]. Data

from analyses of the cell cycle and the expression profiles of human mammary epithelial cells or tumor cells of epithelial origin *in vitro* revealed that the TP53 → CDKN1A ($p21^{WAF1}$)-mediated G_1 checkpoint response was improper even at a DNA damage level of about 180000 *anti*-B[*a*]PDE-DNA adducts/cell [122]. Although TP53 levels were rapidly increasing due to protein stabilization *via* Ser15 phosphorylation, the cells exposed to *anti*-B[*a*]PDE lacked a timely induction of CDKN1A. Whereas the transactivation activity of TP53 was not impaired in the case of several other downstream targets (e.g., *GADD45*, *WIP1*, *p53R2*) [122], a similar *anti*-B[*a*]PDE-induced and TP53-mediated transcriptional repression has been observed at the *BRCA1* locus [123]. The BRCA1 tumor suppressor is involved in DNA damage response, DNA double-strand break and transcription-coupled repair, thereby contributing to the inhibition of genomic instability during the course of malignant cellular transformation [124]. At present, however, the reasons for the differential activity of TP53 at various target gene promotors remain obscure.

Mutational profiles as molecular biomarkers

Members of the oncogene family *Ras* are commonly mutated in human cancers and animal models for chemical-induced carcinogenesis [125]. Codon 12 (in exon 1) of *K-Ras* is the most frequently affected codon in human cancers including human lung adenocarcinomas [126]. Using human bronchial epithelial cells it could be demonstrated that the first dG residue in this codon is a preferential binding site for ultimate DNA-damaging metabolites of various carcinogens such as 2-AAF (*N*-acetoxy-AAF at C8), AFB_1 (AFB_1 *exo*-8,9-oxide at N7), and B[*a*]P (*anti*-B[*a*]PDE at N^2) [127] (cf. Figs 3 and 7). As compared to other sites, the 'hotspot' character of codon 12 in target cells of chemical lung tumorigenesis resulted from a synergism between the preferential binding of the carcinogenic metabolite and a poor repair of those lesions that had been formed [127]. While methylation at CpG sites in the vicinity of codon 12 of *K-Ras* had no influence on the preferential binding of carcinogens at this position [128], the presence of 5-methylcytosines greatly enhanced the binding of the ultimate genotoxic descendants of 4-ABP, 2-AAF, AFB_1 and B[*a*]P at dG residues within the DNA binding domain (exons 5, 7 and 8) of the human *TP53* gene [129–131]. Analysis of the binding profiles of *N*-hydroxy-*N*-acetyl-4-ABP and *anti*-B[*a*]PDE at the *TP53* locus in human bladder cells [132] and bronchial epithelial cells [133], respectively, and comparison to the tissue-specific *TP53* mutational 'hotspot' pattern in tumors from these organs provided strong evidence for an etiological role of these compounds in the causation of the disease. In both cases, the codons and the positions within the codons affected matched between cells exposed to *N*-hydroxy-*N*-acetyl-4-ABP or *anti*-B[*a*]PDE and the *TP53* mutation database of human bladder or lung cancer. Further, and in congruence to the strand bias of G → T transversions in lung cancer of smokers,

anti-B[*a*]PDE-N^2-dG adducts were almost exclusively formed at methylated CpG dinucleotides of the nontranscribed DNA strand, possibly as a result of the slow repair of this strand as compared to its transcribed counterpart [134]. On the other hand, comparison of *TP53* mutational hotspots in hepatocellular carcinoma with AFB_1-induced DNA damage in human liver HepG2 cells rather questions the relationship between compound and disease [135]. This analysis is, however, contrasted by several lines of evidence: (i) a wealth of epidemiological data point to a correlation between AFB_1 exposure and specific mutations in codon 249 of *TP53* [136, 137]; (ii) new insights on the biological activity and repair resistance of a major AFB_1-DNA adduct (the AFB_1-FAPY adduct, cf. Fig. 7) support the notion of its contribution to the occurrence of G → T transversions at the third position in this codon [138]; and (iii) the presence of AFB_1-DNA adducts in hepatocellular tumors is accompanied by a higher frequency of methylated CpG sites in the promotors of the tumor suppressors *p16* and *RASSF1A*, two genes epigenetically silenced in a high percentage (47% and 85%) of liver cancer cases from Taiwan [139].

Risk assessment in chemical carcinogenesis – implications

The toxicological approach

Studies in animal tumor models or in cells *in vitro* lend support to the notion that the level of DNA binding along with the intrinsic (structural) quality of the DNA adducts induced and their qualitative and quantitative distribution both at the organismic level (i.e., within different kinds of organs and tissues) and at the cellular level (i.e., within the cell's genome) are the main determinants for the biological activity of a particular genotoxic carcinogen. Because of this intimate correlation between DNA damage and carcinogenic potency it can be anticipated that differences in the activity of enzymes that produce DNA-reactive intermediates, detoxify these species, or repair the DNA lesions formed, may play a major role in mediating the individual tumor susceptibility in human populations (see below). However, there are essentially three major issues that limit the predictive value of the knowledge obtained in experimental tumor models with regard to human cancer:

1. All XMEs (including polymorphic variants) can contribute to both activation and detoxification of organic carcinogens (e.g., GST enzymes: AFB_1 *exo*-8,9-oxide *versus* haloalkanes/alkenes; see Figs 4 and 7). Occasionally, toxication and detoxication routes of the very same carcinogen are served by the very same enzyme (e.g., mEH: formation of PAH dihydrodiols, which then may be subject to either conjugation → excretion or further activation towards dihydrodiol epoxides; see Figs 6 and 7). An interesting example of these interactions is the protective effect of CYP1A1 and 1A2

in vivo against DNA adduct formation and toxicity of B[*a*]P [140] or 4-ABP [91]. Both enzymes are involved in activation of B[*a*]P (CYP1A1) or 4-ABP (CYP1A2; see Figs 2, 3 and 6), yet animals that lack the corresponding genes have been found to suffer from higher DNA adduct levels in internal organs and increased toxicity. It seems likely that this effect is due to the well-balanced expression levels of CYP1A1 and 1A2 along with detoxifying phase-II enzymes in wild-type animals, but also a result of the overcompensatory induction of other oxidative enzymes, such as CYP1B1 or flavin-dependent monooxygenases, which would substitute for the absence of CYP1A1 and 1A2 [141].

2. Humans are mostly exposed to complex mixtures of compounds rather than to single carcinogens. For instance, due to the manner of their generation (incomplete combustion), more than 100 different PAHs can be detected in airborne particulates [142]. Cigarette smoke entails the risk of being exposed to about 60 known carcinogens from a variety of chemical classes including 4-ABP, B[*a*]P, and traces of metal ions [143]. The interactions of those individual compounds may result in synergistic effects within the biological system. At the level of DNA repair, for example, co-exposure of cells to Ni ions [144], or As and its methylated metabolites [145], was found to enhance B[*a*]P-mediated mutagenesis through inhibition of NER-catalyzed removal of *anti*-B[*a*]PDE-N^2-dG adducts. Even the presence of structurally and stereochemically different DNA lesions in the same genome may cause repair inhibition through sequestration of critical NER subunits by those modifications that are more repair resistant (so-called 'decoy adducts') [146]. Despite being refractory to excision, 'decoy adducts' immobilize NER factors, and may therefore contribute to synergistic interactions between multiple genotoxic agents present in complex environmental mixtures. Thus, the results obtained from single compound experiments would not allow direct extrapolation to the corresponding effects expected to be exerted from mixtures.
3. In experimental tumor models, animals or cells are treated with single compounds in very high doses as compared to the levels of human background exposures. Therefore, regulatory toxicologists have to extrapolate the dose-response relationships found in animals into the low-dose exposure ranges of humans in order to assess the accompanying risk. Depending on the kind of approach applied, the results may differ tremendously, and are often subject to believe or disbelieve [147, 148].

The role of molecular epidemiology

Many studies conducted in recent years tried to explore the influence of metabolic gene polymorphisms in the human population and their interactions on the levels of biomarkers of carcinogen exposure such as urinary metabolites, DNA adducts, chromosomal aberrations and others [149]. The goal of these

studies is to use this information and to identify subpopulations of humans that are likely to be more susceptible ('to be at high risk') of developing tumors in response to carcinogen exposure. Due to their low frequency in Caucasian populations, however, significant contributions of gene polymorphisms related to carcinogen activation/inactivation are usually hard to detect [150–152] (Tab. 1). One exception is the lack of GSTM1 activity (*GSTM1* null genotype), which is most common (50% of Caucasians) and hence entails some marked effects on cancer susceptibility (Tab. 1).

Given the chemical complexity of most environmental matrices, it seems difficult, if not, impossible to uncover any causative connections between certain

Table 1. Genetic polymorphisms of metabolic enzymes related to chemical carcinogenesis and their role in tumor susceptibility (selection)[a]

Genetic polymorphisms → Biological effects/tumor susceptibility (selection)	
Cytochrome P450-dependent monooxygenases (CYP, EC 1.14.14.1)[b]	
CYP1A1	*CYP1A1*2A* (*Msp*I, 3' untranslated region) and **2B* (Ile^{462}Val, exon 7) are associated with increased catalytic activity, higher levels of PAH-DNA adducts and *TP53* mutations in smokers. *CYP1A1 Msp*I combined with *GSTM1 0/0* leads to an 'at risk' genotype for tobacco-accociated DNA damage and lung cancer.
CYP1A2	Limited evidence for higher risk of bladder cancer in smokers with *CYP1A2*1F* (^{163}C→A: SNP intron 1) entailing increased inducibility. Combination effect with slow NAT2 acetylator phenotype.
CYP1B1	Effects of known polymorphisms are only marginal *in vitro*. However, *CYP1B1*3* (Val^{432}Leu) entails a higher frequency of smoking-induced *TP53* mutations [166]. Increased OR with combined *GSTM1*2* or *GSTT1*2*. Limited evidence.
CYP2A6	*CYP2A6*2* (Leu^{160}His) and **3* (exon deletion): impaired activity, yet no *in vivo* evidence.
CYP2E1	*CYP2E1*6* (^{7632}T→A: *Dra*I RFLP intron 6): N7-alkyl levels in lung samples elevated. Inadequate evidence for increased lung and breast cancer risk.
Microsomal epoxide hydrolase (mEH, EC 3.3.2.3)	
mEH	Substrates: arene oxides (e.g., AFB_1, B[*a*]P). Limited evidence for higher AFB_1 protein adduct levels and liver cancer susceptibility in subjects with *mEH* Tyr^{113}His (exon 3 polymorphism). Limited evidence for reduced lung cancer risk in smokers [167].
Sulfotransferases (SULT, EC 2.8.2.1)	
SULT1A1	Substrates: toxication of aromatic *N*-hydroxylamines (e.g., 2-AAF), benzylic alcohols and phenols (e.g., PAHs, benzene); detoxication of hydroxy derivatives (e.g., PAHs). Large interindividual variation in activity. Various alleles, e.g., *SULT1A1*2* (Arg^{213}His) with reduced activity. Limited evidence for a protective role of *SULT1A1*2* in colon, but higher risk in lung.
SULT1A2	Several alleles, e.g., *SULT1A2*2* (Asn^{235}Thr), cause a strong decrease in activity. Functional role *in vivo* unclear.
SULT2A1	Main form in liver. Several rare alleles with moderate effects on enzyme activity (<2).

(Continued on next page)

Table 1. (Continued)

Genetic polymorphisms → Biological effects/tumor susceptibility (selection)	
N-Acetyltransferases (NAT, EC 2.3.1.5)	
NAT1	Predominantly extrahepatic. Substrates: *N*-acetylation (detoxication), and *O*-acetylation or *N*,*O*-transacetylation (toxication) of arylamines/amides and some HCAs. *NAT1*10* allele (elevated activity) entails higher risk for colon and bladder cancer (high rate of *O*-acetylation of HCAs in colon and arylamines in bladder).
NAT2	Predominantly hepatic. Substrates and reactions as NAT1, main form for HCAs, but no *N*,*O*-transacetylation. Several NAT2 alleles (e.g. *NAT2*5B*) are associated with a slow acetylator phenotype. Slow acetylators have higher levels of 4-ABP-hemoglobin adducts and an increased risk of bladder cancer (impaired hepatic *N*-acetylation of arylamine); yet they are at lower risk for colon cancer.
Glutathione *S*-transferases (GST, EC 2.5.1.18)	
GSTM1	Substrates: epoxides of PAHs and olefines (AFB_1, 1,3-butadiene, etc.), arylamine esters. *GSTM1 0/0* genotype (loss of activity): although mainly expressed in liver, higher PAH-DNA adduct levels, cytogenetic damage, and *TP53* mutations in lungs of smokers; effect pronounced with combined *CYP1A1*2A* genotype. Higher 4-ABP-hemoglobin levels in smokers and non-smokers; effect pronounced with combined slow NAT2 acetylator genotype. *GSTM1 0/0* alone is only a weak modifier of lung (OR 1.41) and bladder cancer (OR 1.44). For lung cancer, the effect significantly increases in the presence of an active *CYP1A1 Msp*I genotype (OR 3-10).
GSTT1	Substrates: epoxides of PAHs, 1,3-butadiene, ethylene oxide (detoxication), dihaloalkanes/-methanes (toxication). Accordingly, *GSTT1 0/0* polymorphism entails a higher or lower risk for genomic damage *in vitro*, depending on the substrate. At present, no evidence for modulation of human cancer risk.
GSTP1	Equivocal results *in vitro*. Limited evidence for association between *GSTP1 $Ile^{105}Val$* polymorphism and increased bladder cancer susceptibility. Inadequate evidence for a role in human lung cancer.
Arylhydrocarbon receptor (AHR) pathway	
AHR	Several polymorphisms in humans reported. Combination of $Lys^{554}Leu$ and $Val^{570}Ile$ impairs TCDD-mediated CYP1A1 induction *in vitro*. Strong evidence for a correlation of human lung, laryngeal, and oral cavity cancer with AHR phenotype.
ARNT	Polymorphisms known, but functional roles as yet unknown [169].
DNA repair proteins	
XRCC1/3	Involved in base excision and DNA double strand repair. *XRCC1 $Arg^{399}Gln$* (exon 10) and *XRCC3 $Thr^{241}Met$* (exon 7) are associated with higher 'bulky' DNA adduct levels in non-smokers and both non- and ex-smokers, respectively. Combined effects of multiple gene variants on DNA damage levels observed. As yet only insufficient evidence for an important role in human (lung) cancer susceptibility.
XPD	Helicase involved in NER. *XPD $Asp^{312}Asn$* (exon 10) and *$Lys^{751}Gln$* (exon 23) correlate to significant elevated DNA adduct levels in non-smokers. Combination effects observed. Limited evidence for role in cancer susceptibility: OR (lung cancer) of 1.06-3.2.

[a]Adapted from [159, 166–176]
[b]cf. Figure 2.
Additional websites: *http://www.imm.ki.se/CYPalleles/* (genetic polymorphisms of CYPs); *http://www.louisville.edu/medschool/pharmacology/NAT.html* (genetic polymorphisms of NATs); HCAs, heterocyclic amines; OR, odds ratio; RFLP, restriction fragment length polymorphism.

forms of human cancer and the exposure to particular carcinogenic compounds. In addition to any epidemiological hints, collective evidence obtained from molecular toxicology and molecular epidemiology together may nevertheless be able to uncover the role of individual compounds (or single classes of compounds), and to extract their contribution from the overall human biological response to environmental mixtures. Although highly debated, one of the most well worked-out examples in this regard is the crucial role of carcinogenic PAHs (i.e., B[*a*]P) in the etiology of human lung cancer based on their presence in cigarette smoke. Their importance in tumor initiation is strongly supported by several lines of evidence that can be summarized as follows:

1. As early as 1973, it was reported that the extent of inducibility of 'AHH' (now known as CYP1A1 and 1B1) was increased in lung cancer patients as compared to controls [153]. CYP1A1 and 1B1 are the two major monooxygenases involved in activation of B[*a*]P and other carcinogenic PAHs (Fig. 6), and it appears that CYP1A1 inducibility is related to lung cancer risk in smokers [154].
2. The activity of pulmonary CYP1A1 correlates to 'bulky' *anti*-B[*a*]PDE-DNA adduct levels in human lung tissue from cancer patients [154, 155]. The high variability in lung PAH-DNA adduct levels can be rationalized by large interindividual differences in pulmonary CYP1A1 expression [154], which in turn result from polymorphisms in the regulation of the corresponding gene [156].
3. The levels of *anti*-B[*a*]PDE-DNA adducts in lung tissue *in vivo* are generally higher in smokers as compared to ex- or non-smokers [157].
4. The levels of *anti*-B[*a*]PDE-DNA adducts in lung tissue from individuals carrying a *CYP1A1 Msp*I-*GSTM1*-null genotype are increased as compared to wild-type allele carriers [158] (Tab. 1). This combined 'at risk' genotype correlates to a higher frequency of *TP53* exon 5–8 mutations in lung tumor tissue [159], and is related to high susceptibility for developing lung cancer [152]. Hence, germline polymorphisms of two important genes involved in PAH metabolism (i.e., *CYP1A1*, *GSTM1*) and associated with increased levels of PAH-DNA adducts in lung target tissue, are also related to smoking-associated *TP53* mutations in this tissue.
5. Exposure of human bronchial epithelial cells to *anti*-B[*a*]PDE leads to the formation of *anti*-B[*a*]PDE-N^2-dG adduct hotspots within *K-Ras* (codon 12) and *TP53* (codons 157, 248, 273) at positions identical to mutational hotspots detected in lung tumor tissue from smokers (see above). Both genes are frequently mutated in cigarette smoke-induced lung cancer [126, 160]. In accordance with the preferential formation of *anti*-B[*a*]PDE adducts at N^2-dG sites (Fig. 7), G $\rightarrow$ T transversions were detected as the principal mutations at all of these mutational hotspots.

The coincidence of mutational hotspots in *K-Ras* or *TP53* and *anti*-B[*a*]PDE-N^2-dG adduct hotspots suggests that B[*a*]P is involved in trans-

formation of human lung tissue in smokers. There is no doubt that cigarette smoke contains a highly heterogenous mixture of hundreds of compounds including tobacco-specific *N*-nitrosamines such as NNK, which constitute another major class of strong lung carcinogens present in this matrix (Fig. 1) [161]. However, *N*-nitrosamines predominantly induce G → A and C → T transitions in *K-Ras* and *TP53* of animal models [162]. Although some of these (alkylating) compounds are principally capable of producing G → T transversions, *N*-nitrosamines are activated by CYP2E1, CYP2A6 and some other isoforms, but not by CYP1A1 [46, 163] (Fig. 2), and they barely contribute to 'bulky' DNA adducts induced in the lungs of smokers. The presence of G → A transitions in the *TP53* gene of human lung cancer tissue [164], and the modulating effects of *CYP2E1* gene polymorphisms in human lung cancer risk [165] (Tab. 1), however, suggest that synergistic effects may have a particular importance in chemical-induced lung cancer in man.

Prospects

Background levels of carcinogen-DNA adducts in human tissue samples from unexposed individuals were found to be in the range of 1 per 10^5 (oxidative lesions/human placenta), 1 per 10^7 (B[*a*]P/human placenta), 0.3–3.9 per 10^8 (4-ABP/human bladder), or 0.2 per 10^8 nucleotides (tobacco-specific *N*-nitrosamines/peripheral lung) [177]. The improvement of our knowledge on the efficiency of the enzymatic repair of such lesions and on the various biological effects (modes of action) exerted by genotoxic carcinogens (Fig. 5) is necessary to determine molecularly defined no-adverse-effect levels as the basis for setting 'practicable' thresholds in the human environment [178]. Further, any risk assessment that would not consider the interindividual variability within the human population would be prone to severely underestimate the risks to those subjects who are most vulnerable to carcinogen-induced DNA damage. It therefore becomes important to identify individuals 'at risk' by means of toxicogenetics and molecular epidemiology [179, 180]. This implies that we learn more about the role and interplay of additional, as yet unknown, susceptibility and resistance genes targeted by human carcinogens or involved in modulating human responses to carcinogenic compounds. Beyond the range of known polymorphic enzymes in carcinogen metabolism and repair (Tab. 1), additional genetic variants are likely to contribute to the development of sporadic cancer [181, 182]. The discovery of these variants and the characterization of their interactions with environmental exposures are clearly among the major topics in chemical-related cancer research in the years to come.

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