Genotoxicity: damage to DNA and its consequences

David H. Phillips and Volker M. Arlt

Institute of Cancer Research, Section of Molecular Carcinogenesis, Brookes Lawley Building, Cotswold Road, Sutton, Surrey SM2 5NG, United Kingdom

Abstract

A genotoxin is a chemical or agent that can cause DNA or chromosomal damage. Such damage in a germ cell has the potential to cause a heritable altered trait (germline mutation). DNA damage in a somatic cell may result in a somatic mutation, which may lead to malignant transformation (cancer). Many *in-vitro* and *in-vivo* tests for genotoxicity have been developed that, with a range of endpoints, detect DNA damage or its biological consequences in prokaryotic (e.g. bacterial) or eukaryotic (e.g. mammalian, avian or yeast) cells. These assays are used to evaluate the safety of environmental chemicals and consumer products and to explore the mechanism of action of known or suspected carcinogens. Many chemical carcinogens/mutagens undergo metabolic activation to reactive species that bind covalently to DNA and the DNA adducts thus formed can be detected in cells and in human tissues by a variety of sensitive techniques. The detection and characterisation of DNA adducts in human tissues provides clues to the aetiology of human cancer. Characterisation of gene mutations in human tumours, in common with the known mutagenic profiles of genotoxins in experimental systems, may provide further insight into the role of environmental mutagens in human cancer.

Keywords: Carcinogenicity, mutagenicity, mutation, DNA adduct, micronucleus, chromosomal aberration, transgenic animals, DNA strand break, Ames test, comet assay, ^{32}P-postlabelling, immunoassay, mass spectrometry, human biomonitoring.

Introduction

Cancer is a genetic disease arising from a series of somatic mutations. Mutations in DNA may arise spontaneously, or as a result of chemical action by agents of either endogenous or exogenous origin. Genetic toxicology is the study of agents that can damage the DNA and chromosomes of cells. In eukaryotic organisms, genetic damage in somatic cells may lead to malignancy. In germ cells it may adversely affect reproduction or provoke heritable mutations. Consequently, investigating the genotoxicity of a compound is often carried out in the context of seeking to understand its mechanism of carcinogenicity and this has become an essential component of the process of risk assessment for human exposure to a known animal carcinogen. Investigating genotoxicity is also important in assessing whether or not a new compound is a carcinogen and/or mutagen, and this process contributes to the more fundamental process of hazard identification.

Understanding mechanisms of carcinogenesis often relies on analysis of the molecular and cellular effects of carcinogens in laboratory experiments. This is a necessary simplification of a complex process and while such approaches often provide critical evidence for mechanisms, it must be recognised that laboratory models rarely cover all the possible facets of the process, and there are many instances in which the classification of a carcinogen is not a straightforward matter.

A genotoxic carcinogen typically induces tumours in multiple organs of rodents, may be carcinogenic to more than one species and to both males and females. In addition, there is often evidence of a dose-response relationship for tumour induction, of the type that does not suggest evidence of a threshold. In contrast, non-genotoxic carcinogens are more likely to be characterised by tumour induction in a single species and/or in a single tissue and, commonly, in one sex only, often at low incidence and only at high dose with associated evidence of toxicity. Multi-species, multi-organ carcinogens are more likely to be human carcinogens and, indeed, most agents classified as human carcinogens by the International Agency for Research on Cancer (IARC) are genotoxic. It is thus a general principle that chemicals that are carcinogenic in animals by a

genotoxic mechanism pose a greater potential risk to humans than non-genotoxic carcinogens, and the default assessment of genotoxins is that human exposure at any level poses a risk. For such agents exposure should be As Low As Reasonably Achievable (ALARA). For non-genotoxic carcinogens it may be possible to define a threshold, *i.e.* a level of exposure below which the agent does not present a carcinogenic risk to humans.

In some animal models carcinogenicity can be divided into an initiation phase, involving a single treatment with a genotoxic agent, followed by promotion, involving repeated treatments with a non-genotoxic agent. A common feature is that initiation is considered irreversible, such that the promotion phase can be delayed significantly yet still ultimately result in tumour formation. Furthermore the tumour response may be absent or greatly reduced if the initiator is applied after the promoter, or if treatment is with either initiator or promoter alone. Although this model of initiation and promotion has served well as an experimental system for defining genotoxicity and for some studies of mechanisms of tumour formation, it appears to present an over-simplification of the process, particularly when considering the mechanism(s) of carcinogenesis in humans. Epidemiological evidence on the age distribution of many common cancers suggested that cancer induction is a multi-stage process, involving as many as 5-7 distinct events [1]. Although this conclusion was reached more than 50 years ago, before anything was known about the changes associated with malignancy were identified or understood, genetic analysis of tumours over the last 10 or more years has borne out this out. Current understanding is that the accumulation of a number of mutations (5-7 is a reasonable estimate) in critical genes in progenitor cells leads to the manifestation of the malignant phenotype.

Phenotypically, malignancy is characterised by six essential alterations in cell physiology: (*i*) self-sufficiency in growth signals, (*ii*) insensitivity to growth-inhibitory signals, (*iii*) evasion of programmed cell death (apoptosis), (*iv*) limitless replicative potential, (*v*) angiogenesis and (*vi*) tissue invasion and metastasis [2]. Although there is not yet an exact match between the genotypic

and phenotypic characteristics of tumours, it is logical to conclude that carcinogenesis is driven by the accumulation of critical mutations in cells that converts them and their progeny from normal cells to fully malignant ones. The identification of mutations in specific genes in human cancers has demonstrated that they are associated with both early and late stages of tumour progression [3]. Thus, while it is thought that DNA damage (often involving binding to DNA by carcinogens) occurs in the early, initiating stages of carcinogenesis, it is probable that genotoxic events are also a feature of later stages of the multistage process, now that it is apparent that gene mutation is associated with several stages of carcinogenesis.

Short-term tests for genotoxicity

In the context of short-term tests for mutagenicity and genotoxicity, a genotoxic agent is one that induces point mutations, deletions, insertions, gene amplifications, chromosomal rearrangements or numerical chromosomal changes (aneuploidy). The tests are therefore designed to detect one or more type of genetic alteration. Since such biological properties result directly or indirectly from DNA damage, other assays have been developed to identify this damage directly. No single assay, no matter how extensive the protocol, can detect all genotoxic chemicals [4]. Therefore, it is generally accepted that a number of tests must be conducted to evaluate whether a chemical is genotoxic or not, and often a weight-of-evidence approach must be taken to evaluate the results.

A number of organisations and advisory bodies have produced guidelines in the last ten years. These include the International Programme on Chemical Safety (IPCS), the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), and the International Workshop on Genotoxicity Testing (IWGT). These and other guidelines have been reviewed and compared by Cimino [5].

For a test to be useful it should be both sensitive and specific (see Table 1). Several guidelines recommend that a test battery for genotoxicity should include: 1) a test for gene mutation in bacteria; 2) an *in-vitro* test that includes cytogenetic evaluation of chromosomal damage in mammalian cells or an *in-vitro* mammalian cell mutagenicity test such as the mouse lymphoma *Tk* assay; 3) an *in-vivo* test for chromosomal damage using rodent haematopoietic cells. Some guidelines advise that negative results in both of the first two assays may in some cases (*e.g.* a low-volume chemical where the potential for human exposure is minimal) remove the necessity of conducting the third, *in-vivo*, test. More recently, however, the Seventh Amendment to the European Union Cosmetics Directive that will ban the marketing of cosmetics and personal care products containing ingredients tested in animals has highlighted the need for better *in-vitro* tests for toxicity and genotoxicity [6].

Bacterial mutagenicity testing

The most widely used bacterial assay to detect chemically-induced gene mutations is the Ames Salmonella assay developed by Bruce Ames [7; 8]. *Salmonella typhimurium* strains that contain defined mutations in the histidine locus form the basis of this 'reverse' mutation assay. In the assay, bacteria are incubated with a range of concentrations of the test compound to induce a second mutation that directly reverses or suppresses the original mutations and, thus, restores the biological function to the non-functional histidine gene. Strains of *S. typhimurium* used in the Ames assay are auxotrophic for histidine and revertants are selected by their ability to grow in the absence of this amino acid. Two of the most commonly used *S. typhimurium* strains are TA98 and TA100. *S. typhimurium* TA98 has a *hisD3052* mutation detecting frame-shift reversion events whereas *S. typhimurium* TA100 has a *hisG46* mutation detecting base-pair substitution events [9]. The great strength of the assay is the ability to identify and score a small number of mutants from a relative large population of unmutated cells. However, as bacteria lack many endogenous metabolic

pathways that are required for the bioactivation of the test chemicals, extracts of mammalian liver (usually rat) are incorporated as an exogenous activation system [9]. Fractionated tissue homogenate such as the 9000 *g* supernatant (S9 fraction), prepared from the livers of rats pretreated with Aroclor 1254, provides a rich source of mixed-function mono-oxygenases required for bioactivation.

The Ames assay can either be used to assess the mutagenic potency of a chemical as part of the toxicological screening, or else it can form part of a detailed mechanistic examination of the chemical's mutagenic potential. In the current ICH and OECD Guidelines, the use of 5 tester strains is recommended: TA98, TA100 and TA1535; TA1537 or TA97 or TA97a; TA100 (or alternatively one of several *Escherichia coli* WP2 strains). In order to make the assay more sensitive, these strains contain an *rfa* mutation resulting in defective lipopolysaccharide and increased permeability to large test molecules, or a deletion in the *uvrB* gene making the strains deficient in nucleotide excision repair. Some strains (*e.g. S. typhimurium* TA98 and TA100) include additionally a plasmid (pKM101) containing *umuDC* genes encoding for a translesion-synthesis DNA polymerase that elicits error-prone repair [10]. Using genetically-engineered *S. typhimurium* strains that either overexpress or lack enzymes required for the bioactivation of different carcinogens can provide useful information on their metabolism [11; 12]. Moreover, 'humanised' *S. typhimurium* strains with defined human enzymes have been developed in order to identify which human enzymes are involved in bioactivation and to improve the relevance of Ames Salmonella assay for detecting agents hazardous to humans [13].

Mammalian mutagenicity testing

The mouse lymphoma assay (MLA) is the most widely used mammalian gene mutation assay [14]. It detects various mutation events involving the thymidine kinase (*Tk*) gene in L5178Y/$Tk^{+/-}$ 3.6.2C mouse lymphoma cells [15; 16]. The gene coding for thymidine kinase is on

mouse chromosome 11 and allows the cell to salvage nucleotides from the culture medium for reuse in metabolism but is not essential for cell survival. Since eukaryotic cells are diploid, heterozygous cells are used where two copies of the *Tk* gene are present but one copy has been inactivated. Otherwise, many mutations arising in mammalian cells cannot be selected directly, since the second copy of the gene would complement the first. Mutants in the MLA are detected by plating cells into medium containing trifluorothymidine (TFT), a thymidine analogue [17]. Thus, toxic TFT placed in the medium will be transported into normal $Tk^{+/-}$ (non-mutated) cells that consequently die, while $Tk^{-/-}$ mutants will be resistant to the toxic TFT and survive, and subsequently form clones that can be counted. The L5178Y system is the recommended *in-vitro* mammalian cell mutation assay because it detects a wide range of genetic alterations, including both mutations and chromosomal damage [18].

Transgenic rodent mutation assays

Transgenic rodent mutation assays were first developed in the 1990s [19; 20], Muta™Mouse and Big Blue® Mouse and Rat being the assays most widely used [21; 22]. Muta™Mouse carry a recombinant λ-bacteriophage vector containing the entire *Escherichia coli lacZ* (β-galactosidase) [21]. Mutations occurring in the *lacZ* gene are measured by positive selection of $lacZ^-$ mutants on phenylgalactosidase (P-gal)-containing medium using an indicator bacteria strain (*E. coli* $lacZ^-$ $galE^-$). In the presence of P-gal only $lacZ^-$ bacteria (mutants) will grow and produce plaques, whereas $lacZ^+$ (*i.e.* non-mutants) produce the enzyme β-galactosidase converting P-gal into galactose and subsequently into the toxic intermediate uridine diphosphate (EDP)-galactose, which accumulates in *E. coli* $galE^-$ and kills the cells. In the Big Blue® system, the reporter gene is *lacI* contained in a λ-bacteriophage vector [22]. Mutants occurring in the *lacI* gene are selected on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)-containing medium using an *E. coli* $lacI^-$ indicator strain. Wild-type *lacI* (non-mutants) will repress the *lac* operon encoding for β-

galactosidase forming clear plaques whereas *lacI*⁻ mutants will produce β-galactosidase that uses X-gal as substrate producing blue coloured plaques that can be counted. Since the 'reporter' genes that serve as targets for detecting mutations are incorporated into the chromosomes of the transgenic mice or rats, somatic mutations can be measured within any tissue of the exposed animal, and more importantly enables mutation induction and measurement in the actual target tissue for tumour development. General guidance on recommended protocols has been published [23; 24].

The *lacI*/*lacZ* models are well suited to detect point mutations but unsuited for the detection of large deletion mutations induced by clastogens [19]. However, the coding size of, for instance, the *lacZ* gene is about 3 kb (kilo base pairs), which is not compact enough to routinely identify mutations by DNA sequencing. Thus, a new reporter gene, the *cII* gene of the λ phage, has been used and is applicable to both Muta™Mouse and Big Blue® Mouse systems [25]. The *cII* gene is susceptible to mutagenesis, just as is *lacI* or *lacZ*, but has the advantage over them that the coding region is only 300 base pairs and can easily be sequenced in a single run. An alternative transgenic mouse model, *gpt* delta, is reported to be suitable for the detection of large deletions [26].

Tests for chromosome damage

Structural chromosome changes that can be detected by conventional *in-vitro* cytogenetics are chromosome aberrations (CA), micronuclei (MN) and sister chromatid exchanges (SCE).

Structural CA are changes in normal chromosome structure or number that can occur in cells after chemical exposure or radiation. They result from direct DNA breakage, replication on a damaged DNA template, inhibition of DNA synthesis, and other mechanisms (*e.g.* inhibition of topoisomerase II) [27]. Cells commonly used to measure structural CA are human peripheral blood lymphocytes or established lymphoblastoid cell lines [28]. Peripheral lymphocytes are popular cells for *in-vitro* studies because they are human primary cells, have a low spontaneous rate of chromosomal damage, and can be easily cultured with a stable karyotype ($2n$=46). Structural CA

are generally scored in metaphase-arrested cells after Giemsa staining [29]. For over 30 years structural CA in human peripheral blood lymphocytes have been used in occupational and environmental settings as a biomarker of exposure and a marker of cancer risk [30; 31].

The *in-vitro* micronucleus test allows the detection of both structural (clastogenic) and numerical (aneugenic) chromosome changes using interphase cells [32]. Thus, MN represents a measure of both chromosome breakage and chromosome loss, and an increased frequency of micronucleated cells is a biomarker of genotoxic effects. MN formed by clastogenicity induction can be distinguished from those produced by aneugenic activity by the absence of centrometric DNA or kinetochore proteins in the MN using centrometric probes or kinetochore antibodies [33]. The standard *in-vitro* micronucleus test is usually performed in lymphocytes [34], the cytokinesis-block micronucleus assay being the most widely used method. This assay is specifically restricted to once-divided cells and these cells are recognised by their binucleated appearance after inhibition of cytokinesis by cytochalasin-B [35]. Restricting the scoring of MN to binucleate cells prevents confounding effects that can be a major variable in the assay. The use of MN as a measure of chromosomal damage has become a standard assay both in genotoxicity testing (although an OECD guideline protocol has yet to be adopted) and human biomonitoring studies [36; 37].

The rodent micronucleus test is a widely used and extensively validated assay to assess chromosome damage *in vivo* and has been incorporated into standard rodent toxicology screening assays [38; 39]. For the analysis, immature erythrocytes (*i.e.*, polychromatic erythrocytes; reticulocytes) in either bone marrow or peripheral blood have been found equally acceptable when the rodents have been exposed to the test compound by an appropriate route. A detailed description of the study design and experimental procedure has been published [39; 40].

Comet assay

The comet assay (or single-cell gel electrophoresis assay) is a simple and sensitive method for measuring alkali labile sites and DNA strand breaks in the DNA of mammalian cells [41]. In the assay, a small number of cells suspended in agar are lysed under alkaline conditions, subjected to electrophoresis, neutralised and stained with a fluorescent DNA dye, such as propidium iodide [42; 43]. Cells with increased DNA damage display increased migration of the chromosomal DNA from the nucleus resembling the shape of a 'comet'. Using image analysis software parameters such as percentage of DNA in the tail (percent migrated DNA), tail length, and tail moment (fraction of migrated DNA multiplied by some measure of tail length) can be determined as a measure of DNA damage. As an *in-vivo* genotoxicity assay, it has the advantage that it can be applied to a single-cell suspension of material from any animal tissue, allowing consideration of potential target tissues and also taking account of possible inaccessibility to exposure of tissues (*e.g.* bone marrow) required for other *in-vivo* assays.

Under alkaline conditions, the assay detects overt strand breaks, which can include single and double strand breaks, as well as transient repair-induced breaks. It also detects lesions that are alkali-labile, which includes AP (apurinic/apyrimidinic) sites (see below). More precise information on the nature of the lesions detected can be obtained by the inclusion of lesion-specific endonucleases in the assay protocol, which convert some types of DNA damage to strand breaks [44]. Formamidopyrimidine-DNA-glycosylase (FPG) has been used to detect oxidised DNA damage, principally 8-oxo-dGuo (7, 8-dihydro-8-oxo-2'-deoxyguanosine), with high sensitivity [45]. More recently, it has been reported that FPG also detects some types of alkylation damage, and that the human homologue of FPG, hOGG1, is a more specific endonuclease for oxidation products [46]. Another enzyme, endonuclease III, converts oxidised pyrimidines to strand breaks [44].

The comet assay is used in many studies to assess DNA damage and repair and has widespread application in genotoxicity testing *in vitro* and *in vivo* [47]. Since virtually any cell population or single-cell suspension from any tissue type can be used for analysis, the assay in widely used in environmental biomonitoring and human population monitoring [44; 48; 49].

Correlations of mutagenicity and carcinogenicity

Clearly a major purpose of conducting the foregoing assays is to be able to predict whether or not a chemical is a carcinogen without conducting a costly and time-consuming animal bioassay. The reliability of such tests, both in terms of the specificity and selectivity, is a matter of ongoing debate, subject to the continual accumulation of new data. Where a compound known to be carcinogenic is not detected as a mutagen or genotoxin in such assays, or where a mutagen (or genotoxin) has been found to be non-carcinogenic, some sort of explanation needs to be sought. A carcinogen may be non-mutagenic or non-genotoxic because its mechanism of action does not involve DNA damage (*i.e.* it is a non-genotoxic carcinogen that may, for example, act as a tumour promoter or by inhibiting DNA methylation). On the other hand it may be that mutagenic activity of the genotoxic carcinogen is limited to the chromosomal level, or that unusual metabolic activation for activity is required and that this is not achieved in the *in-vitro* test. The addition of external enzymatic activation systems (*e.g.* rat liver S9) may not be adequate for some compounds, particularly where phase II enzymes are required or where the half-life of the reactive species may be short or its cell permeability limited. Strategies to overcome these shortcomings include the use of human liver S9 [50] and the engineering of bacteria or mammalian cells to express human xenobiotic metabolising genes [13; 51], but these approaches are, as yet, research tools and not part of the regulatory armoury.

Non-carcinogens may test positive as mutagens if the activity in the test system is due to a metabolic pathway not exhibited *in vivo* or due to the absence of a competing detoxification

pathway or lack of DNA repair; or it may be that mutagenicity is limited to a particular type of genetic damage (*e.g.* aneuploidy). Alternatively it may be that mutagenicity is insufficient for carcinogenicity, which may require accompanying cell proliferation in the target tissue; or it may simply be that the *in-vivo* rodent models used for carcinogenicity testing may not be sufficiently sensitive for some weak mutagens.

As stated earlier, it is widely recognised that no single assay can detect all genotoxic carcinogens, hence the evolution of the standard battery of tests. But even then, there are some genotoxins that go undetected by all these assays [52].

DNA adducts formed by chemical carcinogens

Genotoxic chemical carcinogens are either directly or indirectly DNA reactive. Most chemical carcinogens are not chemically reactive as such, but undergo metabolic activation in mammalian cells to reactive intermediates that react with DNA (Table 2), hence the requirement for inclusion of metabolising enzymes in many *in-vitro* genotoxicity assay systems. Carcinogen-induced DNA damage can take several forms. It can result in breaks in the sugar-phosphate backbone of the molecule, either in one of the two strands of the double helix (forming single-strand breaks), or in both (causing double-strand breaks). Covalent binding of the carcinogen results in the formation of a chemically altered base (or, occasionally, phosphate group) in DNA that is termed an *adduct*. Formation of adducts at some positions of the DNA bases (for example at the N7 position of guanine) can render the base-sugar bond unstable and lead to loss of the adducted base (depurination or depyrimidination). The resulting modification to DNA is the formation of an AP site. Some carcinogens are bifunctional and can give rise to both monoadducts and crosslinks in DNA, the latter being either intrastrand or interstrand crosslinks. Many cancer chemotherapeutic agents have this property, and it is widely held that interstrand crosslinks are cytotoxic (accounting

for the therapeutic properties of the drugs), while the monoadducts and intrastrand crosslinks are potentially mutagenic and carcinogenic.

DNA adducts can also originate from endogenous processes, including normal metabolism, oxidative stress and chronic inflammation [53]. The most abundant oxidation lesion in DNA is 8-oxo-dGuo, which can be formed by free radical attack on DNA or through normal aerobic metabolism. It is suspected that some genotoxic carcinogens that do not appear to directly modify DNA instead damage it through inducing oxidative stress leading to increased oxidative damage to DNA.

In experimental studies where multiple doses of carcinogens have been administered to animals and both tumour outcome and DNA adduct levels have been determined, there has in general been found to be a linear relationship between dose and both these parameters at low dose, although deviations from linearity may be observed at higher doses [54]. Nevertheless the low-dose effects are more relevant to human exposure scenarios than the high-dose effects, which may be explained in part by the influence of toxicity.

Methods for adduct detection

A number of sensitive methods have been developed for the detection and characterisation of DNA adducts (Table 3) [54; 55]. For an assay to be applicable to human exposure, it must (*i*) be sensitive enough to detect low levels of adducts; (*ii*) require only microgram quantities of DNA; (*iii*) provide results quantitatively related to the exposure; (*iv*) be applicable to unknown adducts that may be formed from complex mixtures; and (*v*) be able to resolve, quantitate and identify adducts.

Most of the early work on adducts required the use of radiolabelled compounds (labelled either with ^{3}H or ^{14}C) at a position of the molecule where the isotope is not lost during metabolic activation and binding to DNA [55]. The DNA binding is then measured by the detection of

radioactivity in DNA isolated from exposed animals or cells in culture achieving sensitivities of detection of 1 adduct in 10^8 nucleotides with ^{3}H-labelling, although ^{14}C-labelling is less sensitive due to the lower specific activity of ^{14}C-labelled compounds compared with ^{3}H-labelled ones (a consequence of the much longer half-life of ^{14}C compared with that of ^{3}H) [55]. However, due to the highly radioactive test compounds it was not possible to use this approach in studies involving humans.

In 1981, the ^{32}P-postlabelling technique was developed [56-58]. The method comprises a 4-step process that involves (i) DNA digestion, (ii) a procedure that isolates or selects the adducts for preferential labelling, (iii) the introduction of a radiolabel into the DNA adducts using enzymatic [^{32}P]phosphorylation of the nucleotide adduct and (iv) separation of the ^{32}P-labelled adducts using thin layer (TLC) or high performance liquid chromatography (HPLC) [59; 60]. The assay requires only small (1–10 μg) quantities of DNA and is capable of detecting adducts at frequencies as low as 1 adduct in 10^{10} nucleotides, making it widely applicable in human biomonitoring [61]. It can be used for a wide variety of classes of compounds, including polycyclic aromatic hydrocarbons (PAHs), aromatic and heterocyclic amines, unsaturated aldehydes, simple alkylating agents, reactive oxygen species, ultraviolet light (UV) radiation, and for the detection of adducts formed by complex mixtures [54; 62]. A limitation of the method is that it does not provide structural information; identification of adducts is reliant on co-chromatography using characterised synthetic standards [59]. A different approach using a similar experimental protocol is the chemical linkage of a fluorescent dye (*e.g.* BODIPY) to the DNA adducts, which can subsequently be separated by capillary electrophoresis and detected by laser-induced fluorescence (CE-LIF) [63]. Although this methodology is not yet sensitive enough to be applied to human samples (detection limit 1 adduct per 10^7 nucleotides) [63; 64], it has proved to be a suitable technique to determine global DNA methylation levels [65].

Mass spectrometry (MS) coupled with liquid-chromatography-electrospray ionisation spectrometry (ESI-LC-MS) is becoming used increasingly for the detection of DNA adducts providing unequivocal identification of the nature of an adduct [55; 66; 67]. The sensitivities achieved are normally lower than with ^{32}P-postlabelling but, with the detection of 1 adduct per 10^8 to 10^9 nucleotides using 50–100 μg of DNA, they are sufficient to give useful data on human environmental or dietary exposures [67]. Accurate quantitation of DNA adduct levels is achieved by the use of a stable isotope internal standard (*e.g.* labelled either with ^{15}N or ^{13}C). Although mostly applied to the detection of specific well-characterised lesions, more recent techniques allow for the simultaneous detection of multiple adducts, and this 'adductome' approach has potential for the detection and characterisation of DNA adducts in human tissues [68]. Accelerator mass spectrometry (AMS), which measures isotope ratios, represents the most sensitive analytical method so far for detecting DNA adducts, with limits of adduct detection as low as 1 adduct in 10^{11} nucleotides [66; 69]. The main limitation of the technique is that it depends on the presence of an isotope such as ^{14}C or ^{3}H in the compound of interest. However, because of the high sensitivity of AMS, it has been possible to obtain ethical approval to give minute amounts of a radioactive carcinogen, for example, 2-amino-1-methyl-6-phenylimidazo[4, 5-*b*]pyridine (PhIP) or tamoxifen, to human individuals prior to surgery and to detect DNA adducts in the excised tissue [70; 71].

Other physicochemical methods for the detection of DNA adducts are based on the fact that some adducts are highly fluorescent, enabling their detection by fluorescence spectroscopy [55; 72]. Combining the fluorescent characteristics (specific excitation and emission wavelengths) with HPLC separation techniques make it even possible to detect stereoisomers. Adducts with fluorescent properties include those formed by PAHs and aflatoxins, cyclic (etheno) adducts, and some methylated adducts [72]. Other adducts, notably 8-oxo-dGuo, are readily detected by HPLC coupled with electrochemical detection (ECD) [73]. All these methods can provide a sensitivity of detection of around 1 adduct per 10^8 nucleotides, while requiring relative large quantities (100–

1000 μg) of sample DNA. Overall, the major limitations of these methods are the required spectral (*e.g.* intrinsic fluorescence) and physicochemical properties of the adducts.

Immunoassays have also been used for the detection of DNA adducts in human and experimental samples [74; 75]. Antibodies have been raised against a variety of carcinogen-modified DNAs, including those containing adducts of PAHs, aromatic amines, methylating agents, tamoxifen, UV radiation, and oxidative damage. Immunoassays are highly sensitive, but have generally been less sensitive than ^{32}P-postlabelling and usually require more DNA for analysis, although some recent developments have both increased sensitivity and reduced the amount of DNA required, improving the sensitivity to a level closer to that of ^{32}P-postlabelling [76]. When combined with histochemistry, cell-specific localisation of adducts in paraffin-embedded tissue is possible [77; 78]. However, antibodies can show cross-reactivity with adducts formed by the same class of compounds, which can obscure both the nature of the adducts and the levels at which they are present.

Biological significance of DNA adducts

While it is evident that DNA damage and binding by carcinogens occurs in the early, initiating stages of carcinogenesis, it has become increasingly clear that damage to DNA is also a feature of later stages of the multistage process, now that it is known that mutations in some genes are associated with later stages of progression of some types of tumour. It is also evident that the formation of DNA adducts is by no means a sufficient event for carcinogenesis, , as DNA adducts are frequently detectable in both target and non-target tissues. Nevertheless, inhibition of DNA adduct formation will decrease the incidence of tumours formed subsequently, and increasing the adduct levels generally leads to a higher tumour yield. Other evidence that strongly links DNA adduct formation to tumour initiation is the demonstration that XPA knockout mice, which are deficient in nucleotide excision repair, are highly sensitive to tumour induction by carcinogens that

form stable adducts that would be removed from DNA in normal mice by this repair mechanism [79].

That chemical modification of DNA can result in the same alterations as observed in mutated genes in tumours was observed with the H-*ras*-1 proto-oncogene transfected into NIH3T3 cells [80]. Prior modification (by reaction with benzo[a]pyrene diol-epoxide, BPDE, the reactive metabolite of benzo[a]pyrene; see Figure 1) of the plasmid containing the gene resulted in mutations occurring in the DNA after transfection and replication of the host cells, manifested as the appearance of transformed foci. Mutations that activate proto-oncogenes such as *ras* genes occur in a few codons in the gene, so correlations between the sites of mutations in such experiments may not be very informative. In contrast, for tumour suppressor genes there may be many possible sites of DNA damage and mutation that can lead to altered function of the gene product that contributes to malignant transformation. Such a gene is *TP53*, which has been found to be mutated in ~50% of human tumours. Correlations can be usefully sought between the mutation spectra observed in different human tumours in order to provide clues to the nature of the initiating agent(s) [81]. This approach has led to evidence for the involvement of the mycotoxin aflatoxin B_1 in the initiation of liver cancer in regions of high incidence in China, where a G→T transversion in codon 249 of *TP53* is a common mutation in the disease [82]. In lung cancer, codons 157, 248 and 273 of the gene are frequently mutated; G→T transversions are much more common in cases of lung cancer among smokers than among non-smokers, and these types of mutation are characteristic of bulky carcinogens, such as the PAHs, which are present in tobacco smoke. When the sites of DNA adduct formation by BPDE in the *TP53* gene in HeLa cells and bronchial epithelial cells were determined, it was found that codons 157, 248 and 273 were preferentially modified, correlating with the frequently mutated sites in lung tumours of smokers [83].

UV causes DNA damage chiefly by dimerisation of adjacent pyrimidines in the same DNA strand. The biological importance of these lesions is illustrated by the fact that sufferers of

Xeroderma Pigmentosum (XP), who have a deficiency in nucleotide excision repair mechanisms that remove pyrimidine dimers and other bulky adducts from DNA (also deficient in XPA knock-out mice mentioned above), are prone to sunlight-induced skin cancer. Moreover, the type of *TP53* mutation found commonly in such tumours, but rarely in tumours of internal organs, is a tandem mutation occurring at pyrimidine pairs (CC→TT transitions), highly suggestive that it arose from UV-induced pyrimidine dimers [84].

Thus, there are examples of genetic changes in tumours that closely match the genetic changes that can be induced experimentally in cellular DNA by specific genotoxic agents. These tumour-specific mutations in *TP53* and the demonstration that chemically-modified DNA transforms cells show that the mutations observed in human tumours could have arisen from the formation of carcinogen-DNA adducts *in vivo*. Clonal expansion of the mutated cells and the acquisition of further genetic alterations eventually leads to malignancy [3].

Adducts as biomarkers of occupational and environmental exposure to carcinogens

Sensitive DNA adduct detection methods, not requiring the use of radiolabelled carcinogens, make it possible to monitor DNA isolated from human tissues for evidence of prior exposure to carcinogens. Many different tissues have provided DNA for such studies [85], including blood, sputum, buccal mucosa, cervical mucosa, sperm, bladder (exfoliated urothelial cells in urine), placenta and hair roots. DNA from these accessible sources of human cells have been used in many studies, but by far the most commonly used tissue source has been blood cells (either lymphocytes or the whole fraction of nucleated white blood cells). While these are not target cells for malignancy, they are useful surrogates and are known to display evidence of genotoxic exposure using other, less sensitive, endpoints, such as micronucleus formation, chromosomal aberrations and mutation in reporter genes such as *HPRT*.

Heavy industries where an increased risk of lung and other cancers has been observed include iron and steel production, aluminium production, coke ovens and graphite electrode manufacture. The principal genotoxic exposure in these industries is to PAHs. Many studies have investigated DNA adduct formation in workers in these industries, using white blood cells as the monitored tissue. In general, the results of such studies have been the demonstration of statistically significant increases in the level of DNA adducts in the exposed workers, compared with controls [86]. Other industrial workforces studied, with similar results, include roofers, chimney sweeps, incinerator workers, petrol refinery workers, traffic police and bus maintenance workers [86].

DNA adduct detection can also be used to investigate environmental exposure to genotoxic carcinogens [86]. For example, chronic environmental exposure to industrial sources of carcinogens has occurred in Upper Silesia and the Krakow region of Poland, and in Northern Bohemia in the Czech Republic. Studies on these populations have revealed significantly elevated levels of DNA adducts in blood cells compared with control populations from rural areas of the same countries. In Xuan Wei province of China, the practice of using smoky coal for cooking and heating in unventilated houses leads to a high level of smoke indoors and high incidences of lung cancer, particularly in the women (very few of whom smoke tobacco). Placental, blood and lung (from bronchoalveolar lavage) cells have all been used as sources of DNA to compare exposed female residents of Xuan Wei with a control group from Beijing, and in each case evidence for elevated levels of adducts was obtained [87]. In Henan province of China, there is an exceptionally high prevalence of oesophageal cancer and, among several suspected environmental factors, the high content of PAHs in the diet has recently become of interest; evidence that this may play a role in the aetiology of the disease is supported by the observation of high levels of PAH-DNA adducts, detected by immunohistochemistry, in archived surgical specimens of oesophagi from the region [88].

A recent example of the identification of a human carcinogen is aristolochic acid (AA), a constituent of plants of the genus *Aristolochia.* AA is genotoxic, being positive in many short-term tests, and forms covalent DNA adducts in tissues of rodents, and in human cells in culture [89]. An outbreak of renal failure, followed by urothelial cancer in some of the patients, occurred among individuals in Belgium who took a slimming regimen containing Chinese herbs, one of which turned out to be an *Aristolochia* species. ^{32}P-Postlabelling analysis of DNA from the tissues of these patients revealed the presence of AA-DNA adducts, implicating the compound as the genotoxic agent involved in the carcinogenic process leading to urothelial tumours [90]. The renal disease, now known as Aristolochic acid nephropathy (AAN), is pathologically similar to Balkan endemic nephropathy (BEN), in which AA is also implicated [91]. The source is thought to *Aristolochia clematitis,* which grows wild in the Balkans and whose seeds may contaminate wheat flour in the region. The detection of AA-DNA adducts in renal tissues from BEN sufferers provides strong evidence for the involvement of AA in the aetiology of the disease [92]. Furthermore, analysis of *TP53* mutations in BEN tumours, and in one AAN tumour, shows a preponderance of AT-TA transversion mutations, which is the predominant mutation that AA causes in experimental studies [92] [93].

The relationship between DNA adduct formation and tobacco smoking has been widely studied and used to validate the biomarker (see below). Tobacco smoke contains at least 50 compounds that are known to be carcinogenic, including representatives of several distinct classes of compounds (PAHs, aromatic amines, *N*-nitrosamines, aza-arenes, aldehydes, other organic compounds and inorganic compounds). Most of these compounds are genotoxic carcinogens that form DNA adducts. In many studies that have compared DNA from smokers, ex-smokers and non-smokers, higher levels of adducts have been found in many target tissues of smokers: lung, bronchus, larynx, bladder, cervix and oral mucosa [94]. In some of these studies a linear correlation between estimated tobacco smoke exposure and adduct levels has been observed. In tissues of the

respiratory tract adduct levels in ex-smokers tend to be intermediate between smokers and non-smokers, indicating that adducts are removed through DNA repair and/or cell turnover. The half-life of adduct persistence appears to be between one and two years.

For some of these studies specific adducts have been detected, but in others a more general measure of DNA damage has been made, namely aromatic/hydrophobic adducts detected by ^{32}P-postlabelling, or PAH-DNA adducts detected by immunoassay. Recent studies have found that when adduct levels are adjusted to take account of the level of tobacco smoke exposure, lung DNA from women smokers is more highly adducted that that of male smokers. This finding is interesting in view of epidemiological evidence suggesting that women are at a 1.5-2-fold greater risk of lung cancer from smoking. It would appear that the adduct analysis provides biochemical, mechanistic evidence to support the morbidity data [95].

Some, but not all, studies have shown elevated levels of lung adducts in cancer cases compared with controls. The relationship between adduct levels in target tissues (*e.g.* lung) and other tissues (*e.g.* blood) has been investigated to see whether the latter can serve as a valid accessible surrogate source of DNA for the former. Results for smoking-related adducts have been inconsistent [54; 96], perhaps because other sources of exposure to some classes of carcinogens, such as PAHs, which are also ingested as dietary contaminants, may contribute to the overall level of adducts in the blood but not to the same extent in the lung.

DNA adducts in prospective studies

When measuring adducts in smokers at the time of cancer diagnosis (e.g. in case-control studies), investigators are not looking at the biochemical events causal in the initiation of those tumours, as these would have occurred decades earlier. However, because smoking is addictive and habitual for the vast majority of tobacco users, DNA adducts in tumour-adjacent tissue at the time of tumour manifestation can still serve as a useful biomarker that gives an indication of an

individual's probable steady-state level of DNA damage maintained over a long period of time. In order to determine whether DNA adducts have predictive value in cancer risk, it is necessary to conduct prospective studies in which DNA samples are collected and stored from a large cohort of individuals who are then followed up to determine who does and who does not develop cancer in the future. It is then possible to perform a nested case-control study within the cohort to determine whether DNA adduct analysis of the stored samples reveals whether differences between the two groups were evident prior to the onset of disease.

The first example of this approach was a study conducted in Shanghai, China, where a high incidence of liver cancer is associated with dietary exposure to aflatoxin B_1, 18, 244 men provided a single urine sample and provided detailed dietary questionnaire data, in addition to which food analyses were carried out [97]. When 55 cases of liver cancer subsequently arose in the cohort, these were matched to 267 disease-free controls and their urine samples analysed by HPLC-fluorescence to detect the presence of aflatoxin derivatives. A significant association was found between the presence of aflatoxin metabolites, including the aflatoxin-N^7-guanine adduct, and liver cancer. Interestingly, when data obtained from questionnaires and food analyses were considered without the biomarker data, no association between exposure and liver cancer was evident. Thus in this case the power of biomarkers of exposure showed a clear advantage over more traditional means of exposure assessment to show a causal association.

The ability of DNA adducts to predict lung cancer risk was investigated in tobacco smokers [98]. From a follow-up of a cohort of 15, 700 males who had provided blood samples at the outset of the study, 93 cases of lung cancer were identified and matched to 173 controls. Analysis of white blood cell DNA by ^{32}P-postlabelling revealed that smokers who got lung cancer had 2-fold higher levels of bulky/hydrophobic DNA adducts than smokers who did not. The smokers who had elevated levels of adducts were approximately three times more likely to be diagnosed with lung cancer 1-13 years later than the smokers with lower adduct concentrations.

The predictive power of DNA adducts to distinguish groups of individuals who developed cancer from those who did not was also investigated in two recent studies that measured bulky DNA adducts in leukocytes by ^{32}P-postlabelling analysis. In the first, 115 cases of lung cancer were matched with twice the number of controls from European cohorts totalling more than 500, 000 people [99]. Detectable DNA adducts were significantly more common in non-smokers and long-term ex-smokers who developed lung cancer than in those who did not. The second study investigated 245 individuals with lung cancer and 255 without, from a population-based cohort of 53, 689 men and women [100]. The median level of DNA adducts was significantly higher for smokers who developed lung cancer than for those that did not. Although adduct levels were statistically significantly higher in the cases in both these studies, the numerical differences from the controls was somewhat small. Thus the ability to predict cancer risk from DNA adduct measurements on an individual basis will be very limited, despite the collective differences between the cases and the controls. Nevertheless, DNA adduct analysis should have applications in investigating the efficacy of chemoprevention strategies by, for example, documenting a reduction in adduct levels concomitant with a reduction in cancer risk in interventions in an occupationally- or environmentally-exposed population.

Summary

DNA adduct formation, or the causation of DNA damage by less direct means, is an important property of genotoxic agents. The strategies that have been developed for determining the carcinogenic potential of chemicals, using short-term tests, are based on detecting evidence of either DNA damage or its biological consequences. Although it is well recognised that the carcinogenic activity of some chemicals is the result of non-genotoxic mechanisms, the majority of known human carcinogens are genotoxic. Early studies on DNA adducts required use of radioactively labelled compounds, but alternative methods with a high degree of sensitivity and selectivity have

since been developed, enabling their wider application, including the monitoring of human exposure to environmental carcinogens and in providing clues to the aetiology of some cancers. Experimental interventions that reduce DNA adduct formation also reduce carcinogenicity, while enhancing DNA adduct formation has the opposite effect. In prospective studies, elevated DNA adducts have been found in individuals who subsequently developed cancer relative to those who did not. Continuing research into the detection and characterisation of DNA adducts in human tissues will shed further light on the causative agents of human cancers.

Acknowledgements

We thank Dr. Stan Venitt for critical reading of the manuscript. The work of the authors is supported by Cancer Research UK.

References

1 Armitage P, Doll R (1954) The age distribution of cancer and a multi-stage theory of carcinogenesis. *Br J Cancer* 8**:** 1-12.

2 Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100**:** 57-70.

3 Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell* 61**:** 759-767.

4 Kirkland DJ, Hayashi M, MacGregor JT, Muller L, Schechtman LM, Sofuni T (2003) Summary of major conclusions. *Mutat Res* 540**:** 123-125.

5 Cimino MC (2006) Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes. *Environ Mol Mutagen* 47**:** 362-390.

6 Tweats DJ, Scott AD, Westmoreland C, Carmichael PL (2007) Determination of genetic toxicity and potential carcinogenicity in vitro--challenges post the Seventh Amendment to the European Cosmetics Directive. *Mutagenesis* 22**:** 5-13.

7 Ames BN, Durston WE, Yamasaki E, Lee FD (1973) Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc Natl Acad Sci U S A* 70**:** 2281-2285.

8 Maron DM, Ames BN (1983) Revised methods for the Salmonella mutagenicity test. *Mutat Res* 113**:** 173-215.

9 Mortelmans K, Zeiger E (2000) The Ames Salmonella/microsome mutagenicity assay. *Mutat Res* 455**:** 29-60.

10 Mortelmans K (2006) Isolation of plasmid pKM101 in the Stocker laboratory. *Mutat Res* 612**:** 151-164.

11 Einisto P, Watanabe M, Ishidate M, Jr., Nohmi T (1991) Mutagenicity of 30 chemicals in Salmonella typhimurium strains possessing different nitroreductase or O-acetyltransferase activities. *Mutat Res* 259**:** 95-102.

12 Yamada M, Espinosa-Aguirre JJ, Watanabe M, Matsui K, Sofuni T, Nohmi T (1997) Targeted disruption of the gene encoding the classical nitroreductase enzyme in Salmonella typhimurium Ames test strains TA1535 and TA1538. *Mutat Res* 375**:** 9-17.

13 Glatt H, Meinl W (2005) Sulfotransferases and acetyltransferases in mutagenicity testing: technical aspects. *Methods Enzymol* 400**:** 230-249.

14 Clements J (2000) The mouse lymphoma assay. *Mutat Res* 455**:** 97-110.

15 Clive D, Flamm WG, Machesko MR, Bernheim NJ (1972) A mutational assay system using the thymidine kinase locus in mouse lymphoma cells. *Mutat Res* 16**:** 77-87.

16 Clive D, Johnson KO, Spector JF, Batson AG, Brown MM (1979) Validation and characterization of the L5178Y/TK+/- mouse lymphoma mutagen assay system. *Mutat Res* 59**:** 61-108.

17 Moore MM, Clive D, Hozier JC, Howard BE, Batson AG, Turner NT, Sawyer J (1985) Analysis of trifluorothymidine-resistant (TFTr) mutants of L5178Y/TK+/- mouse lymphoma cells. *Mutat Res* 151**:** 161-174.

18 Moore MM, Honma M, Clements J, Bolcsfoldi G, Burlinson B, Cifone M, Clarke J, Clay P, Doppalapudi R, Fellows M, Gollapudi B, Hou S, Jenkinson P, Muster W, Pant K, Kidd DA, Lorge E, Lloyd M, Myhr B, O'Donovan M, Riach C, Stankowski LF Jr., Thakur AK, Van Goethem F (2007) Mouse lymphoma thymidine kinase gene mutation assay: meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment. *Mutat Res* 627**:** 36-40.

19 Nohmi T, Suzuki T, Masumura K (2000) Recent advances in the protocols of transgenic mouse mutation assays. *Mutat Res* 455**:** 191-215.

20 Lambert IB, Singer TM, Boucher SE, Douglas GR (2005) Detailed review of transgenic rodent mutation assays. *Mutat Res* 590**:** 1-280.

21 Gossen JA, de Leeuw WJ, Tan CH, Zwarthoff EC, Berends F, Lohman PH, Knook DL, Vijg J (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations in vivo. *Proc Natl Acad Sci U S A* 86**:** 7971-7975.

22 Kohler SW, Provost GS, Fieck A, Kretz PL, Bullock WO, Sorge JA, Putman DL, Short JM (1991) Spectra of spontaneous and mutagen-induced mutations in the lacI gene in transgenic mice. *Proc Natl Acad Sci U S A* 88**:** 7958-7962.

23 Heddle JA, Dean S, Nohmi T, Boerrigter M, Casciano D, Douglas GR, Glickman BW, Gorelick NJ, Mirsalis JC, Martus HJ, Skopek TR, Thybaud V, Tindall KR, Yajima N (2000) In vivo transgenic mutation assays. *Environ Mol Mutagen* 35**:** 253-259.

24 Thybaud V, Dean S, Nohmi T, de Boer J, Douglas GR, Glickman BW, Gorelick NJ, Heddle JA, Heflich RH, Lambert I, Martus HJ, Mirsalis JC, Suzuki T, Yajima N (2003) In vivo transgenic mutation assays. *Mutat Res* 540**:** 141-151.

25 Jakubczak JL, Merlino G, French JE, Muller WJ, Paul B, Adhya S, Garges S (1996) Analysis of genetic instability during mammary tumor progression using a novel selection-based assay for in vivo mutations in a bacteriophage lambda transgene target. *Proc Natl Acad Sci U S A* 93**:** 9073-9078.

26 Nohmi T, Katoh M, Suzuki H, Matsui M, Yamada M, Watanabe M, Suzuki M, Horiya N, Ueda O, Shibuya T, Ikeda H, Sofuni T (1996) A new transgenic mouse mutagenesis test system using Spi- and 6-thioguanine selections. *Environ Mol Mutagen* 28**:** 465-470.

27 Albertini RJ, Anderson D, Douglas GR, Hagmar L, Hemminki K, Merlo F, Natarajan AT, Norppa H, Shuker DE, Tice R, Waters MD, Aitio A (2000) IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety. *Mutat Res* 463**:** 111-172.

28 Parry EM, Parry JM (1995) In vitro cytogenetics and aneuploidy, in: *Environmental Mutagenesis* (Phillips DH, Venitt S eds), pp 121-139, BIOS Scientific Publishers, Oxford.

29 Dean BJ, Danford N (1984) Assays for the detection of chemically-induced chromosome damage in cultured mammalian cells, in: *Mutagenicity testing - a practical approach* (Venitt S, Parry JM eds), pp 187-232, IRL Press, Oxford.

30 Bonassi S, Ugolini D, Kirsch-Volders M, Stromberg U, Vermeulen R, Tucker JD (2005) Human population studies with cytogenetic biomarkers: review of the literature and future prospectives. *Environ Mol Mutagen* 45**:** 258-270.

31 Norppa H, Bonassi S, Hansteen IL, Hagmar L, Stromberg U, Rossner P, Boffetta P, Lindholm C, Gundy S, Lazutka J, Cebulska-Wasilewska A, Fabianova E, Sram RJ, Knudsen LE, Barale R, Fucic A (2006) Chromosomal aberrations and SCEs as biomarkers of cancer risk. *Mutat Res* 600**:** 37-45.

32 Fenech M, Morley AA (1985) Measurement of micronuclei in lymphocytes. *Mutat Res* 147**:** 29-36; Kirsch-Volders M, Vanhauwaert A, De Boeck M, Decordier I (2002) Importance of detecting numerical versus structural chromosome aberrations. *Mutat Res* 504**:** 137-148.

33 Eastmond DA, Pinkel D (1990) Detection of aneuploidy and aneuploidy-inducing agents in human lymphocytes using fluorescence in situ hybridization with chromosome-specific DNA probes. *Mutat Res* 234**:** 303-318.

34 Van Hummelen P, Kirsch-Volders M (1990) An improved method for the 'in vitro' micronucleus test using human lymphocytes. *Mutagenesis* 5**:** 203-204.

35 Fenech M (2006) Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. *Mutat Res* 600**:** 58-66.

36 Decordier I, Kirsch-Volders M (2006) The in vitro micronucleus test: from past to future. *Mutat Res* 607**:** 2-4.

37 Corvi R, Albertini S, Hartung T, Hoffmann S, Maurici D, Pfuhler S, van Benthem J, Vanparys P (2008) ECVAM retrospective validation of in vitro micronucleus test (MNT). *Mutagenesis*.

38 Hayashi M, Tice RR, MacGregor JT, Anderson D, Blakey DH, Kirsh-Volders M, Oleson FB, Jr., Pacchierotti F, Romagna F, Shimada H, Sutou S, Vannier B (1994) In vivo rodent erythrocyte micronucleus assay. *Mutat Res* 312**:** 293-304.

39 Hayashi M, MacGregor JT, Gatehouse DG, Adler ID, Blakey DH, Dertinger SD, Krishna G, Morita T, Russo A, Sutou S (2000) In vivo rodent erythrocyte micronucleus assay. II. Some aspects of protocol design including repeated treatments, integration with toxicity testing, and automated scoring. *Environ Mol Mutagen* 35**:** 234-252.

40 Hayashi M, Tice RR, MacGregor JT, Anderson D, Blakey DH, Kirsh-Volders M, Oleson FB, Jr., Pacchierotti F, Romagna F, Shimada H, et al. (1994) In vivo rodent erythrocyte micronucleus assay. *Mutat Res* 312**:** 293-304.

41 Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175**:** 184-191.

42 Speit G, Hartmann A (2005) The comet assay: a sensitive genotoxicity test for the detection of DNA damage. *Methods Mol Biol* 291**:** 85-95.

43 Olive PL, Banath JP (2006) The comet assay: a method to measure DNA damage in individual cells. *Nat Protoc* 1**:** 23-29.

44 Collins AR, Oscoz AA, Brunborg G, Gaivao I, Giovannelli L, Kruszewski M, Smith CC, Stetina R (2008) The comet assay: topical issues. *Mutagenesis* 23**:** 143-151.

45 Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol* 26**:** 249-261.

46 Smith CC, O'Donovan MR, Martin EA (2006) hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. *Mutagenesis* 21**:** 185-190.

47 Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 35**:** 206-221.

48 Moller P, Knudsen LE, Loft S, Wallin H (2000) The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. *Cancer Epidemiol Biomarkers Prev* 9**:** 1005-1015.

49 Jha AN (2008) Ecotoxicological applications and significance of the comet assay. *Mutagenesis* 23**:** 207-221.

50 Hakura A, Shimada H, Nakajima M, Sui H, Kitamoto S, Suzuki S, Satoh T (2005) Salmonella/human S9 mutagenicity test: a collaborative study with 58 compounds. *Mutagenesis* 20**:** 217-228.

51 Doehmer J (2006) Predicting drug metabolism-dependent toxicity for humans with a genetically engineered cell battery. *Altern Lab Anim* 34**:** 561-575.

52 Brambilla G, Martelli A (2004) Failure of the standard battery of short-term tests in detecting some rodent and human genotoxic carcinogens. *Toxicology* 196**:** 1-19.

53 Marnett LJ (2000) Oxyradicals and DNA damage. *Carcinogenesis* 21**:** 361-370.

54 Poirier MC, Santella RM, Weston A (2000) Carcinogen macromolecular adducts and their measurement. *Carcinogenesis* 21**:** 353-359.

55 Phillips DH, Farmer PB, Beland FA, Nath RG, Poirier MC, Reddy MV, Turteltaub KW (2000) Methods of DNA adduct determination and their application to testing compounds for genotoxicity. *Environ Mol Mutagen* 35**:** 222-233.

56 Randerath K, Reddy MV, Gupta RC (1981) 32P-labeling test for DNA damage. *Proc Natl Acad Sci U S A* 78**:** 6126-6129.

57 Gupta RC, Reddy MV, Randerath K (1982) 32P-postlabeling analysis of non-radioactive aromatic carcinogen--DNA adducts. *Carcinogenesis* 3**:** 1081-1092.

58 Gupta RC (1985) Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen:DNA adducts. *Cancer Res.* 45**:** 5656-5662.

59 Phillips DH, Arlt VM (2007) The 32P-postlabeling assay for DNA adducts. *Nat Protoc* 2**:** 2772-2781.

60 Phillips DH, Castegnaro M (1999) Standardization and validation of DNA adduct postlabelling methods: report of interlaboratory trials and production of recommended protocols. *Mutagenesis* 14**:** 301-315.

61 Phillips DH (1997) Detection of DNA modifications by the 32P-postlabelling assay. *Mutat Res* 378**:** 1-12.

62 Beach AC, Gupta RC (1992) Human biomonitoring and the 32P-postlabeling assay. *Carcinogenesis* 13**:** 1053-1074.

63 Schmitz OJ, Worth CC, Stach D, Wiessler M (2002) Capillary electrophoresis analysis of DNA adducts as biomarkers for carcinogenesis. *Angew Chem Int Ed Engl* 41**:** 445-448.

64 Wirtz M, Schumann CA, Schellentrager M, Gab S, Vom Brocke J, Podeschwa MA, Altenbach HJ, Oscier D, Schmitz OJ (2005) Capillary electrophoresis-laser induced fluorescence analysis of endogenous damage in mitochondrial and genomic DNA. *Electrophoresis* 26**:** 2599-2607.

65 Stach D, Schmitz OJ, Stilgenbauer S, Benner A, Dohner H, Wiessler M, Lyko F (2003) Capillary electrophoretic analysis of genomic DNA methylation levels. *Nucleic Acids Res* 31**:** E2.

66 Farmer PB, Brown K, Tompkins E, Emms VL, Jones DJ, Singh R, Phillips DH (2005) DNA adducts: Mass spectrometry methods and future prospects. *Toxicol Appl Pharmacol* 207**:** 293-301.

67 Singh R, Farmer PB (2006) Liquid chromatography-electrospray ionization-mass spectrometry: the future of DNA adduct detection. *Carcinogenesis* 27**:** 178-196.

68 Kanaly RA, Hanaoka T, Sugimura H, Toda H, Matsui S, Matsuda T (2006) Development of the adductome approach to detect DNA damage in humans. *Antioxid Redox Signal* 8**:** 993-1001.

69 White IN, Brown K (2004) Techniques: the application of accelerator mass spectrometry to pharmacology and toxicology. *Trend Pharmacol Sci* 25**:** 442-447.

70 Dingley KH, Curtis KD, Nowell S, Felton JS, Lang NP, Turteltaub KW (1999) DNA and protein adduct formation in the colon and blood of humans after exposure to a dietary-relevant dose of 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine. *Cancer Epidemiol Biomarkers Prev* 8**:** 507-512.

71 Brown K, Tompkins EM, Boocock DJ, Martin EA, Farmer PB, Turteltaub KW, Ubick E, Hemingway D, Horner-Glister E, White IN (2007) Tamoxifen forms DNA adducts in human colon after administration of a single [14C]-labeled therapeutic dose. *Cancer Res* 67**:** 6995-7002.

72 Weston A (1993) Physical methods for the detection of carcinogen-DNA adducts in humans. *Mutat Res* 288**:** 19-29.

73 ESCODD (2003) Measurement of DNA oxidation in human cells by chromatographic and enzymic methods. *Free Radic Biol Med* 34**:** 1089-1099.

74 Poirier MC (1994) Human exposure monitoring, dosimetry, and cancer risk assessment: the use of antisera specific for carcinogen-DNA adducts and carcinogen-modified DNA. *Drug Metab Rev* 26**:** 87-109.

75 Santella RM (1999) Immunological methods for detection of carcinogen-DNA damage in humans. *Cancer Epidemiol Biomarkers Prev* 8**:** 733-739.

76 Divi RL, Beland FA, Fu PP, Von Tungeln LS, Schoket B, Camara JE, Ghei M, Rothman N, Sinha R, Poirier MC (2002) Highly sensitive chemiluminescence immunoassay for

benzo[a]pyrene-DNA adducts: validation by comparison with other methods, and use in human biomonitoring. *Carcinogenesis* 23**:** 2043-2049.

77 van Gijssel HE, Divi RL, Olivero OA, Roth MJ, Wang GQ, Dawsey SM, Albert PS, Qiao YL, Taylor PR, Dong ZW, Schrager JA, Kleiner DE, Poirier MC (2002) Semiquantitation of polycyclic aromatic hydrocarbon-DNA adducts in human esophagus by immunohistochemistry and the automated cellular imaging system. *Cancer Epidemiol Biomarkers Prev* 11**:** 1622-1629.

78 Pratt MM, Sirajuddin P, Poirier MC, Schiffman M, Glass AG, Scott DR, Rush BB, Olivero OA, Castle PE (2007) Polycyclic aromatic hydrocarbon-DNA adducts in cervix of women infected with carcinogenic human papillomavirus types: an immunohistochemistry study. *Mutat Res* 624**:** 114-123.

79 van Steeg H, Klein H, Beems RB, van Kreijl CF (1998) Use of DNA repair-deficient XPA transgenic mice in short-term carcinogenicity testing. *Toxicol Pathol* 26**:** 742-749.

80 Marshall CJ, Vousden KH, Phillips DH (1984) Activation of c-Ha-ras-1 proto-oncogene by in vitro modification with a chemical carcinogen, benzo(a)pyrene diol-epoxide. *Nature* 310**:** 586-589.

81 Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, Olivier M (2007) Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Hum Mutat* 28**:** 622-629.

82 Besaratinia A, Pfeifer GP (2006) Investigating human cancer etiology by DNA lesion footprinting and mutagenicity analysis. *Carcinogenesis* 27**:** 1526-1537.

83 Denissenko MF, Pao A, Tang M, Pfeifer GP (1996) Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. *Science* 274**:** 430-432.

84 Dumaz N, Drougard C, Sarasin A, Daya-Grosjean L (1993) Specific UV-induced mutation spectrum in the p53 gene of skin tumors from DNA-repair-deficient xeroderma pigmentosum patients. *Proc Natl Acad Sci U S A* 90**:** 10529-10533.

85 Phillips DH (1996) DNA adducts in human tissues: biomarkers of exposure to carcinogens in tobacco smoke. *Environ. Health Perspect.* 104, Suppl. 3**:** 453-458.

86 Phillips DH (2005) Macromolecular adducts as biomarkers of human exposure to polycyclic aromatic hydrocarbons, in: *The Carcinogenic Effects of Polycyclic Aromatic Hydrocarbons* (Luch A ed), pp 137-169, Imperial College Press, London.

87 Mumford JL, Lee X, Lewtas J, Young TL, Santella RM (1993) DNA adducts as biomarkers for assessing exposure to polycyclic aromatic hydrocarbons in tissues from Xuan Wei women with high exposure to coal combustion emissions and high lung cancer mortality. *Environ Health Perspect* 99**:** 83-87.

88 van Gijssel HE, Schild LJ, Watt DL, Roth MJ, Wang GQ, Dawsey SM, Albert PS, Qiao YL, Taylor PR, Dong ZW, Poirier MC (2004) Polycyclic aromatic hydrocarbon-DNA adducts determined by semiquantitative immunohistochemistry in human esophageal biopsies taken in 1985. *Mutat Res* 547**:** 55-62.

89 Arlt VM, Stiborova M, Schmeiser HH (2002) Aristolochic acid as a probable human cancer hazard in herbal remedies: a review. *Mutagenesis* 17**:** 265-277.

90 Nortier JL, Martinez MC, Schmeiser HH, Arlt VM, Bieler CA, Petein M, Depierreux MF, De Pauw L, Abramowicz D, Vereerstraeten P, Vanherweghem JL (2000) Urothelial carcinoma associated with the use of a Chinese herb (Aristolochia fangchi). *N Engl J Med* 342**:** 1686-1692.

91 Arlt VM, Ferluga D, Stiborova M, Pfohl-Leszkowicz A, Vukelic M, Ceovic S, Schmeiser HH, Cosyns JP (2002) Is aristolochic acid a risk factor for Balkan endemic nephropathy-associated urothelial cancer? *Int J Cancer* 101**:** 500-502.

92 Grollman AP, Shibutani S, Moriya M, Miller F, Wu L, Moll U, Suzuki N, Fernandes A, Rosenquist T, Medverec Z, Jakovina K, Brdar B, Slade N, Turesky RJ, Goodenough AK,

Rieger R, Vukelic M, Jelakovic B (2007) Aristolochic acid and the etiology of endemic (Balkan) nephropathy. *Proc Natl Acad Sci U S A* 104**:** 12129-12134.

93 Arlt VM, Stiborova M, vom Brocke J, Simoes ML, Lord GM, Nortier JL, Hollstein M, Phillips DH, Schmeiser HH (2007) Aristolochic acid mutagenesis: molecular clues to the aetiology of Balkan endemic nephropathy-associated urothelial cancer. *Carcinogenesis* 28**:** 2253-2261.

94 Phillips DH (2002) Smoking-related DNA and protein adducts in human tissues. *Carcinogenesis* 23**:** 1979-2004.

95 Mollerup S, Ryberg D, Hewer A, Phillips DH, Haugen A (1999) Sex differences in lung CYP1A1 expression and DNA adduct levels among lung cancer patients. *Cancer Res* 59**:** 3317-3320.

96 Perera FP (2000) Molecular epidemiology: on the path to prevention? *J Natl Cancer Inst* 92**:** 602-612.

97 Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD (1994) A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. *Cancer Epidemiol. Biomarkers Prev.* 3**:** 3-10.

98 Tang D, Phillips DH, Stampfer M, Mooney LA, Hsu Y, Cho S, Tsai WY, Ma J, Cole KJ, She MN, Perera FP (2001) Association between carcinogen-DNA adducts in white blood cells and lung cancer risk in the physicians health study. *Cancer Res* 61**:** 6708-6712.

99 Peluso M, Munnia A, Hoek G, Krzyzanowski M, Veglia F, Airoldi L, Autrup H, Dunning A, Garte S, Hainaut P, Malaveille C, Gormally E, Matullo G, Overvad K, Raaschou-Nielsen O, Clavel-Chapelon F, Linseisen J, Boeing H, Trichopoulou A, Trichopoulos D, Kaladidi A, Palli D, Krogh V, Tumino R, Panico S, Bueno-De-Mesquita HB, Peeters PH, Kumle M, Gonzalez CA, Martinez C, Dorronsoro M, Barricarte A, Navarro C, Quiros JR, Berglund G, Janzon L, Jarvholm B, Day NE, Key TJ, Saracci R, Kaaks R, Riboli E, Vineis P (2005) DNA adducts and lung cancer risk: a prospective study. *Cancer Res* 65**:** 8042-8048.

100 Bak H, Autrup H, Thomsen BL, Tjonneland A, Overvad K, Vogel U, Raaschou-Nielsen O, Loft S (2006) Bulky DNA adducts as risk indicator of lung cancer in a Danish case-cohort study. *Int J Cancer* 118**:** 1618-1622.

101 Anon (1999) Consensus report, in: *The Use of Short- and Medium-term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation* (McGregor DB, Rice JM, Venitt S eds), pp 1-18, IARC, Lyon.

102 Shelby MD, Purchase IFH (1981) Assay systems and criteria for their comparisons, in: *Evaluation of Short-term Tests for Carcinogens* (de Serres FJ, Ashby J eds), pp 16-20, Elsevier/North Holland, New York.

103 Phillips DH (2008) Biomarkers of exposure: adducts, in: *Molecular Epidemiology of Chronic Diseases* (Wild CP, Vineis P, Garte S eds), pp 111-125, Wiley.

Table 1. Performance terms for short-term tests for genotoxicity

Test outcome	**Carcinogen**		
	Yes	**No**	**Total**
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	c + d	N = a + b + c + d

Term	**Definition**	**Description**
Sensitivity	a/(a + c)	$\frac{\text{number of carcinogens found positive}}{\text{number of carcinogens tested}}$
Specificity	d/(b + d)	$\frac{\text{number of non-carcinogens found negative}}{\text{number of non-carcinogens tested}}$
Positive predictivity	a/(a + b)	$\frac{\text{number of carcinogens found positive}}{\text{number of positive results obtained}}$
Negative predictivity	d/(c + d)	$\frac{\text{number of non-carcinogens found negative}}{\text{number of negative results obtained}}$
Accuracy	(a + d)/N	$\frac{\text{number of correct test results}}{\text{number of chemicals tested}}$

Adapted from Anon [101] and Shelby and Purchase [102]

Table 2: Some representative carcinogens, their environmental sources, their active metabolites, sites of modification of DNA, and major type of induced mutation

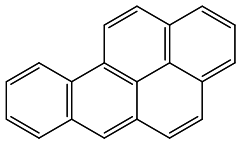
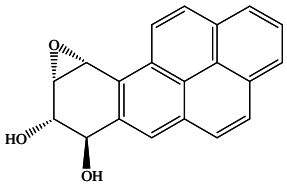
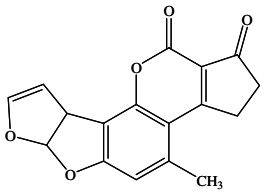
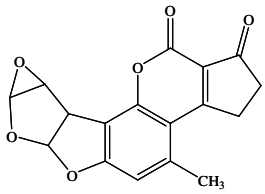
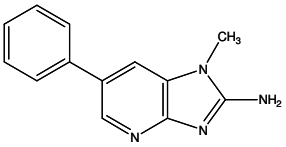
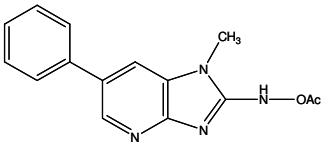
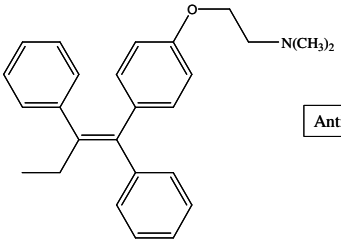
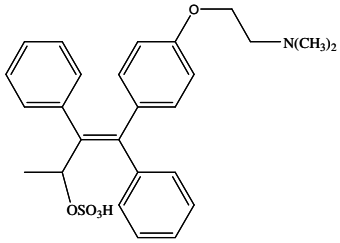
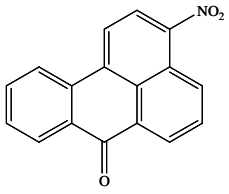
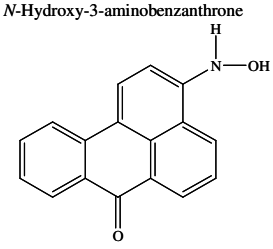
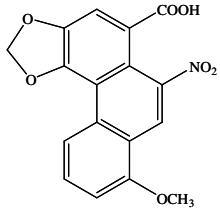
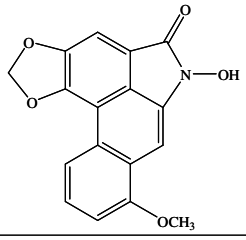
Carcinogen	Environmental source	Major active metabolite	Sites of modification of DNA	Major type of mutation
Benzo[*a*]pyrene (BaP) 	Tobacco smoking Combustion processes	BaP 7,8-dihydrodiol 9,10-oxide (BPDE) 	N^2-Guanine N^6-Adenine	GC → TA
Aflatoxin B_1 (AFB_1) 	Mycotoxin	AFB_1 8,9-epoxide 	N^7-Guanine	GC → TA
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) 	Food processing	*N*-Acetoxy-PhIP 	C8-Guanine	GC → TA
Tamoxifen 	Anticancer drug	α-Hydroxytamoxifen sulphate 	N^2-Guanine N^6-Adenine	GC → TA
3-Nitrobenzanthrone 	Diesel exhaust Urban air pollution	*N*-Hydroxy-3-aminobenzanthrone 	N^2-Guanine C8-Guanine N^6-Adenine	GC → TA
Aristolochic acid I (AAI) 	*Aristolochia* species	*N*-Hydroxyaristolactam I 	N^2-Guanine N^6-Adenine	AT → AT

Table 3. DNA adduct detection methods applicable to human biomonitoring and their limits of detection

Method	Variations	Amount of DNA required	Approximate detection limits
^{32}P-postlabelling	Nuclease P_1 digestion, butanol extraction, HPLC	1-10 μg	1 adduct per 10^9-10^{10} nucleotides
Immunoassay	ELISA, DELFIA, CIA, IHC	20 μg	1.5 adducts per 10^9 nucleotides
Fluorescence	HPLC fluorescence, SFS	100-1000 μg	1 adduct per 10^9 nucleotides
Mass Spectrometry		Up to 100 μg	1 adduct per 10^8 nucleotides
AMS[a]		Up to 100 μg	1 adduct per 10^{11}-10^{12} nucleotides

[a] Accelerator mass spectrometry. Requires use of radiolabelled compounds
Reproduced from [103]

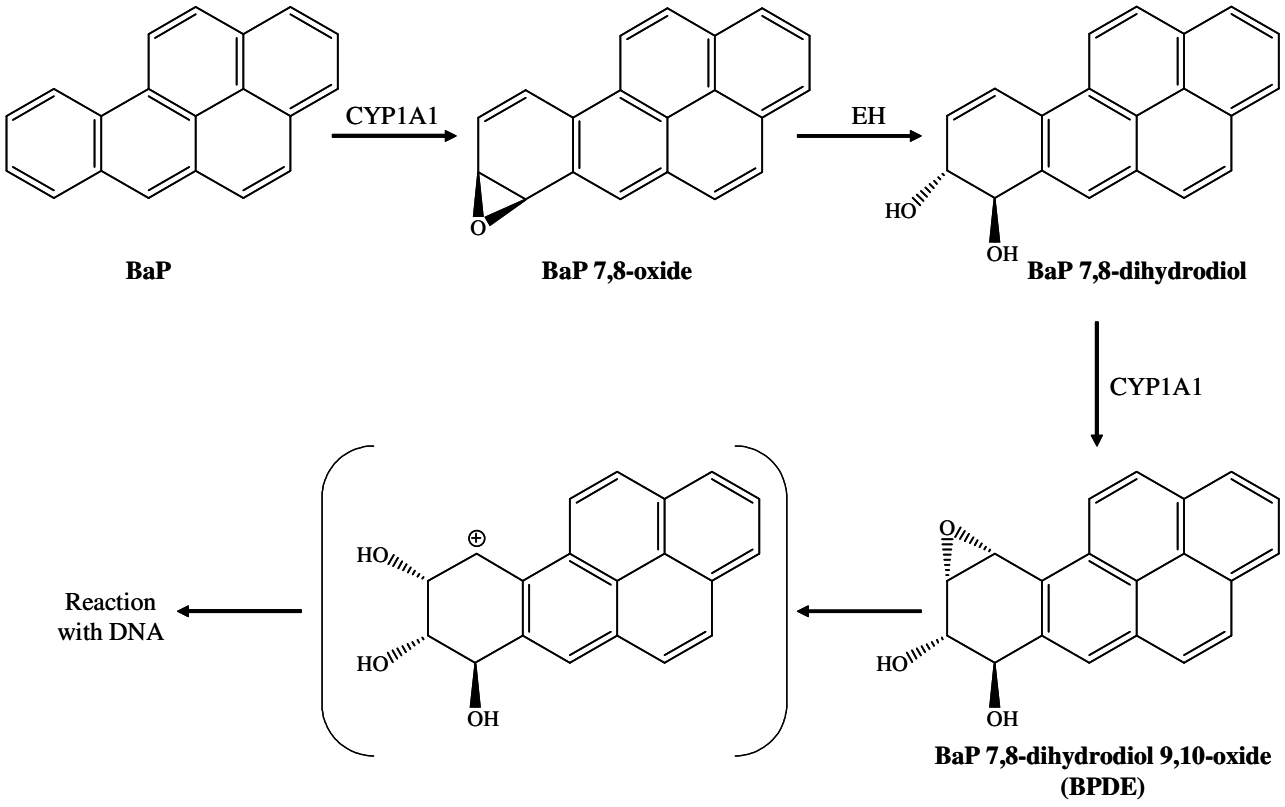


Figure 1: Major pathway of metabolic activation and DNA adduct formation of benzo[*a*]pyrene (see text for details). CYP1A1, cytochrome P450 1A1; EH, epoxide hydrolase.