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Mechanisms of DNA-reactive and epigenetic chemical carcinogens: applications to carcinogenicity testing and risk assessment

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Chemicals with carcinogenic activity in either animals or humans produce increases in neoplasia through diverse mechanisms. One mechanism is reaction with nuclear DNA. Other mechanisms consist of epigenetic effects involving either modifications of regulatory macromolecules or perturbation of cellular regulatory processes. The basis for distinguishing between carcinogens that have either DNA reactivity or an epigenetic activity as their primary mechanism of action is detailed in this review. In addition, important applications of information on these mechanisms of action to carcinogenicity testing and human risk assessment are discussed.

Introduction

Chemicals with carcinogenic activity, *i.e.*, the property of increasing neoplasia,[1] in either animals or humans, can be broadly categorized on the basis of their primary mechanism of action. One type of carcinogen is characterized by covalent reaction with nuclear DNA.[2] The other type exerts epigenetic (non-genotoxic) effects produced by modifications of cellular macromolecules which regulate gene activity or by perturbation of cellular regulatory processes. Substantial progress has been made in the understanding of these diverse mechanisms of chemical carcinogenicity. Here we review the current knowledge for identification of mechanistically different types of carcinogen and the applications of this information to carcinogen testing and human risk assessment.

Critical effects of chemicals in the process of rodent carcinogenesis

Investigations of the pathogenesis of experimental cancer induced by chemicals have established that the process consists of two necessary and mechanistically distinct sequences of events.[3] The two stage phenomenon of carcinogenesis was first formulated by Rous and co-workers,[4] who studied sequential application of carcinogenic substances to rabbit skin and from the results postulated that "carcinogenesis was composed of an initiating process, responsible for the conversion of normal into latent tumor cells, and a promoting process, whereby these latent tumors were made to develop into actual tumors". In concurrent similar experiments on mouse skin, Berenblum developed the same concept of two sequential phenomena which he termed "precarcinogenic" and "epicarcinogenic" actions.[5] These two stages of carcinogenesis are now known each to comprise multiple molecular and cellular events. To encompass this complexity, the first sequence of carcinogenesis has been designated as neoplastic conversion and the second as neoplastic development (Fig. 1).[1]

The sequence of neoplastic conversion consists of the inception of neoplasia. This entails the alteration of the genome of a normal cell through oncogenic mutations, either gene or chromosomal, and gene dysregulation to yield a cell with an abnormal phenotype and growth behaviour. With the acquisition of sufficient genetic alterations, estimated to be approximately four,[6] including altered expression of oncogenes or tumor suppressor genes, neoplastic conversion culminates in the emergence of a neoplastic cell with the capacity for progressive growth and tumor formation.

The sequence of neoplastic development consists of the clonal expansion of preneoplastic or neoplastic cells and their evolution (progression) into tumors. The growth of the neoplastic population can result from either disruption of homeostasis,[7,8] which may involve impaired intercellular communication due to cell membrane changes, or increased responsiveness of altered cells to endogenous or exogenous proliferation factors. To achieve growth, a neoplasm must develop a neovasculature (angiogenesis) to sustain the expanding cellular mass. The cells of a developing neoplasm can progressively deviate from the normal phenotype through alterations in gene expression, achieving independence from homeostasis and

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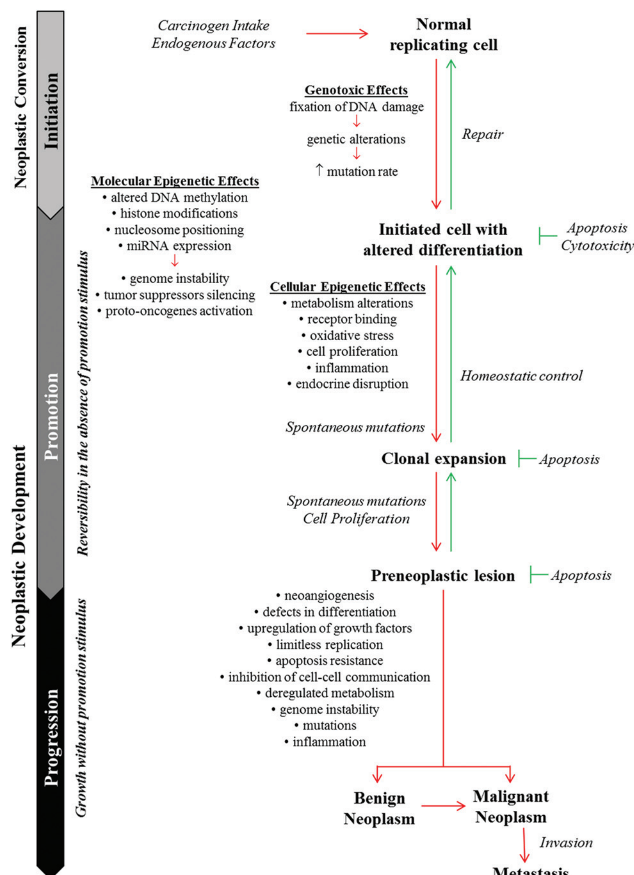

Fig. 1 Process of carcinogenesis.

acquiring malignant properties, such as invasion and metastasis. The events involved in these two sequences can be accomplished through a variety of chemical effects.

In the sequence of neoplastic conversion, change in the genome can be effected by chemicals in several ways, mainly through alteration in the structure[9–11] or function[12,13] of DNA. Most carcinogens that produce neoplastic conversion act, either in their parent form or after metabolic activation, as electrophilic reactants to produce DNA damage. DNA replication is required for the conversion of DNA damage to mutations. In studies in mammalian cell culture, actively proliferating cells displayed greater sensitivity to chemical-induced cell transformation[14] and mutagenicity.[15] Specifically, chemical DNA damage that is incurred during the S-phase of DNA synthesis is more mutagenic than that occurring in other phases of the cell cycle.[16] Also, increased cell proliferation in tissues with low proliferative activity, such as the liver, increases susceptibility to induction of carcinogenicity by DNA-reactive chemicals.[17–19] Accordingly, cell proliferation is regarded as a determining factor in carcinogenesis.[20–23]

In the second sequence of carcinogenesis, neoplastic development, a variety of chemical effects can lead to selective growth of preneoplastic cells (Fig. 1). Epigenetic molecular changes can affect gene expression, leading to abnormal proliferation, without altering the sequence of DNA. A cellular epigenetic mechanism for facilitating abnormal cell proliferation is the disruption of homeostatic control of proliferation of preneoplastic and neoplastic cells, which can be produced by inhibition of cell–cell communication.[24] A variety of other cellular epigenetic activities contribute to the enhancement of tumor development (Fig. 1), including cell receptor-mediated induction of cell proliferation,[25] cell injury (irritation) leading to regenerative cell proliferation,[26] hormonal perturbation with stimulation of cell proliferation,[27] and immunosuppression, all allowing emergence of abnormal cells.[28,29]

Based upon the accruing evidence of differences in the mechanisms by which chemicals induce cancer, a proposal was advanced beginning in 1977 at several meetings[30–33] to distinguish between two fundamentally different types of carcinogen. One was termed genotoxic,[34] or later, DNA-reactive,[35] to designate the capability of this type of agent to react with nuclear DNA directly and produce structural changes (*i.e.*, mutations). The other type, termed epigenetic,[35] or otherwise referred to as non-genotoxic, was conceived to lack the property of reacting covalently with DNA (or other cellular nucleophiles), but rather to exert other types of biological effects, mentioned above, as the basis of their carcinogenicity. Using available information, various carcinogens were assigned to specific classes within these two categories, while carcinogens for which the information required for classification was not sufficient, remained unclassified.[2]

A current classification of DNA-reactive and epigenetic carcinogens[1] is shown in Table 1. Formation of covalent adducts in nuclear DNA defines DNA-reactive carcinogens, but both types of carcinogen can contribute to carcinogenicity in an epigenetic manner, by causing alterations in gene expression or cellular homeostasis. Currently, it is generally recognized that both genetic alterations and epigenetic perturbations are equally important in a multistage development of cancer.[36–39] Nevertheless, there are important implications of the different mechanisms of action for testing strategies and risk assessment.

DNA-reactive (genotoxic) carcinogens

In the categorization of carcinogens elaborated by Weisburger and Williams (1981),[2] shown in Fig. 1, the classical organic carcinogens that form electrophilic reactants were designated as genotoxic, or later DNA-reactive. The rationale for substituting the term DNA-reactive has been described.[35] Basically, the term DNA-reactive is more specific to the description of carcinogens that undergo chemical reaction with nuclear DNA thereby forming chemical-specific covalent adducts, whereas the term genotoxic is often used to denote any positive result in a genotoxicity assay, most of which do not directly measure DNA reactivity.

To categorize a carcinogen as DNA-reactive, the only definitive proof of this property is the demonstration of the for-

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Table 1 Classification of chemicals with carcinogenic activity

A. DNA-reactive (genotoxic) chemicals

- ***1. Activation independent***
  - *Alkylating agents*: Nitrogen mustards, cyclophosphamide
  - *Epoxides*: Ethylene oxide
- ***2. Activation dependent***
  - *Alkenylbenzenes*: Methyl eugenol, safrole, estragole
  - *Aliphatic halides*: Vinyl chloride
  - *Aromatic amines, aminoazo dyes and nitro-aromatic compounds*: *o*-Toluidine, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP), polycyclic 4 aminobiphenyl, benzidine, dimethylaminoazobenzene, 1-nitropropane (nitroalkane)
  - *Hydrazine derivatives*: 1,2-Dimethylhydrazine, azoxymethane, methyl-azoxymethanol
  - *Mycotoxins*: Aflatoxin B1, pyrrolizidine alkaloids, ochratoxin
  - *N-Nitroso compounds*: Dimethylnitrosamine, *N*-nitrosonornicotine
  - *Pharmaceuticals*: Chlorambucil, tamoxifen
  - *Polycyclic aromatic hydrocarbons*: Benzo(*a*)pyrene, 7,12-dimethylbenzo[*a*]anthracene
  - *Triazines (diazoamino compounds)*: 3,3-Dimethyl-1-phenyltriazene

B. Epigenetic (non-genotoxic) chemicals

- ***1. Promoter***
  - *Liver enzyme-inducer type hepatocarcinogens*: Chlordane, DDT, pentachlorophenol, phenobarbital, polybrominated biphenyls, polychlorinated biphenyls
  - *Urothelial cell proliferation enhancers*: Sodium saccharin
  - *Skin tumor enhancers*: Croton oil
- ***2. Endocrine-modifier***
  - *Antiandrogens*: Finasteride, vinclozolin
  - *Antithyroid thyroid tumor enhancers*: Thyroperoxidase inhibitors (amitrole, sulfamethazine); thyroid hormone conjugation enhancers (phenobarbital, spironolactone)
  - *β_2-Adrenoreceptor agonists in female rats*: Soterenol, salbutamol
  - *Gastrin-elevating inducers of gastric neuroendocrine tumors*: Omeprazole, lansoprazole, pantoprazole, alachlor, butachlor
  - *Estrogenic hormones*: Estrogens, diethylstilbestrol, and hormone modifiers atrazine and chloro-*S*-triazines
  - *Neuroleptics* (dopamine inhibitors), *gonadotrophin releasing hormone-like drugs* (goseline)
- ***3. Immunomodulator***
  - Cyclosporine
  - *Purine analogs*: Azathioprine
- ***4. Cytotoxin***
  - *Forestomach toxicants*: Butylated hydroxyanisole, propionic acid, diallyl phthalate, ethyl acrylate
  - *Male rat $\alpha 2\mu$-globulin nephropathy inducers*: D-Limonene, *p*-dichlorobenzene
  - *Nasal toxicants*: Chloracetanilide herbicides (alachlor, butachlor)
  - *Liver toxicants*: Carbon tetrachloride
  - *Renal toxicants*: Potassium bromate, nitrilotriacetic acid
- ***5. Peroxisome proliferator-activated-receptor (PPAR) α/γ agonist***
  - *Hypolipidemic fibrates*: Ciprofibrate, clofibrate, gemfibrozil
  - *Miscellaneous*: Lactofen
  - *Phthalates*: Di(2-ethylhexyl) phthalate (DEHP), di(isononyl) phthalate (DINP)
- ***6. Inducer of urine pH extremes***
  - Melamine, saccharin, dietary phosphates, carbonic anhydrase inhibitors

C. Inorganic compounds

- ***1. Metal or metal salt***
  - Beryllium, cadmium, chromium, nickel, silica
- ***2. Fiber***
  - Asbestos

D. Unclassified

- Acrylamide, acrylonitrile, benzene, dioxane, dioxin, furan, methapyrilene, nucleoside analogs (entecavir, zidoriudine, zalcitabine)

mation of a chemical-specific covalent adduct(s) in nuclear DNA in the target tissue of carcinogenicity.[40] Measurements of adducts can now be made with a variety of sensitive techniques.[41–46] The structures of DNA-reactive carcinogens have been extensively elucidated[1,35] and for a carcinogen with a structure compatible with the formation of an electrophile, unequivocal positive results in assays that measure genotoxicity can serve as a strong indication of DNA reactivity.[47] Nevertheless, short-term assays are not the primary or the sole basis on which the distinction between DNA-reactive and other types of carcinogens should be made.

The structures of DNA-reactive carcinogens are such that they form electrophilic reactants, either from chemical reactions or following bioactivation. The structures of the five known carcinogenic electrophiles are depicted in Fig. 2. Most DNA-reactive carcinogens form either carbonium or nitrinium ions. The presence in a molecule of these electrophiles or precursor structures is a structural indication of potential DNA reactivity. The exact chemical structure of the adducts formed in DNA has been demonstrated for many DNA-reactive carcinogens.[48–51] Most DNA-reactive carcinogens give rise to more than one type of adduct in DNA and, hence, a continuing challenge in establishing the basis for the carcinogenicity of individual DNA-reactive chemicals has been to determine which type of adduct or combination of adducts and at which locations in the genome they are formed are critical to carcinogenicity.[52]

DNA-reactive carcinogens are both synthetic (*e.g.* benzidine) and naturally occurring (*e.g.* aflatoxin) (Table 1). Most of the widely studied experimental organic carcinogens, such as polycyclic aromatic hydrocarbons, aromatic amines and nitrosamines, are DNA reactive. Some are carcinogenic without biotransformation, that is, are activation independent (*e.g.* alkylating agents), but most require bioactivation to one of the five electrophilic structures shown in Fig. 2 and hence are activation dependent.

The property of DNA reactivity is clearly the essential effect of carcinogens of this type in producing cancer. Thus, DNA-reactive carcinogens induce neoplasia largely as a consequence of their reaction with nuclear DNA, resulting in neoplastic conversion of the affected cells in the first sequence of carcinogenesis (Fig. 1).

DNA-reactive carcinogens also can react with other molecular components of the cell, as documented by Miller and Miller[53] in their studies of protein binding of aminoazo dyes. Non-DNA macromolecule binding could exert cellular and tissue effects that facilitate either sequence of carcinogenesis. Among the epigenetic effects contributing to carcinogenesis are enhanced cell proliferation and promotion. It is well-established that enhanced cell proliferation increases the susceptibility of cells to both the carcinogenic[17–19] and mutagenic[16] effects of chemicals. Cell proliferation can enhance neoplastic conversion by creating the possibility for errors during DNA replication in the newly synthesized strand at sites opposite to unrepaired carcinogen-induced DNA damage. Some of the differences in the potencies of DNA-reactive carcinogens are likely to

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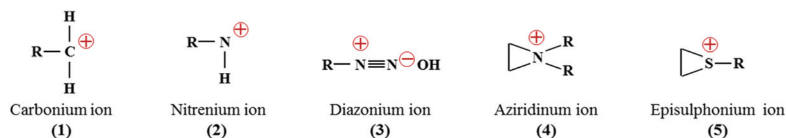

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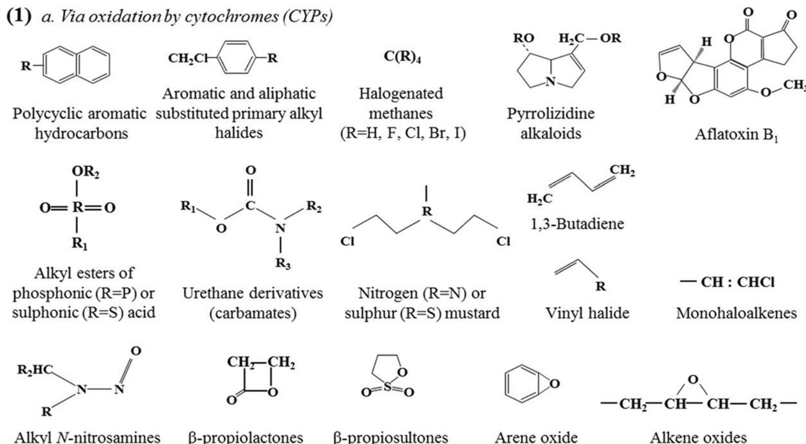
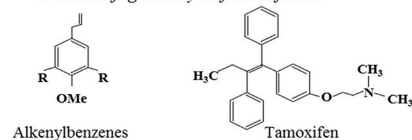


b. Via conjugation by sulfotransferase



(2) *Via oxidation by cytochromes (CYPs)*

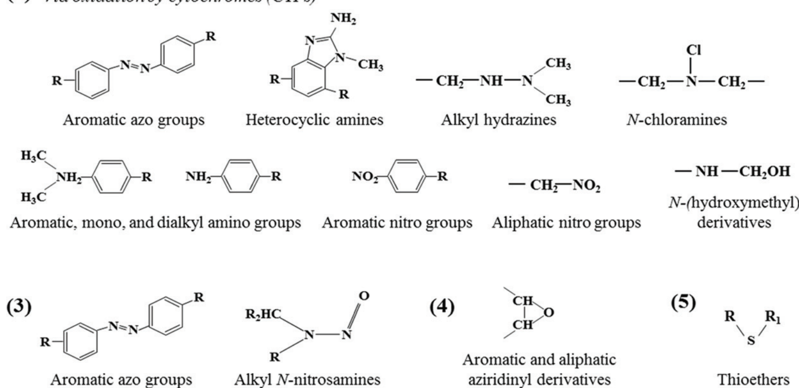


Fig. 2 Structures of reactive electrophiles and DNA-reactive carcinogens.

be attributable to their promoting activity or other epigenetic effects,[54] in addition to induction of neoplastic conversion.[55]

As a consequence of the mechanism of action of DNA-reactive carcinogens, many carcinogens of this type exert potent carcinogenic effects in experimental systems, including induction of tumors in several tissues (Table 1).

Because of their mechanism of action, DNA-reactive carcinogens, have been assumed not to have a cancer threshold. However, current research using rigorous approaches reveals no-effect levels for DNA-reactive carcinogens.[56–58] Nevertheless, the features of DNA-reactive carcinogens are indicative of a high degree of hazard to potentially exposed humans (see below) and it is probably for this reason that the majority of carcinogens recognized by the International Agency for Research on Cancer (IARC)[59] as having caused human cancer is of the DNA-reactive type (Table 2).

Epigenetic (non-genotoxic) carcinogens

The category of epigenetic carcinogens is comprised of chemicals that elicit neoplasia through molecular or cellular epigenetic effects, which do not involve DNA reaction. Epigenetic

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Table 2 Chemicals with sufficient evidence for carcinogenicity in humans

Agent	Target organ	GTX	Agent	Target organ	GTX
4-Aminobiphenyl[a]	Urinary bladder	+	Ethylene oxide[a]	Hematopoietic system	+
Acetaldehyde associated with consumption of alcoholic beverages[a]	Upper GI tract, colon, liver, breast (females)	±	Etoposide alone, and in combination with cisplatin and bleomycin	Hematopoietic system	+
Aflatoxins[a]	Liver	+	Formaldehyde[a]	Nasopharynx, hematopoietic system	+
Arsenic and inorganic arsenic compounds[a]	Urinary bladder, skin, lungs	Oxidative DNA damage	Lindane	Hematopoietic system	−
Azathioprine[a]	Hematopoietic system, skin	+	Melphalan[a]	Hematopoietic system	+
1,3-Butadiene[a]	Hematopoietic system	+	Methoxsalen (8-Methoxypsoralen) plus ultraviolet A radiation[a]	Skin	+
1,4-Butanediol dimethanesulfonate (Busulphan, Myleran)[a]	Hematopoietic system, lungs	+	Methyl-CCNU (1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; Semustine)[a]	Hematopoietic system	+
Benzene[a]	Hematopoietic system	+	MOPP and other combined chemotherapy including alkylating agents	Hematopoietic system, lungs	+
Benzidine[a]	Urinary bladder	+	Mustard gas (sulfur mustard)[a]	Lungs	+
Beryllium and beryllium compounds[a]	Lungs	Oxidative DNA damage	2-Naphthylamine[a]	Urinary bladder	+
Bis(chloromethyl)-ether[a]	Lungs	GTX in humans	Nickel compounds[a]	Nasal cavity, paranasal sinus, lungs	Oxidative DNA damage
Cadmium and cadmium compounds[a]	Lungs	Oxidative DNA damage	Pentachlorophenol (PCP)	Hematopoietic system	Oxidative DNA damage
Chlorambucil[a]	Hematopoietic system	+	Phenacetin	Renal pelvis and ureter	+
Chlornapazine (*N*,*N*-bis (2-chloroethyl)-2-naphthylamine)	Urinary bladder	+	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)[a]	Lungs, soft tissue sarcoma, multiple sites	Aryl hydro-carbon receptor binding
Chloromethyl methyl ether[a]	Lungs	+	*o*-Toluidine[a]	Urinary bladder	+
Chromium(VI) compounds[a]	Lungs	+Oxidative DNA damage	Tamoxifen[a]	Endometrium	+Estrogen receptor modulator
Cyclophosphamide[a]	Hematopoietic system, urinary bladder	+	Thiotepa (1,1′,1″-phosphinothioylidynetrisaziridine)[a]	Hematopoietic system	+
Cyclosporine[a] (ciclosporin)	Hematopoietic system, skin	Oxidative DNA damage	Treosulfan	Hematopoietic system	+
Diethylstilboestrol[a]	Breast, uterine cervix and vagina (exposure *in utero*)	+Estrogen receptor modulator	Trichloroethylene[a]	Kidney, hematopoietic system, liver	+
Estrogen only therapy, postmenopausal[a]	Endometrium, ovary	+Oxidative DNA damage Estrogen receptor modulator	Vinyl chloride[a]	Liver	+

Chemicals provided in this table are assigned to IARC Group 1 carcinogens (carcinogenic to humans). [a] Also mentioned in 14th report on carcinogens by NTP (2016).[215] GTX, genotoxicity; +, positive; + /-, equivocal; -, negative.

carcinogens have structures, including that of their metabolites, which are not compatible with formation of a likely electrophilic reactant (Fig. 3). In other words, they lack the structural features of DNA-reactive chemicals illustrated in Fig. 2. Epigenetic agents generally have been inactive in short-term assays for genotoxicity, especially assays for DNA reactivity.[60] Moreover, the formation of adducts in nuclear DNA by such carcinogens, which is the definitive evidence of DNA reactivity,

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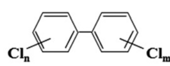

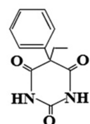

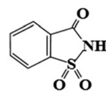

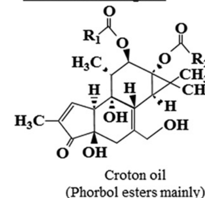

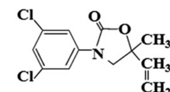

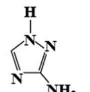

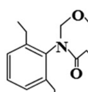

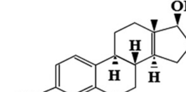

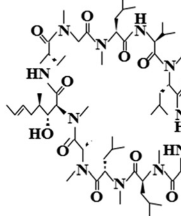

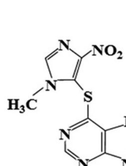

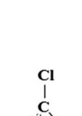

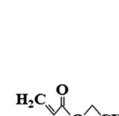

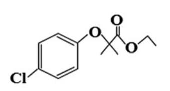

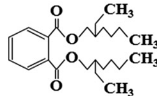

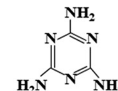

Fig. 3 Structures of prototype epigenetic carcinogen and molecular mechanisms involved in their carcinogenesis.

has not been found in cells of their target tissues using sensitive techniques.[61,62]

Epigenetic carcinogens must also be demonstrated to exert other primary molecular or cellular effects in their target tissues that are the basis for their carcinogenicity through actions in either of the two sequences of carcinogenesis (Fig. 1).[29,63–65] It is important to note that "non-genotoxic" carcinogens for which an epigenetic effect that could be the basis of carcinogenicity has not been reasonably established, remain unclassified since absence of genotoxicity is not sufficient for assignment to the epigenetic category. Moreover, since absence of genotoxicity does not establish a mechanism of action; the term "non-genotoxic" is not equivalent to epigenetic.

Epigenetic carcinogens can affect gene expression in the absence of affecting the DNA sequence. Thus, genome-wide or gene specific DNA methylation and lysine methylation of certain sites on histone proteins, are often implicated in repression of transcription, while other histone modifications, such as acetylation, phosphorylation and arginine methylation, promote transcriptional activation. Expression of non-coding regulatory RNAs (miRNA) is associated with posttranscriptional silencing of target mRNA. In the process of carcinogenesis, the aforementioned changes in the epigenome contribute to aberrant activation of silenced tumor-promoting genes, while suppressing the activity of tumor-suppressor genes, leading to chromosomal and genomic instability. These molecular changes are produced by many established epigenetic carcinogens, including furan, arsenic, phenobarbital, methapyrilene and others.[39,54,66,67,216]

A variety of other cellular epigenetic effects underlie carcinogenicity, often through induction of cell proliferation. In the first sequence, neoplastic conversion, DNA replication allows fixation of spontaneous mutations.[68] In the second sequence, neoplastic development, epigenetic carcinogens can enhance tumour formation through disruption of tissue homeostasis.[69] Also, neurohormonal, or immunosuppressive effects facilitate the emergence of cryptogenically formed, *i.e.* background, neoplastic cells.

Some well-established examples of epigenetic carcinogens are given in Table 1. Epigenetic carcinogens are both synthetic (*e.g.* dichlorodiphenyltrichloroethane (DDT)) and naturally occurring (*e.g.* D-limonene).

The structures of epigenetic carcinogens underlie their mechanisms of epigenetic effects. Prototype structures for different classes of epigenetic carcinogens are shown in Fig. 3.

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Unlike DNA-reactive carcinogens, which all ultimately form an electrophile, the structures of epigenetic carcinogens are highly diverse. Nevertheless, some common features exist. For example, DDT, polychlorinated biphenyls, and chlordane which are all halogenated polycyclic compounds, accumulate in the liver and act as liver tumor promoters,[70,71] facilitating liver tumor development from pre-existing transformed cells.[72] Another critical structural feature is phenoxyacetic acid moieties present in fibrates, which are the basis for biding to the peroxisome proliferator activated receptor alpha (PPARα) leading to enhanced liver cell proliferation and development of liver tumors.[73–75]

A common feature of many epigenetic carcinogens, as noted, is the induction of cell proliferation, which is a contributing or even the major component in their carcinogenicity.[76] For several epigenetic carcinogens the enhancement of cell proliferation is a consequence of the binding of the carcinogen to cellular receptors.[25] Some of the receptors that bind carcinogenic ligands are given in Table 3. The role of receptor binding in carcinogenicity is complex; it may well be essential, but not sufficient for the carcinogenicity of some ligands. For example, for the rodent liver carcinogen tamoxifen, which binds to the liver estrogen receptor, such binding is apparently not the primary mechanism of action of hepatocarcinogenicity since an analogue, toremifene, which also is an estrogen agonist in the liver,[77] is not hepatocarcinogenic.[78] Thus, other cellular effects may contribute to carcinogenicity during cell proliferation. For example, the carcinogenicity of PPARα agonists may involve a combination of induced cell proliferation and oxidative damage to DNA resulting from induced peroxisomal generation of reactive oxygen species (ROS), *e.g.*, hydrogen peroxide.[79]

Another mechanism that characterizes many tumor promoters is inhibition of gap junctional communication.[80,81] As noted above, this interferes with cellular and tissue homeostasis, allowing expansion of altered cell populations.

A feature of some epigenetic carcinogens, as mentioned above, is that they cause increased production of ROS leading to oxidative DNA damage.[82–84] DNA oxidation, however, is weakly mutagenic[85,86] and there is some evidence that it may be more important in promotion (neoplastic development) than in initiation (neoplastic conversion), which, nevertheless, can lead to tumorogenicity.[87]

Table 3 Hepatocellular xenobiotic nuclear receptors

	Ligands		
Receptor	Endobiotics	Xenobiotics	Genes activated by xenobiotics
Ahr – arylhydrocarbon		Polycyclic aromatic hydrocarbons Omeprazole	CYP1A
Adopted orphan receptors (low-affinity ligands)			
CAR[a] – constitutive androstane	Androstanes	Phenobarbital	CYP2B CYP2C
PXR[a] – pregnane X	Progesterone Pregnenolone	Dexamethasone Rifampicin Hyperforin	CYP3A4
PPAR[a] – peroxisome proliferator activated (α)	Fatty acids	Fibrates Phthalates	Peroxisomal enzymes
PPAR (γ)	1,5-Deoxy-delta 12,14-Prostaglandin J2	Troglitazone	CYP4A
LX[a] – liver x (α, β) FXR[a] – farnesoid X	Oxysterols Bile acid Farnesoids	Geranylgeranyl phosphate Cafestol Chenodeoxycholic acid	CYP7A CYP7A1
RXR – retinoid x (α, β, γ)	9-*cis* Retinoic acid	Retinoids Rexinoids Docosahexaenoic acid	>300 including Transcription factors Cell surface receptors Structural proteins
Hormone receptors (high-affinity lipophilic ligands)			
ER – estrogen (α, β)	Estrogens	Tamoxifen Genistein	
AR – androgen GR – glucocorticoid PR – progesterone VDR – vitamin D	Testosterone Glucocorticoids Progesterone Vitamin D	Polychlorinated biphenyls Dexamethasone Polychlorinated biphenyls Cholecalciferol	 CYP2B6 CYP2C9

[a] Heterodimerize with RXR.

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Table 4 Characteristic of carcinogenicity of DNA-reactive and epigenetic carcinogens

DNA-reactive carcinogens	Epigenetic carcinogens
• DNA-reactive - DNA adducts formation - Other types of direct DNA damage • Potentially mutagenic or cytotoxic	• Indirectly produce DNA alterations/damage - Mitogens stimulate proliferation - Cytotoxic chemicals induce regeneration - Alter DNA repair or cause genomic instability - Modulate receptor-mediated effects - Alter cell proliferation, cell death - Induce chronic inflammation or immunosuppression - Inhibit cell–cell communication - Induce oxidative stress
• Most require metabolic activation to become reactive, some act as an electrophile directly • Neonates often more sensitive • Some exhibit transplacental carcinogenicity	• Shifts in sites of tumor induction by modifiers of biotransformation not reported • Little evidence of enhanced susceptibility of neonates, except saccharin • Little evidence for transplacental carcinogenicity, except diethylstilbestrol and saccharin
• Many are active at low dosage	• May be active at low dosage, but require a level and duration of exposure to produce relevant cellular effect
• Represent human hazards • Additivity of carcinogenicity possible	• Human carcinogenicity seen with hormonal or immunosuppressive agents • Additivity of carcinogenicity uncertain: some can inhibit one another

Six different types of epigenetic carcinogens have been distinguished according to their primary cellular effect (Table 1). The nature of these effects has been described in detail in other papers.[35,88] The characteristics of the carcinogenicity of epigenetic agents, in general, are quite different from that of DNA-reactive carcinogens (Table 4). Epigenetic carcinogens typically require high level or long duration exposure to produce an increase in tumors. An exception is carcinogens which bioaccumulate, such as lipohilic polyhalogenated compounds, where dosing of short duration can lead to a sustained internal dose. The dependence of the carcinogenicity of most epigenetic agents on extended dosing is a consequence of the necessity for them to exert, for some prolonged duration, the primary molecular or cellular effect that underlines their ability to increase neoplasia. Accordingly, it is to be expected that agents of this type would have a threshold for carcinogenicity coinciding with the threshold for their epigenetic effects. In fact, this has been shown for a variety of epigenetic carcinogens.[89] As a consequence of their mechanism of action, epigenetic carcinogens are less potent compared to DNA-reactive. They often affect only a single tissue where their epigenetic effect is produced.

Only a few epigenetic agents, mainly hormonal or immunosuppressive agents have been recognised by the IARC as human carcinogens (Table 2). These induce neoplasia only under conditions where they overtly produce their epigenetic effect.

Applications

Carcinogen testing and hazard identification

A major application of the concept of mechanistically distinct carcinogens is in approaches to carcinogen identification. For this purpose, Weisburger and Williams[2,35,90] introduced the Decision Point Approach (DPA) to carcinogen testing, outlined in Fig. 4. The DPA is a systematic process for the acquisition of critical data for both DNA-reactive and epigenetic effects that are known to be associated with the carcinogenic activity of chemicals.[1,91]

The DPA aims to provide reliable data enabling evaluation of the potential hazard of a test substance at the earliest possible stage to avoid human exposure to harmful chemicals. Also, an expedited approach to testing can assist in prioritizing from the large space of existing untested chemicals and in selecting desirable new chemical or molecular entities, both small and large (*e.g.*, protein) molecules. The following discussion is restricted to testing of small molecules; the testing of biotechnology products and other testing requirements are detailed in Williams *et al.*[1]

The endpoints comprising the DPA provide guidance in identifying a potentially carcinogenic test substance, but negative results do not preclude carcinogenicity, and thus, carcinogenicity testing is the last stage in the approach. The value of obtaining critical data prior to the conduct of a carcinogenicity bioassay is supported by an analysis of data on a large number of pharmaceuticals which reported that a positive finding for either genotoxicity, histopathological changes indicative of pre-neoplasia or hormonal perturbation could be highly predictive of carcinogenic activity.[92] A review of the FDA/CDER database of pharmaceuticals, however, concluded that short-term toxicity studies, including in transgenic mice, do not accurately and reliably predict neoplastic findings in long-term assays.[93]

Stage A of the DPA consists of evaluation of the chemical structure. Among both DNA-reactive and epigenetic carcinogens, numerous types share common structural features (Fig. 2 and 3). The presence of one of these features in a new molecular entity of unknown carcinogenicity suggests potential activity.

For DNA-reactive organic carcinogens, five types of electrophiles, are involved in chemical reactivity and hence DNA

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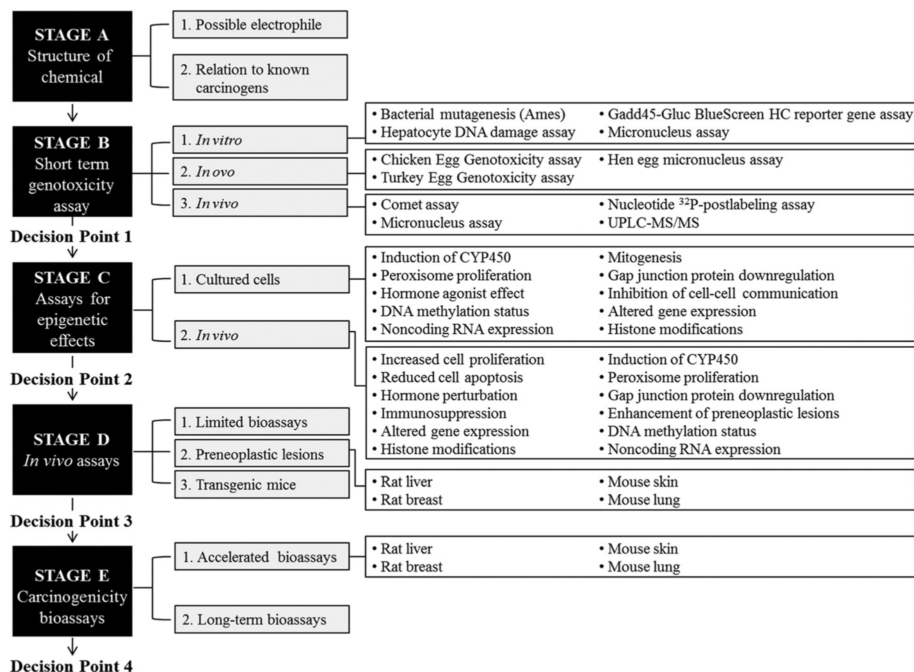

Fig. 4 Decision point approach (DPA) in carcinogen testing.

binding.[1] Examples of the structures of carcinogens which give rise to these electrophiles are shown in Fig. 2. Such molecular features also have been referred to as structural alerts for mutagenicity.[94] The presence of one of these structures is highly suggestive of potential carcinogenic activity. An example is tamoxifen, a polyphenylethylene estrogenic agent with an ethyl side chain which can be sulphated and thereby activated (Fig. 2). The structural analogue, toremifene, has a chlorine substitution on the ethyl side chain which blocks activation. As a consequence, tamoxifen is DNA-reactive and hepatocarcinogenic in rats, whereas toremifene is not.[95]

Structural analysis can also reveal the absence of structures associated with DNA reactivity. For example, aspartame, a methyl ester of aspartic acid (phenylalanine dipeptide) and glyphosate, a glycine derivative, lack electrophile precursors and hence do not exhibit DNA reactivity.[96,97]

There are also variety of structural clues to epigenetic carcinogenicity, as shown in Fig. 3. Examples include halogenated polycyclic compounds, such as DDT, polychlorinated biphenyls, and chlordane which are liver tumor promoters[70,71] and facilitate liver tumor development from pre-existing transformed cells.[72] Compounds with phenoxyacetic acid moieties are possibly PPARα agonists, which act as liver carcinogens through enhanced cell proliferation.[73–75]

The FDA Center for Food Safety and Nutrition has grouped food substances into classes according to chemical structure and estimated their potential toxicity,[98,99] similar to the classes delineated by Cramer *et al.*[100] These structural classes are used for assignment to levels of concern. Substances with functional groups of high probable toxicity are assigned to Category C; substances of intermediate or unknown toxicity are assigned to Category B; and substances of low probable toxicity are assigned to Category A. The recognition of potential toxicity can provide a guide to possible epigenetic carcinogenicity. Several artificial intelligence systems (*in silico*) for assessing potential toxicities related to structures are available.[101–103]

Stage B consists of short-term assays for genetic endpoints. A large number of such assays has been developed over the years,[103] but relatively few are currently used routinely. All regulatory agencies have specific testing recommendations or requirements, which may extend beyond the prediction of potential carcinogenicity. The most widely used *in vitro* assays include bacterial mutagenicity (Ames), mammalian cell mutagenicity/chromosome aberration, and the TK6 cell or human lymphocyte micronucleus.[103–105] These assays differ in their endpoints, but all commonly used *in vitro* assays depend on an exogenous biotransformation system (usually an S9 preparation obtained from induced rat liver) for bioactivation of test chemicals, although biotransformation capable cell lines are available.[106] Biotransformation by subcellular preparations and cell lines, however, is not as complete as that of isolated intact cells,[32,107] which must be considered in extrapolation of results to animals and humans. Each of these assays can yield false positive and false negative results in relation to carcinogenicity.[108]

The predictivity of most genotoxicity assays (*i.e.*, the percentage of positive chemicals that prove to be carcinogens) is limited largely to DNA-reactive carcinogens as a consequence of the fact that DNA alteration is the mechanism of action of this class of carcinogen.

A bacterial mutagenicity assay[109] is required in all testing batteries and has reasonably high predictivity for carcinogeni-

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city.[103,110] A substantial number of bacterial mutagens, however, is non-carcinogenic,[111] for example the plant flavonol, quercetin.[112] The ability of Salmonella mutagenicity to identify carcinogens is not increased by obtaining data in certain other standard *in vitro* assays, such as mammalian cell mutagenicity and chromosome aberration assays,[110,113] which may reflect deficiencies in biotransformation.

To reinforce assurance of the predictiveness of a positive Salmonella mutagenicity finding, in the DPA, another well-established assay, the hepatocyte/DNA damage assay,[114] can be used. This assay, unlike most others, provides intrinsic enzymatic bioactivation of a normal biotransformation-proficient cell type and hence is complimentary to assays that are dependent on exogenous bioactivation. Originally, the hepatocyte DNA damage assay involved autoradiographic measurement of DNA repair synthesis and this is still a useful method,[115] especially for chemicals that may produce unstable adducts. Moreover, repair synthesis amplifies the signal from DNA damage. Currently, several other methods for detection of DNA damage are available, including the alkaline single cell gel electrophoresis (comet) method for detection of single strand breaks[116,117] and the nucleotide postlabelling (NPL) methods for DNA adducts.[41,45,118] In cultured hepatocytes, NPL has been reported to mirror DNA repair synthesis,[115] as would be expected. In general, a relationship exists between DNA binding and carcinogenicity,[119,120] although not all DNA binding is necessarily mutagenic[121] or carcinogenic,[55] since adducts can be unstable or involve sites on bases that are not involved in base pairing. In one substantial data set, a very high correlation with carcinogenicity was provided by positive results in bacterial mutagenicity and hepatocyte DNA repair assays.[122] Other assays for DNA damage include assessment of expression of DNA damage response genes, as with the Blue Screen assay.[123] The features of these assays have been reviewed.[46] The utility of hepatocytes is that they provide intrinsic bioactivation for both phase I and II metabolism. Also, hepatocytes from various species,[124] importantly humans,[115,125] can be used. Clear positive results in both bacterial mutagenicity and hepatocyte DNA damage assays, therefore, raise a strong presumption of potential carcinogenicity.

If testing at this level yields equivocal findings, other *in vitro* assays are available. These include micronucleus assays in the 3 dimensional human skin or peripheral human lymphocytes, which detect clastogenic and aneugenic events.[126] Also, *in vivo* assays for DNA reactivity can be used.

A variety of *in vivo* genotoxicity assays are available. Some assays which are included in recommended batteries, such as the mouse bone marrow micronucleus assay,[127] are not specific for DNA reactivity and interrogate only a single tissue, which in the case of bone marrow is of low chemical biotransformation capability. Where possible, it is desirable to conduct *in vivo* assays in rats, as most toxicity and chemical kinetic studies will be performed in this species. *In vivo* DNA binding can be assessed if radiolabeled test substance is available.[42] Otherwise, assays for DNA damage which can be applied include the comet assay for DNA breakage,[117,128] and NPL or ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for adduct formation.[41,118,129,130] The comet assay and UPLC-MS/MS are indicated where the potential electrophile might be a small alkylating agent, which would not be detected by NPL.

In addition to conventional *in vivo* mutagenicity assays, other models for *in vivo* mutagenicity include genetic-engineered animals such as the Muta™ Mouse and Big Blue® rat models.[103,131,132] These models allow for detection of mutations in various tissues and can provide information on the molecular nature of induced mutations.

A non-animal model for detection of DNA damage in an intact organism is avian eggs.[133–138] In the *in ovo* model, effects are measured in the developing liver. An advantage of the *in ovo* model is that it employs an intact organism which is not a live animal and hence can be used for testing where use of animals is undesirable or precluded. Either chicken or turkey eggs are employed for assays referred to as the Chicken or Turkey Egg Genotoxicity Assay (CEGA or TEGA, respectively). Both have been documented to possess extensive liver biotransformation capability.[133,139] The dosing and maintenance phases of the assays require only a polystyrene incubator and can be conducted in a conventional laboratory. The test substance is injected through the shell into the air sac. Prior to hatching (termination day 11 for chicken and 24 for turkey), livers are harvested for the comet assay, which detects DNA fragmentation, and for the NPL assay for DNA adducts. The model can also be used for assessment of carcinogenic activity (see below). Diverse DNA-reactive carcinogens have been positive in these models.[135,138,140]

At this stage (*i.e.* A and B) a decision can be made on the potential carcinogenicity of the test substance. The FDA/CDER has issued a guidance for integration of results from genetic toxicology studies.[141] Compelling evidence for DNA reactivity is highly suggestive of potential carcinogenicity. If evidence of DNA reactivity is reasonably excluded, there still remains the possibility of epigenetic carcinogenicity.

Stage C of the DPA consists of assays for epigenetic effects that could result in an increase in neoplasms in rodents with long-term dosing. Certain histopathological findings in standard toxicity assays can suggest potential carcinogenicity through epigenetic mechanisms. For example, rodent liver enlargement induced by a chemical is associated with a likelihood of liver carcinogenicity.[142,143] A study by the National Toxicology Program (NTP) found that certain *in vivo* effects, such as increased liver weight and hepatocellular hypertrophy (for both mice and rats) and hepatocellular necrosis (for mice), are better predictors for liver carcinogenicity than bacterial mutagenicity.[143] Also, measurement of enhancement of preneoplastic lesions can indicate promoting effects.[144,145]

Specific assays for epigenetic effects (Fig. 4) are applied selectively depending on the properties of the chemical, such as structure, biologic/pharmacologic action, and toxicity. Many assays for epigenetic activities can be conducted in cultured cells, particularly hepatocytes, which provide intrinsic bioactivation.

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A variety of techniques have been developed to assess molecular changes produced by epigenetic carcinogens, for example, bisulfite genomic sequencing to assess DNA methylation status, western blot or Chromatin immunoprecipitation (ChiP) techniques to evaluate presence of histone modifications, and sequencing technologies to determine miRNA expression profile.

Measurement of gene deregulation is useful for identification of epigenetic effects.[103,146–148] The pattern of changes in gene expression can provide an indication of potential carcinogenicity.[149] For example, increased expression of specific genes, such as hepatic acyl-CoA oxidase, which is increased by PPARα agonist liver oncogens or hepatic cytochrome P450s, which is increased by a variety of liver neoplasm promoters, are linked to epigenetic carcinogenesis, and thus these methods have utility for screening.

Assessment of inhibition of gap junctional communication in cell cultures has been applied to identification of tumor promoters.[24,80] Liver culture systems have been extensively used for this purpose.[72,81,150] However, neoplastic development is a tissue specific event and promoters for specific tissues may not be detected in available systems.

Even when applied *in vivo*, assays for epigenetic effects are of short duration, except for those for promoting activity, which are described below. Positive results indicate potential oncogenicity, in which case the potency relative to that of established carcinogens with a similar mechanism and organotropism provides a guide to risk assessment. A particularly valuable endpoint is monitoring of cell proliferation, because, as discussed above, this is a common response to a variety of epigenetic effects.

At this stage (A–C), a decision can be made on potential carcinogenic activity, both DNA-reactive and epigenetic.

These epigenetic assays can also be applied to elucidation of the mechanism of action of chemicals with known carcinogenic activity. It is critical that studies for this purpose be conducted using the dosing conditions that produced tumor increases in the bioassay and that the epigenetic mechanism be demonstrated in the target tissue(s) of carcinogenicity.

Stage D deploys limited carcinogenicity bioassays (LCB). These are based on induction or enhancement of either neoplasms or established preneoplastic lesions as their endpoint.[144,145,151] These assays can be applied as initiation assays for neoplastic conversion in which the test substance is assessed for its ability to induce the endpoint lesion, or as promotion assays for neoplastic development, in which the test substance is administered after an agent that induces the endpoint lesion to determine the ability of the test substance to enhance the development of the lesion.[151,152] In early experimental studies of initiation of skin carcinogenesis, initiation was achieved with a single administration.[153] Although this is possible with potent DNA-reactive agents, repeated dosing at the maximum tolerated dose for at least 4 weeks is necessary for an adequate assessment of initiation by a test substance. Because promotion requires an even longer time for expression, more extensive dosing, up to 6 months, is required for an adequate test. Essentially, an assay for initiation activity is directed largely toward assessing potential *in vivo* genotoxicity of the test substance, whereas assays for promotion activity assesse an epigenetic mechanisms of action. Accordingly, assays for promotion activity can also be deployed in the DPA at Stage C *in vivo* assays for epigenetic effects (Fig. 4).

An outline for initiation and promotion assays is shown in Fig. 5. The most extensively validated and applied model for an LCB is the rat liver hepatocellular focus assay.[144,151,154,155] This assay in the liver takes advantage of the extensive capability of chemical biotransformation in this organ and the availability of sensitive and reliable markers for preneoplastic lesions.[156] Other commonly used LCBs are the mouse skin papilloma/carcinoma, the mouse lung adenoma/carcinoma, and the rat mammary gland adenoma/carcinoma assays.[90,144,145] Each of these assays has advantages for specific types of chemicals; for example, mouse skin is very responsive to polycyclic aromatic hydrocarbons and is appropriate for assessment of topical products.

Positive findings for initiation are considered to be highly indicative of potential carcinogenic activity,[157] likely through DNA reactivity. For example, the rodent hepatocarcinogen tamoxifen, which is DNA-reactive,[95] produced a rapid initiating effect in rat liver.[158] Promoting activity also suggests a potential for carcinogenic activity,[157] likely through epigenetic effects. In either case, it is possible to establish dose-effect data and no-effect levels to direct the design of chronic bioassays for risk assessment.

Another type of LCB, employs genetically engineered mouse models[159,160] in some of which the principal genetic targets include specific oncogenes (H-ras model), tumor suppressor genes (p53 model), or the entire genome in DNA-repair deficient animals (XPA −/− deficient model). Four models that have been most frequently used are the p53 heterozygous mouse (p53±), the Tg·AC mouse, the CB6F1-Tg ras H2 mouse, and the XPA−/− mouse. The studied transgenic models have responded appropriately to a number of carcinogenic, mostly DNA-reactive, and non-carcinogenic compounds.[159,161] Accordingly, they have been proposed as alternatives to the cancer bioassay in mice and accepted as providing evidence of carcinogenicity.[157]

The newborn mouse also has been used as a LCB.[162] In this model, newborn mice of any strain are administered the test substance by intraperitoneal injection or gavage usually at days 8 and 15 after birth and then held for observation for up to 1 year of age. The model exhibits high sensitivity to DNA-reactive carcinogens but is unlikely to respond to epigenetic agents because of the limited dosing used.

Another model is the *In Ovo* Carcinogenicity Assay (IOCA) using turkey eggs in which histological effects in the embryo-fetal liver are assessed.[163–165] The test conditions are similar to TEGA, described above, except that dosing is done on day 1 to provide the longest possible duration for liver lesion development. Fetal livers are harvested prior to hatching for histopathological examination for preneoplastic/neoplastic-like lesions. The key histopathological features are foci and

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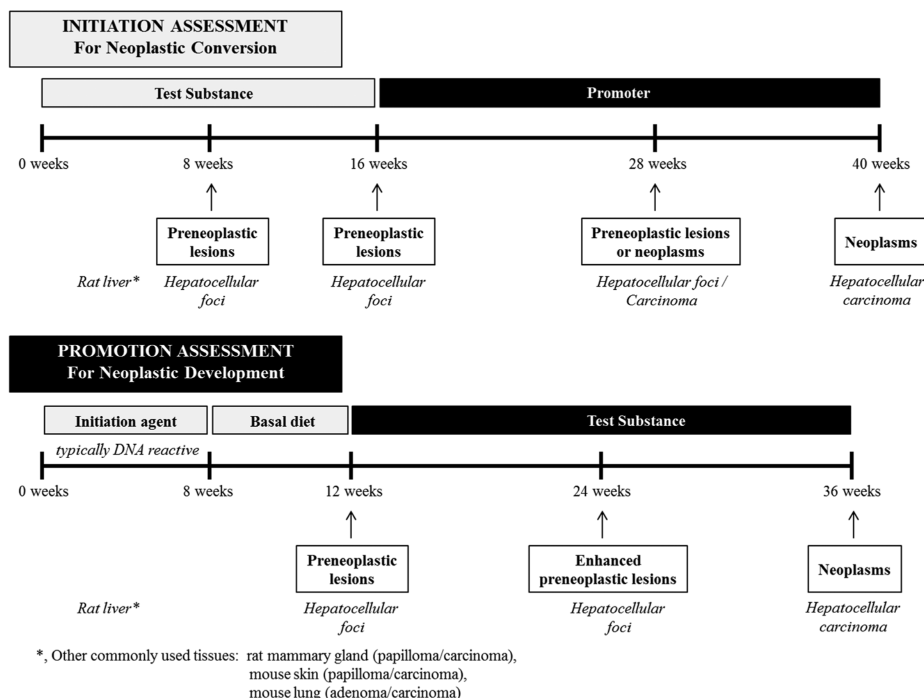

Fig. 5 Limited carcinogenicity bioassay (LCB) for initiation and promotion.

nodules of altered hepatocytes, displaying the phenotypic alterations of established carcinogen-induced lesions in rodent liver.[163,164,166] An inter laboratory validation of this assay has been reported, documenting responsiveness to DNA-reactive agents.[167] This assay allows monitoring of the processes of hepatic proliferation, differentiation, cellular migration, apoptosis, and developmental effects.[135,166] It also has the advantage of being defined as a non-animal method for carcinogenicity testing.

At this stage (A–D), data on potential carcinogenicity is available to assist in making a decision.

Stage E involves cancer bioassays. One is the accelerated cancer bioassay (ACB) model which can be used to obtain data on carcinogenicity when there is not a requirement for a full bioassay or there is need to rapidly secure data.[168] It also can be used as an alternative cancer bioassay in a second species. The ACB is essentially a composite of six or more initiation/promotion LCBs for rodent organs in which carcinogenicity has been found for known human carcinogens (*i.e.*, liver, lung, kidney, urinary bladder, stomach, hematolymphoreticular tissue and mammary gland). The protocol consists of two segments: one in which the test substance is administered at the maximum tolerated dose for 16 weeks in an initiation segment followed by promoters for the relevant target organs, and a second part in which the test substance is administered in a promotion segment at the maximum tolerated dose for 24 weeks after administration of initiating agents for the target organs and tissues to be interrogated (Fig. 6). The test substance is also given alone for 40 weeks to assess carcinogenicity.

The ACB has a number of valuable features: (1) it takes less time than the chronic bioassay, as the name implies; (2) it provides mechanistic data on initiation/promotion; and (3) the animals exhibit much less age-related pathology at termination since they are less than 1 year of age at the end of the study whereas background rodent neoplasms occur predominately after 50 weeks.[1] The principal limitation is that the ACB is not as comprehensive as the chronic bioassay, although it has been calculated that the initiating/promoting model is as sensitive as a chronic bioassay because of the promoting stimulus for tumor development.[56,169] Moreover, the ACB, with the addition of bone marrow, interrogates all target tissues affected in rodents by known human DNA-reactive carcinogens (Fig. 6).

If testing progresses to stage E, the chronic bioassay, well defined protocols must be used.[1] Usually, cancer bioassays are required to be conducted in rats and mice, although other species (*e.g.*, hamster) may be used when appropriate. For pharmaceuticals, the value of a mouse cancer bioassay has been questioned.[170] Nevertheless, a mouse cancer bioassay would be indicated when (1) the biological effect of the chemical is best expressed in mice, (2) the chemical has a pharmacologic effect on the gall bladder (present in mice but not in rats), or (3) mice are more representative of human chemical kinetics.[93]

A comprehensive evaluation of carcinogenicity studies of medicinal products that were reviewed in the European Union has been compiled for the years 1995–2009.[171] Due to the high number of rodent tumor findings with unlikely relevance for humans, the value of the currently used testing strategy for carcinogenicity appears questionable. A revision of the carcinogenicity testing paradigm which presently relies primarily on the chronic bioassay is warranted, and should take into account the mechanistic considerations discussed above.

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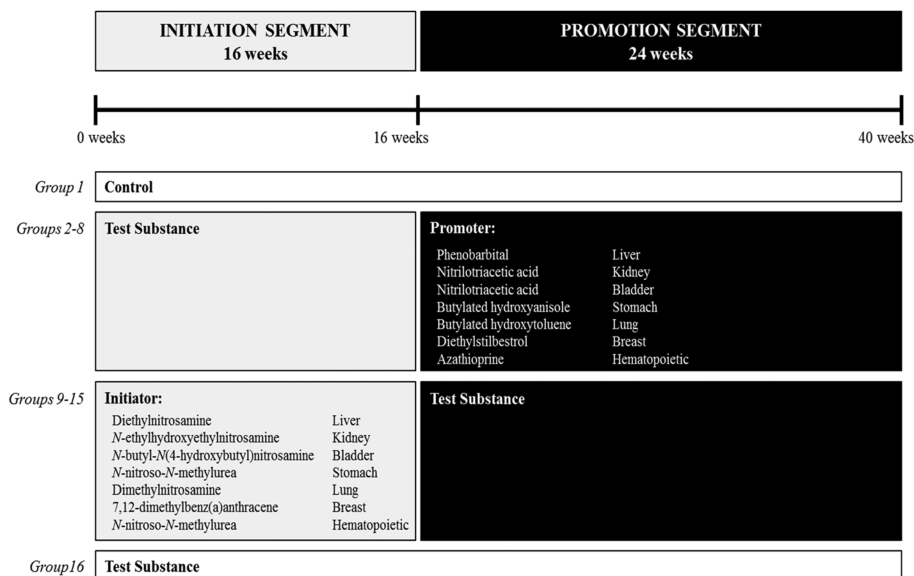

Fig. 6 Accelerated carcinogenicity bioassay (ACB).

Human risk assessment

The knowledge that carcinogens differ in their mechanisms of action directs the requirement for appropriate means for assessing the potential risks to humans of specific carcinogen intake, as opposed to the common assumption that all rodent carcinogens represent human hazards. In human risk assessment, there are two principal considerations: (1) relevance of the carcinogenic mechanism of action to humans and (2) the margin of exposure between the lowest active dose in rodent carcinogenicity studies and the highest human intakes. For example, chemicals such as the pharmaceuticals omeprazole, phenobarbital and clofibrate are carcinogenic in rodents but not in humans with substantial medical use. This reflects the fact that they must produce the cellular effects stemming from substantial intakes in order to express carcinogenic activity in animals. Thus, they do not represent the same potential hazard to humans, for example, as the DNA-reactive industrial agents bis(chloromethyl) ether and vinyl chloride, which are carcinogenic in humans with occupational exposures (Table 2). It is undoubtedly for this reason that drugs with epigenetic mechanisms of carcinogenicity in rodents, although used extensively as human medications, have not been associated with risk of human cancer, in contrast to the occupational DNA-reactive chemicals.[59]

As detailed above, most human carcinogens are DNA-reactive (Table 2) and thus, this type of carcinogen clearly represents a potential human cancer hazard.[35,88] Not all exposures to such agents, however, lead to cancer.[172] This is because thresholds exist for their effects (see papers in Greim and Albertini,[173] and Nohmi and Fukushima[58]). The concepts of dose-effect relationships and thresholds are discussed in detail by Calabrese.[174] The most extensive investigations of thresholds have been in rat liver carcinogenesis where no-observed-adverse-effect-levels (NOAEL) have been documented for DNA adduct formation,[57] induced cell proliferation,[56] formation of preneoplastic lesions[56,175,176] and development of promotable neoplasms.[169] The accumulated data base supports the concept of thresholds for effects of DNA-reactive carcinogens.[89]

Several epigenetic carcinogens, either neuro-hormonal-active substances or immunosuppressants, when administered to humans under therapeutic conditions, have been associated with increases in cancer (Tables 1 and 2). These few chemicals were carcinogenic to humans at intakes at which they produced the cellular effects required for carcinogenicity in animal models, including enhanced cell proliferation.[177] Nevertheless, humans are exposed to numerous epigenetic rodent carcinogens, both synthetic (*e.g.* the antioxidant butylated hydroxyanisole) and naturally occurring (*e.g.* limonene), without evidence of adverse effects. In some cases this is due to the fact that the mechanisms of action for rodent carcinogenicity are not operative in humans, as with limonene, an α2μ-globulin nephropathy inducer.[178] Also, most human intakes, with the exception of some drug treatments, do not exceed the threshold for the cellular or tissue effect leading to rodent carcinogenicity.

A variety of approaches to risk assessment have been proposed, ranging from linear no-threshold (LNT) extrapolation to sophisticated physiologically-based pharmacokinetic (PBPK) models.[174,179,180] Given the current knowledge of mechanisms of carcinogenesis, risk assessment procedures must be based on mechanistic considerations and involve a case-by-case analysis entailing evaluation of all relevant biological effects.[181–183] The US EPA[184] and other bodies have adopted procedures involving mechanistic considerations in assessing risk. Current mechanism-based methods for risk assessment are described in several publications.[185–187]

Table 5 Toxicologically insignificant daily intake (TIDI) for carcinogens

$$\text{Toxicologically insignificant daily intake(TIDI)}^a = \frac{\text{No adverse effect level (NOAEL)}}{\text{Safety margin (SM)}}$$

where:

• NOAEL is a no adverse effect level for the molecular/cellular effect that is the basis for carcinogenicity, *i.e.*, DNA adducts or epigenetic effects
• Safety margin (SM) is a multiple of uncertainty factors (UF) for short term to long term intake not needed if molecular/cellular effect has reached steady state:
- 10 for species to species extrapolation
- 10 for individual variation

[a]Based on Williams (2008).[168]

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The prevalence of intake of a specific chemical largely varies among populations and a complexity of modifying factors can be simultaneously present in the environment. This makes it difficult to precisely evaluate the contribution of a single chemical to global public health. In order to improve exposure assessment, molecular cancer epidemiology can be useful in development and validation of new biomarkers of exposure,[188] including epigenetic "fingerprints".[189] In addition, even when an association between an environmental chemical and cancer development is established, such association does not usually provide a mechanistic explanation for the adverse effect.[189] Thus, the Next Generation of Risk Assessment initiative was developed by U.S. Environmental Protection Agency in an effort to incorporate the most recent molecular, computational and systems biology approaches for enhanced risk assessment.[190]

An approach to estimating human intakes that do not convey a significant cancer risk, was suggested by Williams[168,191] to be the calculation of a "toxicologically insignificant daily intake" (TIDI), given in Table 5. The TIDI, uses mechanistic data. For an epigenetic agent, increased cell proliferation is often the most sensitive endpoint to establish a NOAEL for calculation of the TIDI, whereas for a DNA-reactive carcinogen, the molecular effect for a NOAEL that would be used to calculate the TIDI is DNA binding. A level of binding of less than 1 in 10^9 nts,[168] can be assumed to be biologically insignificant based on the fact it represents only 7 adducts per cell, which is well below the level of endogenous DNA modification of over 10 000 bases per cell per day.[192–195]

Human cancer causation

The IARC and NTP have identified over one hundred human carcinogens, including specific chemicals (Table 2) and mixtures.

The first identified chemical causes of human cancers were occupational exposures.[196] Further investigation revealed that some of the most important causative agents in human cancer were voluntary lifestyle practices. This was detailed in 1931 in a much neglected meeting report by Hoffman,[197] a biostatistician for the Prudential Insurance Company who was a pioneer in the study of cancer mortality.[198] Based on data collected from cancer patients and analysis of cancer occurrence patterns, he concluded that "smoking habits unquestionably increase the liability to cancer of the mouth, the throat, the esophagus, the larynx and the lungs". Later work by the epidemiologist Doll and coworkers of Oxford University confirmed the conclusions of Hoffman. Meanwhile, the team of Wynder and Hoffmann of the Sloan-Kettering Institute for Cancer Research and later the American Health Foundation, undertook extensive analysis and bioassay of tobacco smoke components to identify carcinogenic substances therein.[199,200] Using mainly the mouse skin model of carcinogenicity, they found both initiating and promoting effects of tobacco smoke components, indicating the presence of what were later identified as DNA-reactive and epigenetic agents.

Hoffman also implicated other lifestyle factors in cancer etiology, including heavy alcohol use. Subsequently, consumption of diets high in fat and low in fiber in Western communities[198] or diets with excessively salted, pickled or smoked foods in the Orient and in northern and Eastern Europe were identified as risk factors.[201] Burkitt, a physician practicing in Africa, who first described what is now known as Burkitt's lymphoma,[202] called attention to the high dietary fiber consumption in populations with low prevalence of diseases common in Western population including colon cancer.[203]

In a comprehensive analysis of epidemiologic data from the 1970s, Doll and Peto[204] quantified the avoidable risks for cancer in the United States population. They found that 30% of current US cancer deaths (respiratory tract) was due to tobacco. Apart from this, the common types of cancer were not particularly modern diseases and were proposed to result from some long established lifestyle factors, *i.e.* diet. The proportion of cancer deaths attributed to occupational factors was estimated at 4%.

In a follow up to the analysis of Doll and Peto, in 2010, Parkin *et al.*[205] estimated that past exposures to 14 lifestyle factors was responsible for the development of over 40% of cancers in the United Kingdom. Among these factors, tobacco smoking, alcohol consumption, diet and obesity were considered to be responsible for almost one third of all cancer incidences.

Some lifestyle practices result in exposures to DNA-reactive carcinogens,[206] as is the case with tobacco smoke[207] and pickled or smoked foods and broiled meat.[208] Other lifestyle practices, such as consumption of a high fat diet, contribute epigenetic enhancing agents.[198,206] Although practices entail-

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ing exposure to either type of agent can be associated with an increased risk of cancer, an important feature of epigenetic effects is that with cessation of intake, tumor progression is arrested and is potentially reversible, even at a stage at which cellular alteration is present.[209,210] There is also evidence that such effects in humans are reversible; for example, regression of liver tumors has been observed following cessation of use of oral contraceptives[211] and rapid reduction in risk of endometrial cancer was reported after decrease in estrogen use.[212] In contrast, with limited exposure of animals to carcinogens of the DNA-reactive type, cancer occurrence increases with time after exposure.[213,214] This reflects the fact that after neoplastic conversion, passage of time allows for the development of neoplasms from initiated cells. Likewise, with significant human exposures to DNA-reactive carcinogens, risk remains elevated with time, even after cessation of intake (*e.g.* cigarette smoking). Thus, while efforts must be directed toward identifying both types of carcinogen and minimizing their intake by humans, control of DNA-reactive carcinogens should have greater priority and be more stringent than for epigenetic factors. Thus, the distinction between mechanistically different types of carcinogenic agents has important implications for public health practices.

Conclusions

It is now well established that chemicals with carcinogenic activity operate through different mechanisms of action, which can be grouped as DNA reactive or epigenetic (non-genotoxic). Methods for identification of carcinogens of these two types are available, as detailed in Carcinogen Testing and Hazard Identification. DNA-reactive and epigenetic agents represent different types of hazards to humans and this should inform public health practices.

Abbreviations

ACB	Accelerated cancer bioassay
CEGA	Chicken egg genotoxicity assay
Comet	Alkaline single cell gel electrophoresis assay
DDT	Dichlorodiphenyltrichloroethane
DPA	Decision point approach
IARC	International Agency for Research on Cancer
IOCA	*In ovo* carcinogenicity assay
LCB	Limited carcinogenicity bioassays
miRNA	microRNA
NOAEL	No-observed-adverse-effect-level
NPL	Nucleotide P^{32}-postlabeling assay
NTP	National Toxicology Program
PPARα	Peroxisome proliferator activated receptor alpha
ROS	Reactive oxygen species
TEGA	Turkey egg genotoxicity assay
TIDI	Toxicologically insignificant daily intake
UPLC-MS/MS	Ultra performance liquid chromatography-tandem mass spectrometry

Conflicts of interest

There are no conflicts to declare.

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