Approaches to the Risk Assessment of Genotoxic Carcinogens in Food: A Critical Appraisal

ANNEX
Introduction to Genetic Toxicology


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Abstract

This review document addresses the processes of DNA damage, repair and mutation as key steps on the pathway towards cancer development and also briefly describes the most common genotoxicity tests used in the evaluation of whether a compound is genotoxic or not. Knowledge that the carcinogen under evaluation is genotoxic or non-genotoxic is a vital stage of the risk assessment and influences the handling of the human health issues arising from the compound. It is not possible within the scope of this document to provide guidance on the strategies used for concluding whether a compound is genotoxic as this can be a very complex evaluation and is best conducted with expert input. However it is hoped that this review provides an insight into the background behind genotoxicity testing, leading to a better understanding of the genotoxicity tests involved, what they can and cannot predict and the underlying processes that lead to the data produced.

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1 Genotoxicity, DNA Damage, Repair and Mutation

1.1 Introduction

All living organisms are continuously exposed to both endogenous and exogenous agents that can interact with the vital cellular components. Genotoxicity is a general term referring to agents that interact with DNA or the cellular machinery that regulates the fidelity of the genome. A mutation is defined as a permanent change in the amount or structure of the genetic material of an organism which may result in a heritable change in the characteristics of the organism involving single genes, blocks of genes or whole chromosomes. While DNA damage and mutation are both genotoxic endpoints, the presence of genotoxicity such as DNA damage is not indicative of mutation(s). Mutations can lead to a permanent change in the protein transcribed from the mutated gene resulting in an abnormal or inactive protein. If the mutation occurs within a gene and/or the important areas related to their transcription, it can result in a dormant gene being switched on or to an active gene being switched off. Two important types of gene are targets for mutations leading to neoplasia: proto-oncogenes and tumour-suppressor genes. Proto-oncogenes are genes coding for proteins controlling, for example, signalling, growth, or the cell cycle. Once activated a proto-oncogene becomes an oncogene resulting in permanent over-expression of a protein resulting in cell division. An example of an oncogene is the ras gene. Tumour-suppressor genes code for proteins that inhibit cell proliferation. The best known is p53. Mutations of the p53 gene are found in ~60% of human tumours.

Gene mutations are changes in the base pair sequence of the DNA strands. Chromosome aberrations can either be structural or numerical. Structural chromosomal aberrations are breaks in chromosomes. These breaks can give rise to chromosomal rearrangements if the ends of broken chromosomes fuse to each other in an incorrect manner. Numerical chromosomal aberrations (aneuploidy) are changes in the number of chromosomes per cell due to loss or gain of chromosomes during cell division. It is thought there are no chemicals that induce exclusively gene mutations or chromosome aberrations, but compounds can have predominantly one effect.

Maintenance of the integrity of DNA is essential to ensure sustained life of the cell and its optimal function according to the given cell type and developmental state. As a result of this, the cell has developed a variety of protective mechanisms to preserve the integrity of its genetic information and its function for DNA. These protection mechanisms are

i) The prevention of DNA damage e.g. anti-oxidant mechanisms to protect against oxidative agents;
ii) DNA repair pathways to remove damage after it has occurred;
iii) Cell cycle arrest to allow the cell time to repair the DNA damage before replication, and;
iv) Apoptosis, or programmed cell death, which allows multicellular organisms to selectively remove excessively damaged cells.

However, none of these protective mechanisms is completely effective and unrepaired DNA damage can be converted to a mutation by DNA replication. If the mutation occurs in the non-coding area of the genome and not in the areas regulating transcription, it is unlikely to have any affect on the phenotype of the cell and thus
mutations in these areas are of less consequence to the overall health of the organism. Mutations within genes are known to accumulate over the lifespan of the cell and/or organism.

1.2 DNA Damage

The human genome consists of approximately $3 \times 10^9$ bases, in a sequence produced from only four bases, namely adenine (A), cytosine (C), guanine (G) and thymine (T). 5-Methylcytosine (MeC) is an analogue of cytosine, base-pairs as if it is cytosine and has an important role in regulating the transcription of genes.

DNA is formed of two strands of bases bound via the strict pairing of the bases: A binds only to T and C only to G. This pairing is essential for the mechanism of DNA replication, where a new strand is formed by “reading” the opposite strand and inserting the correctly pairing base. These bases are converted into proteins using sets of three bases, known as a triplet or codon, each of which codes for one of the 20 amino acids DNA damage normally affects a single base but can affect more than one at a time, up to and including the whole chromosome. Cells within the human body are continually exposed to DNA damaging agents, from either endogenous or exogenous sources.

The main sources of endogenous DNA damage are hydrolytic damage and oxidative damage resulting from the by-products of intermediary metabolism.

DNA damage can occur from exposure to exogenous as agents that are both natural and man-made including, dietary contaminants, natural constituents and chemicals that are known to interact with DNA. In addition, DNA damage and mutations may be induced by radiation such as UV light. The main sources of the exogenous DNA damage afforded by chemicals are alkylating agents, amines, episulphonium ions, bulky adduct formers, cross-linkers, topoisomerase II inhibitors and oxidizing agents. Some of the DNA damaging agents act directly, whereas others are formed via metabolic activation reactions including oxidation and conjugation pathways such as with GSH (glutathione). A diversity of chemical classes have been implicated in DNA damage. Structure-activity relationships may permit the identification of agents with potential to cause DNA damage by a variety of mechanisms.

Alkylating Agents - Alkylating agents (e.g. diethylnitrosamine) transfer an alkyl group, normally methyl or ethyl, to nucleophilic sites on one of the bases. Alkylation that prevents correct base pairing, such as methylation at the O$^6$ of G, is consequently mutagenic whereas methylation at the N-7 of G does not affect base-pairing and is not mutagenic per se even though it may result in apurinic sites which in turn are targets for chromosome breaks. Thus the total DNA methylation does not necessarily yield information on the level of mutation.

Amines - Aromatic amines (e.g. 2-acetylaminofluorene), heterocyclic amines (e.g. 2-amino-3,4-dimethyl[4,5-f]quinoline (MeIQ)) and polycyclic aromatic amines (e.g. 2-
aminoanthracene) react with DNA via a series of reactions starting with an oxidation reaction generating a hydroxylamine and culminating in the electrophilic nitrenium ion, RNH\(^+\) (Colvin et al., 1998).

**Molecules that are activated by GSH**

For the majority of compounds, glutathione conjugation is a detoxification pathway but, for some chemicals, it can result in transformation into a mutagen. For instance, dibromoethane is metabolised via two routes, the second of which results in nucleophilic substitution of thiolate for halide via a reaction catalysed by glutathione S-transferase (GST) to produce an S-haloalkylglutathione conjugate. This can then spontaneously rearrange to eliminate another halogen atom, creating an episulphonium ion (RSG\(^+\)), which reacts with DNA (Sherratt et al., 1998).

**Bulky adduct formers** - These chemicals normally bind as the whole chemical to one of the bases (e.g. at the N-2 of G) forming a large structure e.g. oxidation products of aflatoxin B\(_1\) and benzo(\(a\))pyrene. This large structure does not normally affect the direct binding of the base pairs but instead alters the gross structure of the double strand of DNA.

**Crosslinkers** - These chemicals crosslink either adjacent bases in the DNA strand (intrastrand) or two opposite bases (interstrand) e.g. mitomycin C. Intrastrand crosslinks are seen by the DNA replication machinery as a single basepair. The interstrand crosslinks prevent the two DNA strands separating to allow replication and transcription of the DNA strand.

**Topoisomerase II Inhibitors** - Topoisomerase II is an enzyme which cleaves DNA to facilitate unwinding of the strands for replication or transcription. An example of a topoisomerase inhibitor is etoposide. If inhibited, replication and transcription are compromised and DNA strand breaks are often formed.

**Oxidizing Agents** - The effects of oxidizing agents have been described under endogenous agents above. Hydrogen peroxide and glyoxal are examples of exogenous chemicals that can also arise endogenously.

### 1.3 DNA Repair and Apoptosis

Cells have evolved various mechanisms of DNA repair, to address the many types of damage inflicted on the DNA. Some repair mechanisms are very specific to distinct types of DNA damage, whereas others will detect a broad range of types of DNA damage. Repair mechanisms fall into 5 classes - base excision repair, nucleotide excision repair, direct damage reversal, chromosome break repair and mismatch repair (Mullaart et al., 1990). These pathways work together to reduce the spontaneous mutation rate to approximately 1 base-pair change in 10\(^9\) to 10\(^12\) (Friedberg et al., 1995).

Base excision repair involves the direct removal of a single affected base pair and its replacement with the correct base. The enzymes involved in this process are specific to particular types of damage. Nucleotide excision repair (NER) recognizes and removes a wide spectrum of lesions that cause large local distortions of the DNA structure, including bulky chemical adducts and some DNA crosslinks (reviewed by Wood, 1997). The
damaged DNA is excised as an oligonucleotide and the resulting gap is filled by DNA polymerases and DNA ligase, using the undamaged strand as a template.

Base excision repair (BER) involves the removal of a single modified base. BER is initiated with the release of altered bases by DNA glycosylases after hydrolytic cleavage. Direct damage reversal involves the enzymatic conversion of damaged nucleotides to their original intact state e.g. the direct removal of the methyl group from O\textsuperscript{6}-methyl adducts by methylguanine methyltransferase. Two repair systems are responsible for correcting chromosomal breaks: homologous recombination repair (HR) and non-homologous end joining (NHEJ). HR is considered an error-free system that restores the chromosome break on the basis of homologous sequence from the sister chromatid or the homologous chromosome. In contrast, NHEJ can give rise to deletions or chromosomal rearrangements since it directly ligates broken ends at regions of little or no homology. Mismatch repair is a poorly defined process occurring principally post-DNA replication to correct bases that are not pairing correctly or where there are small insertions or deletions in the DNA strand.

Once DNA damage is detected in the cell, signalling proteins delay the cell cycle to allow time for DNA repair to occur. This signalling is a vital step to minimize the number of mutations in the cell but can be over-ridden by agents that force the cell into replication (mitogens) or that interfere with the signalling process.

Although not strictly a repair mechanism, apoptosis prevents DNA damage from being passed on to other cells in the whole organism. It is cell death initiated by the cell itself and contrasts with necrosis, cell death caused by damage or infection. Apoptosis is initiated in response to high levels of DNA damage only in the cells of multi-celled organisms and, rather than waste resources on a large amount of DNA repair, the cell is destroyed for the apparent good of the whole organism. The signal transduction pathways leading to apoptosis are not fully understood, and it is probable that some of the genes involved are not currently known. One pathway involves p53 and, as this is the pathway involved in the induction of apoptosis following DNA damage, it is of primary interest in carcinogenesis. However, the function of p53 varies between cell types and other pathways also initiate apoptosis. Mutations in proteins involved in apoptosis can be highly deleterious, as the cell cannot enter apoptosis. An absence of apoptosis has been strongly associated with cancer, as can be demonstrated by the correlation between mutated p53 genes and cancer in humans, whereby mutated p53 genes are present in ~ 60% of cancers, a figure that rises to 90% for skin cancers (IARC TP53 Mutation Database http://www-p53.iarc.fr).

1.3.1 Targeting of DNA Repair
DNA repair is preferentially targeted to the essential areas, such as areas of the genome undergoing transcription and, within that, to the strand of DNA undergoing transcription. Next to this transcription-coupled repair, the integrity of the genome is preserved regularly by genome overall repair. It should be noted, however, that the majority of this evidence originates from nucleotide excision repair research.

Efficient removal of DNA damage from transcribed sequences is thought to enhance cell survival by enabling cells to express essential genes before removal of all DNA damage has been completed. Repair of damaged DNA is further targeted within the transcriptionally active strand of the gene (Mellon et al., 1987; Mellon and Hanawalt, 1989), although some does occur on the non-transcribed strand, albeit at a low level. In
addition to strand specificity, the rate of repair is determined by a complex interplay between adduct structure, the accessibility to repair enzymes, the ability to arrest transcription and the local DNA conformation (McGregor et al., 1995).

1.4 Mutation

If DNA damage is not repaired or if the cell is stimulated to divide prior to completion of DNA repair, it is likely that the DNA replication machinery will convert the DNA damage into a mutation. When DNA replicates, it uses one strand of the DNA helix as a template on which to copy the opposite strand, using the unique pairing of the bases as a guide. For the majority of damaged cells attempting to replicate, the machinery stalls at the damage as it attempts to insert the correct base opposite the damaged one. The damage means the base pairing will appear incorrect, and the replication machinery will remove the base and insert another. This continues for a finite time until the damage is bypassed and a base is inserted at random, to prevent cell death. Mutation can be divided into:

**Point Mutation**: Mutation of a single base at a time. If a purine/pyrimidine base pair is replaced with a base pair in the same purine/pyrimidine relationship (e.g. GC with AT) it is called a transition. A transversion is a nucleotide-pair substitution involving the replacement of a purine with a pyrimidine (GC with TA) or vice versa.

**Frameshifts** - Insertion/deletion of one or two bases, altering the reading frame of the DNA subsequent to the mutated site.

**Insertions/Deletions** - Insertion/deletion of more than two bases, which may change the reading frame of the DNA.

Many of the methods of genotoxicity testing used to predict carcinogenic potential detect mutations resulting from the above events (see Section 2). In addition, the following events may result in mutations:

**Chromosomal breaks** – Exposure to chemicals may result in chromatid or chromosome breaks. Although cells with substantial breaks generally die before mutational events occur, incorrect repair of breaks can lead to deletions or chromosomal rearrangements (e.g. translocation, see Fig. 1 and dicentric chromosomes), which may result in activation or deactivation of relevant genes. A well-known example of a translocation is the Philadelphia chromosome, whereby part of chromosome 9 becomes attached to the end of chromosome 22 and is associated with chronic myeloid leukaemia.
Inversions  - An inversion is a chromosomal mutation involving the removal of a chromosome segment, its rotation through 180 degrees, and its reinsertion in the same location, in other words a section of a chromosome in the reverse orientation.

Chromosome Loss or Gain (Aneuploidy) - Interference of chemicals (e.g. carbendazim) with proteins involved in correct segregation of the chromosomes on cell division may cause chromosome loss or gain in the resulting daughter cells. Chromosome loss/gain is known to be a common state for tumorigenic cells, although whether aneuploidy is a cause or an effect is not known. Aneuploidy leads to loss of, under- or over-expression of genes (see Fig. 2).

Even if a mutation occurs in a coding region of the DNA or in one that controls gene transcription the mutation may not have a neoplastic effect. For point mutations, there is an in-built redundancy in the codons that code for some of the amino acids, such that the final base can vary while still coding for the same amino acid, thus not changing the protein. These types of mutation are known as silent or neutral mutations. Alternatively the codon may change to code for a different amino acid, which in turn will adversely affect the activity of the protein, or may change to the one of the STOP codons, TAA, TAG or TGA, which will lead to a truncated protein. On the other hand, for the ras oncogenes, deleterious mutations are clustered in three areas - codons 12-16, 59-63 and 116-119 – and a single mutation in these areas can serve to activate the normally inactive gene. For frameshifts, insertions and deletions, it is almost certain that the effect of the change in amino acid sequence will alter the resulting protein. Thus, as
most mutagens do not act selectively on specific targets, there is a stochastic element to determining whether a mutation will result in neoplasia or not.

Activation of oncogenes or deactivation of tumor suppressor genes can be particularly deleterious to the overall integrity of the cell as it affects its susceptibility to further mutation and, eventually, to tumorigenesis. Oncogenes are involved in signalling pathways that stimulate proliferation and, thus, increase tumor susceptibility e.g. \textit{ras}. Tumor suppressor genes are those involved in the checkpoints for cell proliferation or cell death and, thus, decrease tumor susceptibility e.g. \textit{p53} and \textit{Rb}. Although the above describes mutation, a similar effect is observed with aneuploidy, whereby the transcription regulation functions for an oncogene or tumor suppressor gene, or the gene itself, may be lost on the missing chromosome.

It should be noted that it is also possible that the mutation will result in direct cell death. If the mutation results in the inactivation of a gene critical for survival, either through direct inactivation (methylation, large deletions or aneuploidy) or through adverse effects on the resultant protein, the daughter cells will not survive. Although this cell death is not a deliberate form of protection against tumorigenesis, and is separate to the apoptosis induced by extensive DNA damage described earlier in this section, it nonetheless plays an important role in minimizing the overall rate of mutation in the living cells of the human body.

1.5 The Pathway to Cancer

The relationship between the number of mutations in a cell and its stage on the path to cancer are not subject to a formulaic relationship. A cell may contain many mutations but if these are not situated in critical genes, there will be little effect on the overall characteristics of the cell. Conversely, the cell could contain one or a few mutations but if these are located in critical genes, the overall characteristics of the cell could be vastly changed such that the cell is on the path of neoplastic development.

It is now acknowledged that cancer is a multi-step sequence, which involves multiple mutations in genes that control cell replication (so that the cell escapes the normal restrictions on growth) and govern the phenotype of the cell (such that they may secrete proteins atypical of their original cell type).

To present the pathway to a cancer cell in a simplified manner, there are 5 major processes that must be activated or inactivated to allow the formation of a cancer cell (amended from Bertram, 2001):

i. enhanced growth stimulatory signals (e.g. activated oncogenes)

ii. insensitivity to growth inhibitory signals (e.g. inactivated tumor suppressor genes)

iii. resistance to apoptosis (e.g. over-expression of \textit{BCL-2})

iv. infinite proliferative capacity (e.g. telomere length is retained)

v. angiogenic potential i.e. the capacity to form new blood vessels and capillaries.

Only once all these are fulfilled will the mutated cell become a malignant cancer cell able to form a tumor and invade surrounding tissue.
As has been discussed previously, DNA damage is not problematic *per se*. It is only when the damage is converted into a mutation following DNA replication and cell division that adverse consequences may occur. Proliferation is vital, first to fix the mutation in the cell and then to expand the number of cells bearing the mutation. Cells normally have multiple pathways to control cell division and, thus, transformed cells must accumulate multiple mutations in the critical genes controlling cell division to allow their autonomous replication.

It should also be noted that any DNA damage induced by exogenous factors is in addition to other DNA damage/mutation already present in the cell from endogenous factors or from previous exogenous factors.

Mutation in critical genes may provide the cell with the first three attributes of a cancer cell as described in the above list. In order to achieve the fourth attribute, cells must prevent the shortening of the telomeres, a process that normally results in cell death. The telomeres are found on the ends of chromosomes and consist in vertebrates of numerous repeats of \textit{T\textsubscript{TAGGG}} up to 6-12 kb long. Over the lifespan of a normal cell, the telomeres are gradually eroded, leading to chromosome instability, and this is a crucial event in limiting the lifespan of cells. Telomere length is monitored by \textit{p53}, which triggers apoptosis or senescence when a critical degree of shortening has occurred (Vaziri and Benchimol, 1999). Telomeres are maintained in essential cells such as the germ cells and cells of the haematopoietic system (Yashima et al., 1998) by an enzyme known as telomerase, but this enzyme is not expressed in healthy cells. Most human malignancies possess active telomerase, although it is not clear whether they have altered the activation of genes that control expression of telomerase or whether they originated from a stem cell with telomerase activity. It is thought that this may differ from organ to organ. At this stage in the process, the cell has become immortal and faces one last challenge – to ensure sufficient nutrients are available to allow continued growth. This can only be achieved through growing in suspension, e.g. ascites in the abdomen or circulatory system, or by angiogenesis, the creation of a new blood supply. Angiogenesis occurs in response to extra-cellular signals, such as vascular endothelial growth factor, and is balanced by inhibitors of angiogenesis, such as thrombospondin. Tumor cells can enhance expression of the pro-angiogenic factors and suppress expression of the inhibiting factors (Hahnfeldt et al., 1999). It appears that oncogenes and tumor-suppressor genes are also involved in this process; e.g. \textit{p53} is responsible for the expression of thrombospondin and, in its absence, expression is downregulated.

From all the above, it is clear that the tumor cell is the result of a considerable number of mutations that must occur in several critical genes within a given cell. Since these mutations are random, and only the successful ones will go forward into a neoplastic cell, each tumor cell clone is likely to contain a unique set of mutations. Furthermore, due to the genomic instability of cancer cells, this uniqueness will continue to evolve as the neoplastic cells progress and form a tumor. The pathology of the tumor and its effects on the whole organism will not be discussed here.
2. Genetic toxicology testing

2.1 Introduction
In genotoxicity testing there are three “endpoints”, gene mutations (comprising the previously described point mutation, frameshifts and small insertions/deletions), chromosomal aberrations (comprising the previously described large insertions/deletions and chromosomal breaks) and aneuploidy.

Genotoxicity testing is usually conducted within a strategy rather than as individual tests. Two major types of genotoxicity test strategies can be distinguished: a battery approach and a phased or tiered approach. In a battery approach 3-5 tests have to be carried out in order to produce a genotoxicity profile of the test substance. The result of each individual test is important and the final conclusion is based on a consideration of all the test results. The suggested standard set of assays does not imply that other genotoxicity tests are considered inadequate. Such tests may serve as options in addition to standard battery tests if further testing is needed in the case of positive results.

In a phased or tiered approach, further testing is dependent on the use of the compound and on the outcome of any previous tests. It starts with in vitro tests which are, when positive, followed by in vivo assays.

Before in vivo testing is undertaken it should be demonstrated, for instance by toxicokinetics or toxicodynamic studies, that the chemical under investigation reaches the target cells of the in vivo test. If it cannot be reasonably expected from all the properties of the test compound that the specific target tissue of a test will be adequately exposed, further investigation with in vivo tests is not meaningful.

If in vivo assays are positive, in vivo germ cell mutagenicity tests have to be considered to evaluate the putative genetic risk in future generations. However, germ cell mutagenicity will not be discussed further.

2.2 Genotoxicity tests
Although more than 100 different test-systems exist, only a limited number have been fully validated and are recommended for use. They were selected for reasons such as degree of standardisation, simplicity and reproducibility. Since 1980, member countries of the Organization of Economic Cooperation and Development (OECD) have prepared and adopted guidelines on these tests (Table 1). Of these 15 tests with an OECD guideline only a limited number is commonly used. The most commonly used assays are shown in bold italics and will be discussed further below.

In Table 1 these tests are divided in four groups. The first two comprise assays for measuring the induction of gene mutations and the induction of chromosome aberrations, thus covering two important endpoints of genotoxicity. The third group includes assays for measuring DNA effects, while a fourth group lists novel assays currently without an OECD guideline. These tests determine pre-mutagenic lesions and are therefore also known as indicator tests.
Table 1: OECD classification of mutagenicity assays and corresponding guidelines. In general these tests are accepted within EU countries.

<table>
<thead>
<tr>
<th>Assays for measuring the induction of gene mutations</th>
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<tbody>
<tr>
<td><strong>Test No. 471:</strong> Bacterial reverse mutation assay</td>
</tr>
<tr>
<td><strong>Test No. 476:</strong> In vitro mammalian cell gene mutation test (N.B. this assay also detects chromosome aberrations)</td>
</tr>
<tr>
<td>Test No. 477: Sex-linked recessive lethal test in Drosophila melanogaster</td>
</tr>
<tr>
<td>Test No. 480: Saccharomyces cerevisiae, gene mutation assay</td>
</tr>
<tr>
<td>Test No. 484: Mouse spot test</td>
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<table>
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<tr>
<th>Assays for measuring the induction of chromosome aberrations</th>
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</thead>
<tbody>
<tr>
<td>Test No. 473: In vitro mammalian chromosome aberration test</td>
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<tr>
<td><strong>Test No. 474:</strong> Mammalian erythrocyte micronucleus test</td>
</tr>
<tr>
<td><strong>Test No. 475:</strong> Mammalian bone marrow chromosome aberration test</td>
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<tr>
<td>Test No. 478: Rodent dominant lethal test</td>
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<tr>
<td>Test No. 483: Mammalian spermatogonial chromosome aberration test</td>
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<tr>
<td>Test No. 485: Mouse heritable translocation assay</td>
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<table>
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<tr>
<th>Assays for measuring DNA effects</th>
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<tr>
<td><strong>Test No. 479:</strong> In vitro sister chromatid exchange assay in mammalian cells</td>
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<tr>
<td>Test No. 481: Saccharomyces cerevisiae, mitotic recombination assay</td>
</tr>
<tr>
<td><strong>Test No. 482:</strong> DNA damage and repair/unscheduled DNA syntheses in mammalian cells in vitro</td>
</tr>
<tr>
<td>Test No. 486: Unscheduled DNA synthesis test with mammalian liver cells <em>in vivo</em></td>
</tr>
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**Novel Assays currently without an OECD guideline**

- *In vitro* micronucleus assay in mammalian cells (draft guideline nr 487)
- *In vivo* Comet assay
- Transgenic mouse/rat mutation assay

All three groups consist of *in vitro* as well as *in vivo* tests. Cells *in vitro* generally possess limited metabolising capacity towards most promutagens. Therefore, tests conducted *in vitro* require the use of an exogenous source of metabolising enzymes. *In vitro* metabolic activation systems cannot mimic entirely the mammalian *in vivo* conditions. Therefore, *in vitro* tests do not always provide information on the mutagenic potential of a substance in mammals. Care should also be taken to avoid conditions that would lead to results not reflecting intrinsic mutagenicity, for instance positive results that arise from changes in pH, osmolarity or high cytotoxicity.

2.2.1 Assays for measuring the induction of gene mutations

The bacterial reverse mutation assay (OECD 471), also known as the Ames test or the Salmonella test, is the most commonly used genotoxicity test. It uses primarily strains of *Salmonella typhimurium* but may also use strains of *Escherichia coli* (Ames et al., 1975; Maron and Ames, 1983). All strains contain a mutation, which makes them amino-acid dependent (histidine in *Salmonella*). The principle of this assay is that genotoxins induce reverse-mutations, which restore the capability to synthesize the essential amino acid.
Some bacterial strains have additional genetic adaptations, which make them more sensitive for the detection of mutations, possibly leading to an overestimation of mutagenic activity. A disadvantage of the assay is its use of prokaryotic cells, which differ from mammalian cells in metabolism, chromosome structure and DNA repair. The bacterial reverse mutation test may not be suitable for the evaluation of some classes of chemicals, e.g. antibiotics or compounds which interfere with the mammalian cell replication system, such as topoisomerase inhibitors. In such cases tests with mammalian cells are more appropriate.

The *in vitro* mammalian cell gene mutation test (OECD 476) detects chemically induced gene mutations in mammalian cells (Moore et al., 1987; Chu and Malling, 1968; Liber and Tilly, 1982). The commonly used cell lines measure mutations at the thymidine kinase (*tk*) or the hypoxanthine-guanuine phosphoribosyl transferase (*hprt*) gene. The principle of the test is that, following exposure to genotoxins, gene function is lost which makes the cells resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (selection for the *tk* gene) or the purine analogues 6-thioguanine or 8-azaguanine (selection for the *hprt* gene). An advantage of the selection using the *tk* gene is that colony sizing allows differentiation between the induction of gene mutations (large colonies) and chromosomal aberrations (small colonies).

Cells are exposed to a range of test concentrations that should cover a wide range of cytotoxicity; exposure to the highest concentration should result in approximately 10-20% relative survival.

### 2.2.2 Assays for measuring the induction of chromosome aberrations

The aim of the *in vitro* mammalian chromosome aberration test (OECD 473) is to determine chemical-induced structural chromosomal aberrations (Evans, 1976; Galloway et al., 1987). Assessment of the chromosome aberration test is made in cells arrested in metaphase by treatment with colchicine. The percentage of cells with aberrations, rather than the total number of aberrations, is used as the measure of genotoxicity. Although indications of aneuploidy (e.g. polyploidy) may occur, the test is not a definitive assessment of aneugenicity.

The mammalian bone marrow chromosome aberration test (OECD 475; Adler, 1984; Preston et al., 1987) is an *in vivo* form of the previous *in vitro* test. The test is routinely performed in rodents. Prior to sacrifice the animals are treated with colchicine to stop cell division of the target cells at metaphase. The target cells are bone marrow cells, since the bone marrow is highly vascularised (and therefore highly likely to be exposed to a test substance in the systemic circulation) and contains a high portion of rapidly dividing cells.

The test suffers from a tissue limitation (bone marrow). Another difficulty is that the active form of the test substance must reach the target cells in order to be effective. Without clear evidence of such exposure (e.g. decrease in mitotic index or toxicokinetic analysis of plasma concentrations) the value of the test is limited.

An alternative to the *in vitro* chromosomal aberration test is the *in vitro* micronucleus test (Fenech and Morley, 1985; Kirsch-Volders et al., 2003). Micronuclei are formed when chromosomal fragments or chromosomes remain behind during cell division, in the latter case, because the formation of the microtubuli, which pull the chromosomes
to the poles, or the attachment of the microtubuli to the centromeres is impaired. Micronuclei are usually, but not always, evaluated in binucleated cells that are formed following treatment with the actin inhibitor cytochalasin B. Cytochalasin B inhibits cell division but not nuclear division resulting in binuclear cells. As cell division is an essential step prior to formation of micronuclei, scoring of micronuclei in binucleated cells increases the sensitivity of the assay. A draft OECD guideline (Test No. 487) is available. In a retrospective validation study an expert panel (Albertini et al., in press) concluded that the in vitro micronucleus test can be regarded as sufficiently validated and can be recommended as an alternative/replacement for the in vitro chromosome aberration test.

The mammalian erythrocyte micronucleus test (OECD 474) is the in vivo form of the previous test (Heddle et al., 1983). The measurement of micronuclei in peripheral blood cells is as acceptable as the bone marrow test, provided the animal does not remove micronucleated peripheral blood cells via the spleen. The pitfalls of this test are identical to those of the mammalian bone marrow chromosome aberration test (OECD 475).

The big advantage of the micronucleus test, either in vivo or in vitro, is the possibility to determine both structural as well as numerical chromosomal aberrations. In order to differentiate between these two endpoints, fluorescence in situ hybridization (FISH) and specific DNA probes can be used. For FISH, the presence of a whole chromosome within a micronucleus is determined using centromere-specific stains (centromeres form the center of the chromosome). Thus micronuclei with centromere staining should contain whole chromosomes, indicative of aneuploidy, and micronuclei without centromere staining should contain chromosome fragments, indicative of structural aberrations. Alternatively, chromosome specific probes can identify specific chromosome loss or gain from individual cells.

2.2.3 Assays for measuring DNA effects

The in vitro sister chromatid exchange (SCE) assay in mammalian cells (OECD 479) utilizes the reciprocal exchanges of DNA between two sister chromatids of a duplicated chromosome at apparently homologous loci (Perry and Thomson, 1984). Chemically-induced DNA damage results in increased occurrence of exchanges possibly due to repair processes. The disadvantages of the SCE test are that it is an indicator test and that the mechanism and relevance of SCE induction are unknown.

The basis of the DNA damage and repair/unscheduled DNA synthesis test in mammalian cells in vitro (OECD 482; Cleaver and Thomas, 1981; Cleaver, 1984) and the unscheduled DNA synthesis test (UDS) in mammalian liver cells in vivo (OECD 486; Ashby et al., 1985) is repair of damaged DNA. During the repair processes the damaged DNA is excised after which DNA polymerases fill the gap. If labelled nucleosides are present they will be introduced into the DNA. The quantity of radioactivity is visualized as grains of silver stain and the number of grains are indicative for DNA damage and thus for genotoxicity. Cells in scheduled cell division can be readily excluded from the analysis as they are fully stained. The in vitro test is predominantly performed in primary hepatocytes; the in vivo one uses the liver of treated rats. However, other cell types or other tissues may be used.
Since a standardized in vivo gene mutation test is still lacking, the UDS test is often used as surrogate test for an in vivo gene mutation test. The UDS test is particularly valuable to detect repair of 20-30 base pairs. The sensitivity decreases if the repair comprises lesser numbers of base pairs. As for other in vivo assays, the in vivo UDS assay also suffers from tissue restriction as it is predominantly used in the liver. Next the UDS test suffers from a compound restriction since only those compounds can be recognized as DNA damaging which induce damage repaired by transcription repair. As before, indications of tissue exposure are necessary as measured by toxicity to the hepatocytes or toxicokinetic analysis of the plasma of exposed animals.

2.2.4 Other assays to detect DNA effects
The lack of an in vivo gene mutation assay makes judgment of the genotoxic potency of some chemicals problematic. Modern molecular techniques made it possible to develop transgenic mice. These animals contain many copies of a transgene, lacZ or lacI of E. coli, as a reporter gene, which is transmitted by the germ cells and thus is present many-fold in all cells of the body. Two transgenic mice models (Gossen et al., 1989; Kohler et al., 1991) and one transgenic rat model (Dycaico et al., 1994) are commercially available.

Tests with transgenic animals have several advantages. Theoretically there is no tissue restriction since the transgene is transmitted by the germ cells. The models appear to have good reproducibility but with a high spontaneous mutation frequency. These commercial models only detect point mutations, insertions and small deletions, but not large deletions. In contrast to the above-mentioned bacteriophage-based models, plasmid vector based models (Gossen et al., 1993) allow the detection of large deletions. No OECD guidelines are available although a review paper for OECD containing a consensus protocol was published recently by Lambert et al. (2005).

The single-cell gel electrophoresis assay or ‘comet’ assay allows the determination of single or double-strands DNA breaks, phosphotriesters and alkali labile sites (Tice et al., 1990) in every tissue from which a single cell suspension can be generated. The comet assay can be performed in vitro as well as in vivo. The basis of the comet assay is that, broken DNA fragments, in cells that have undergone lysis followed by DNA unwinding, move under the influence of an electric field from the nucleus in the direction of the anode resulting in the formation of DNA tails (“comets”). The test is very sensitive but care is needed in the experimental design and in the choice of parameters to be measured (Kim et al., 2002) and to ensure cytotoxicity does not interfere with the evaluation.

The production of DNA adducts can be measured using the covalent binding of radiolabelled test substances or using the 32P-postlabelling assay. 32P-postlabelling involves the digestion of tissue following exposure to unlabelled test substances and the labelling of the nucleoside 3-monophosphates with 32P. The adduct-containing nucleosides may be detected by chromatography and autoradiography.

2.3 Evaluation and interpretation of genotoxicity results
Evaluation of genotoxicity data should be done with care. At first, one has to be sure that individual tests were carried out properly and that the conclusions are justified. Concerning the performance of the different tests, criteria are described in the OECD
guidelines. For tests without an OECD guideline, it might be possible to propose criteria derived from existing guidelines of comparable tests or from a review of the published literature, although this would be a labour-intensive task.

For all in vivo tests, the most important criterion is exposure of target cells or tissues. Conclusions on tests without clear target cell exposure may easily lead to false negatives. Consequently, in every test actual exposure of target cells has to be demonstrated, for instance by measuring toxicity or from toxicokinetic analysis.

There are several criteria to describe a positive response, such as a dose response relationship or a reproducible increase in one single measure-point. For detailed information on the statistical treatment of mutagenicity data, see the monograph by Kirkland (1989). Biological relevance should always be considered first and is more important than statistical significance. Statistical significance should never be the only determining factor.

Several other factors may question the value of a positive response, such as changes in pH, osmolarity or positive results exclusively at very high exposure levels. Also for negative results, factors other than exposure and toxicity should be accounted for such as the volatility and the reactivity of the chemical and the possibility of an inappropriate metabolism system in in vitro tests.

It is not always easy to conclude whether a compound is genotoxic or not because three endpoints, structural and numerical chromosome aberrations and gene mutations, have to be covered and often several genotoxicity tests have been performed. It certainly is not a question of counting the positive and negative outcomes. The conclusion should be reached by a weight of evidence approach. If the conclusion is not immediately unambiguous, expert judgment may be necessary.

It should be kept in mind that not all tests have identical weight. Tests determining one of the endpoints of genotoxicity, chromosome aberrations or gene mutations, are considered of more importance than indicator tests such as SCE or UDS test. A compound should not automatically be considered as genotoxic on results of indicator tests alone. Also, in vivo tests are generally more valuable than in vitro tests: a compound should never be considered as a genotoxic carcinogen on results from in vitro tests alone, although such compounds are usually considered as a potential genotoxic carcinogen until other data are available that influence the conclusion.

Occasionally further testing may be necessary in order to make a conclusion on genotoxicity. Further testing is only justified if the additional test has an added value and may aid in reaching a final conclusion. This is due to the fact that some tests are highly correlated in their outcomes. If there are no criteria for further testing; a case-by-case approach will be necessary to indicate which tests may be best selected. Repetition of a (well-performed) test is of no value unless the experimental conditions of the test change to such an extent that it gets an unequivocal added weight. It is obvious that additional testing should be restricted to the endpoint for which the uncertainties exist.

Genotoxicity testing results in hazard identification, the first step in risk assessment. The ultimate endpoint is not DNA damage but the consequences of the DNA damage e.g. cancer or a genetic disease. The relationship between mutagenic potency predictors and quantitative carcinogenicity is weak (Fetterman et al., 1997). Thus, genotoxic
endpoints cannot normally be used directly for risk characterisation/assessment. The exceptions to this rule are thresholded mutagens but a full explanation of the use of genotoxicity testing for these types of compound falls outside the remit of this review. Reviews of this topic can be found within a special issue of Mutation Research (Issue 464, 2000). Thus, for genotoxic carcinogens, risk assessment should be performed on the disease itself using endpoints such as tumor formation.

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