Guideline for the classification of carcinogenic substances

Guide for classifying substances in terms of their carcinogenic properties, and for assessing their genotoxicity

By the Subcommittee on the Classification of carcinogenic substances
of the Dutch Expert Committee on Occupational Safety

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Health Council of the Netherlands

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foreword

At the request of the Minister of Social Affairs and Employment, the Health Council of the Netherlands evaluates the toxicity, mutagenicity and carcinogenicity of substances to which people can be exposed in the workplace.

The Subcommittee on the Classification of Carcinogenic Substances prepares the classification proposals for germ cell mutagens and carcinogens. The present guidance is a general document outlining the procedures used by the Health Council for recommendations on the classification of substances and the assessment of the carcinogenic mode of action. In specific cases, the Committee can decide to deviate from this guideline when scientifically substantiated.

01 Introduction

1.1 Background

Exposure to carcinogenic substances or processes in the work environment can pose serious health risks. Accordingly, the Working Conditions Decree includes rules on working safely with such substances (or processes). As a general rule, all carcinogenic substances should be replaced by substances with less hazardous properties if possible. Substances that should be regarded as carcinogenic are listed by the Ministry of Social Affairs and Employment (SZW).

At the request of the SZW, the Health Council evaluates the mutagenicity and carcinogenicity of substances to which workers can be exposed in the workplace. Classification proposals for germ cell mutagens and carcinogens are prepared by the Subcommittee on the Classification of Carcinogenic Substances, a Subcommittee of the Dutch Expert Committee on Occupational Safety (DECOS). The criteria for the classification categories are based on the Globally Harmonized System, which has been incorporated into the system and guideline used by the European Union (Regulation (EC) No 1272/2008) for the classification, labelling, and packaging of substances and mixtures (the CLP regulation).

The Committee notes that besides this regulation, the International Agency on the Research of Cancer (IARC) also provided an important framework for the evaluation of carcinogenic substances.[1] The IARC also applies classification categories. However, the regulatory purpose differs from the CLP regulation and different classification categories are applied.

The proposals are based on a hazard assessment, in which the genotoxic and carcinogenic properties are extensively evaluated in a weight-of-evidence approach. The Subcommittee also assesses the mechanism(s) that may account for the carcinogenicity of a given substance. Based on this information, the DECOS decides whether to derive a threshold-hold based recommended occupational exposure limit or to calculate cancer risk values. For the latter, the Economic and Social Council subsequently recommends on the technical and economic feasibility of these cancer risk values. The Ministry of Social Affairs and Employment ultimately sets a statutory limit value.

The present guidance is a technical document used by the Health Council for recommendations on classification of mutagenic and carcinogenic substances, and the assessment of the carcinogenic mode of action.

1.2 The Committee

This guideline was drawn up by the Subcommittee on the Classification of Carcinogenic Substances, of the Dutch Expert Committee on Occupational Safety (DECOS), hereafter referred to as the Committee.

1.3 Structure of the guideline

In Chapter 2 and 3 respectively, the Committee's considerations for the classification of mutagenic and carcinogenic properties are described.

In addition, the Committee can also receive a request from the DECOS to assess the carcinogenic potential of a substance and to determine its mode of action. Dependent on the genotoxic mode of action, the DECOS will derive a threshold-based or a risk-based advisory value. Experimental approaches to distinguish different modes of action are described in chapter 4.

02 classification for mutagenicity

The Committee's recommendation for the classification for germ cell mutagenicity is based on a hazard assessment, in which the genotoxic properties are extensively evaluated in a weight of evidence approach (See *Section 03 quality of the individual studies* of the *Guidance for recommending classifications and health-based occupational exposure limits* for more details).

Although the criteria in the EU Regulation are set for substances that are evaluated according to the CLP regulation, the Committee also considers the criteria useful in recommending classifications for individual substances, mixtures and emissions for which the Regulation does not apply.

2.1 Classification criteria for mutagenic substances

The criteria for the classification categories for germ cell mutagenicity are based on the Globally Harmonized System, which has been incorporated into the system and guideline used by the European Union for the classification, labelling, and packaging of substances and mixtures (Regulation EC 1272/2008, CLP: Section 3.5, Germ cell mutagenicity).

In addition to the EU categories, the Committee added two categories for substances that cannot be classified for mutagenic properties to provide additional clarity as to why a substance is not recommended to be classified. This is the case when available data are insufficient to evaluate the mutagenic properties of the substance (category 3) or when the substance is considered as probably not mutagenic to humans (category 4).

The Committee expresses its conclusions in standard phrases:

Table 1 Hazard categories for germ cell mutagens

Categories	EU classification categories	EU hazard statement codes
CATEGORY 1:	Known or presumed cell mutagen A substance may be further distinguished as: Category 1A: substances known to induce heritable mutations in the germ cells of humans	H340 May cause genetic effects
	Category 1B: substances to be regarded as if they induce heritable mutations in the germ cells of humans	H340 May cause genetic effects
CATEGORY 2:	Substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans	H341 Suspected of causing genetic effects
CATEGORY 3[a]	The available data are insufficient to evaluate the cell mutagenic properties of the substance	
CATEGORY 4[a]	The substance is probably not mutagenic to man	

[a] Not an EU classification category.

The Committee uses the following criteria specified in Regulation EC 1272/2008 for the various categories:

- Category 1A:
  Positive evidence from human epidemiological studies[a].
- Category 1B:
  - Positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals; or
  - Positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity/ genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or
  - Positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people.
- Category 2:
  Positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from:
  - Somatic cell mutagenicity tests in vivo, in mammals; or
  - Other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays.
- Category 3:
  The substance is classified into this category if there is insufficient, good quality human and experimental data on a substance's mutagenicity.
- Category 4:
  A substance is placed in this category when there is sufficient data from both epidemiological studies and experimental animal studies to indicate that mutagenicity in man is unlikely.

[a] Epidemiological studies have been to date unable to provide evidence to classify a substance as a Category 1A mutagen. Hereditary diseases in humans for the most part have an unknown origin and show a varying distribution in different populations. Due to the random distribution of mutations in the genome it is not expected that one particular substance would induce one specific genetic disorder. Therefore, it is unlikely that such evidence may be obtained by epidemiological studies to enable classification of a substance as a Category 1A mutagen. However, in the future novel technology may be implemented that enables category 1A classification, e.g., deep sequencing in germ cells revealing elevated mutagenesis.

2.2 Assessment of study results

Evaluation of epidemiologic studies

For most agents, only observational studies are available that require careful evaluation of study quality before evidence from these studies is deemed sufficient for classification. While many standardized tools for epidemiological study quality evaluation have been developed, these typically do not work well for the evaluation of observational studies assessing carcinogenic effects. The Committee therefore prefers to use an approach that is based on a transparent inventory of all study aspects that have a strong impact on overall study quality. This approach also implies that when deriving evidence from epidemiological studies the complete set of studies is taken into account, rather than evaluating studies one by one. DECOS and its subcommittees follow the same approach which is described in Section 3.2.1 Epidemiological studies of the *Guidance for recommending classifications and health-based occupational exposure limits*, which can be found on the council's website www.healthcouncil.nl.

Evaluation of experimental studies

For recommending a classification, the Committee considers test results from experiments determining mutagenic and/or genotoxic effects in germ and/or somatic cells of exposed animals. Mutagenic and/or genotoxic effects determined in in vitro tests shall also be considered.
The Committee classifies on the basis of well conducted, sufficiently validated tests, preferably as described in Regulation (EC) No 440/2008 adopted in accordance with Article 13(3) of Regulation (EC) No 1907/2006 (Test Method Regulation).

The Committee takes into account that many in vitro and in vivo genotoxicity tests have been developed over the years (OECD guidelines) and used to classify a substance for its genotoxic properties. The OECD guidelines are periodically updated to include new scientific insights.
As a result, the OECD has withdrawn some of these tests or downgraded them to indicator tests in recent years, because questions have arisen on their validity. Examples are tests for unscheduled DNA synthesis, sister chromatid exchanges, and the mouse heritable translocation test.
The Committee still includes studies using these tests in its evaluation, but gives them a lower weight in the evidence synthesis for genotoxicity.
The current status of the OECD Test Guidelines for genetic toxicology can be found in Guidance Document on Revisions to OECD Genetic Toxicology (OECD 2015).

To assess the acceptability of an individual test, and to assess whether a result is positive or negative, the Committee takes into account the general assessment criteria according to the OECD. Several aspects are important to take into consideration, including testing an appropriate number of doses and analysing sufficient number of cells, proper dose selection and correct statistical analysis. Importantly, exposure of the

target cells or target tissue has to be demonstrated. ADME data, obtained using the same route and same species, can be used to demonstrate bone marrow exposure.

03 classification for carcinogenicity

The Committee's recommendation for the classification of carcinogenic substances is based on a hazard assessment, in which the carcinogenic properties and mode of action are extensively evaluated in a weight of evidence approach (See Section 03 quality of the individual studies of the *Guidance for recommending classifications and health-based occupational exposure limits* for more details).

Classification for carcinogenic substances mainly follows European guidelines.

In 3.1, the EU classification criteria for carcinogenicity is given and explained.

In 3.2, the assessment of study results will be discussed. Since the classification criteria are based on data of human and animal studies, the Health Council uses a decision table (Table 3) that simplifies the classification based on the outcome of both human (epidemiological) and animal studies, which is further explained in 3.3.

3.1 Classification criteria for carcinogenic substances

The Committee bases its classification categories for carcinogenicity on the European legislation for the classification, labelling, and packaging of substances and mixtures (Regulation EC 1272/2008, CLP: Section 3.6, Carcinogenicity). In addition to the EU categories, the Committee added two categories for substances that cannot be classified for carcinogenic properties to provide additional clarity as to why a substance is not recommended to be classified. This is the case when available data is insufficient to evaluate the carcinogenic properties of the substance (category 3) or when the substance is considered as probably not carcinogenic to humans (category 4).

The Committee expresses its conclusions in standard phrases:

Table 2 Hazard categories for carcinogens

Categories	EU classification categories	EU hazard statement codes
CATEGORY 1:	Known or presumed human carcinogens A substance is classified in Category 1 for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as:	
	Category 1A: known to have carcinogenic potential for humans, classification is largely based on human evidence.	H350 May cause cancer
	Category 1B: presumed to have carcinogenic potential for humans, classification is largely based on animal evidence.	H350 May cause cancer
CATEGORY 2:	Suspected human carcinogens	H351 Suspected of causing cancer
CATEGORY 3[a]	The available data are insufficient to evaluate the carcinogenic properties of the substance.	not applicable
CATEGORY 4[a]	The substance is probably not carcinogenic to man.	not applicable

[a] Not an EU classification category

The Committee uses the following criteria for the various categories:

- Category 1A:
  There is sufficient evidence from epidemiological studies to support the existence of a causal relationship between human exposure and the development of cancer. In addition, there is a plausible explanation for a causal relationship between exposure and effect[a]. In some cases, a substance for which there is only limited evidence from epidemiological studies to support a relationship between exposure and the development of cancer can still be placed in this category if the studies in question are complemented by sufficient evidence from animal studies to establish the existence of such a relationship.
- Category 1B:
  There is sufficient evidence to suggest that human exposure results in an increased risk of cancer developing in those exposed.
  Positive epidemiological data are lacking, but based on chronic animal experiments and other relevant information, it is likely that the substance causes cancer in man. A substance is considered carcinogenic to man if a marked increase in the number of malignant tumours has been obtained in at least two experimental animal species, or in a single species in two or more independent studies. In certain cases, the Committee may decide to also include non-malignant tumours. If – in addition to two positive studies – negative studies are available, the Committee may decide to place the substance in Category 2.
- Category 2:
  There is evidence from experimental animal studies showing that exposure to this substance can cause cancer. However, the information is insufficient to classify the substance as is presumed to be carcinogenic to man. There are a number of possible reasons for a substance to be classified into this category:
  - Only one, good quality experimental animal study is available. This animal study demonstrated positive results.
  - Several experimental animal studies are available, all with positive results. These studies, however, are of less quality which precludes classification into Category 1B.
  - Good-quality experimental animal studies have been conducted, but either the results do not give a clear picture or the data are open to interpretation. This is the case if, for example:
    - tumour incidence in animals is only increased with borderline significance, which can be due to high spontaneous tumour incidence.
    - in animals, tumour incidence is increased following exposure via routes that are less relevant to the human situation (e.g. intraperitoneal or intravenous).

In exceptional cases, a positive result in only a single animal species can nevertheless lead to the substance being classified as is presumed

[a] Criteria have been developed to assess whether an epidemiological association also implies the existence of a causal link (see Hill published in 1965).[2]

to be carcinogenic to man (Category 1B). This is the case when there is a substantial amount of supportive evidence, such as (a) positive genotoxicity data, (b) evidence of carcinogenicity or genotoxicity from metabolic or biochemical studies, (c) induction of benign tumours in a second animal species, (d) structural similarity with known carcinogens (Category 1A or Category 1B).

- Category 3:
  There is insufficient, good quality human or experimental animal data on the carcinogenic potential of the substance.
- Category 4:
  There is sufficient data from both epidemiological studies and experimental animal studies showing the absence of a relation between exposure and tumour development. A number of good epidemiological studies and experimental animal studies have been published. In these studies either no exposure-induced tumours were observed, or the tumours (including the mode of action) in the animal study were not relevant to man.

3.2 Assessment of study results

Beyond the determination of the strength of evidence for carcinogenicity, a number of factors need to be considered that influence the overall likelihood that a substance poses a carcinogenic hazard in humans. The full list of factors that influence this determination would be very lengthy, but some of the more important ones are considered below.

To assess the results of available studies, the Committee takes the following factors into account (adapted from ECHA (2017) Guidance on the Application of the CLP Criteria; Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures):

a. Tumour type and background incidence:
   Carcinogenic effects in experimental animals are considered relevant to humans by default. They are considered for classification unless there is sufficient evidence to show that certain types of tumours are not relevant to humans.
   It is generally considered positive evidence of carcinogenic activity if there is a statistically significant increase in tumour incidence, especially when a dose-response relationship is present. However, when the spontaneous tumour incidence is high, increased incidence of tumours in treated animals may lie at the borderline of biological and/or statistical significance. In these cases robust statistics must be availabe.
b. Multisite responses:
   Generally, two-year bioassays in mice or rats are used to determine whether chemicals have carcinogenic potential. Chemicals induce a wide range of responses, ranging from no effects in either species to the induction of multiple malignant neoplasms in both species. Between these two extremes, a wide range of responses can be observed in tissues, sexes, and species, demonstrating important differences

among the carcinogens and between the species tested. In order to determine which classification is appropriate, it is important to consider the tumour profile observed with a substance. If a substance causes tumours in more than one species and/or at multiple sites, it usually provides strong evidence of carcinogenicity.

c. Progression of lesions to malignancy:
Generally, when a substance increases tumour incidence in response to treatment, it will meet the criteria for classification as a carcinogen. Demonstrating that a substance causes malignant tumours will usually be sufficient to support a Category 1B classification (CLP Annex I, 3.6.2.2.3). Currently, benign tumour induction is considered to provide a lower strength of evidence than malignant tumour induction and usually supports a Category 2 classification (CLP Annex I, 3.6.2.2.3). Nonetheless, benign tumours can also pose significant concerns, and the strength of evidence should be evaluated using expert judgement regarding their significance. For example, any indication that the observed benign tumours resulted from mutation induction may increase concern and dictate Category 1B classification.

d. Reduced tumour latency:
The latency of tumour development i.e., the rate at which a substance induces tumours, is often a reflection of the potency of a carcinogen, but is currently not routinely included in classification and hazard categorisation. However, a substance causing unusual tumor types or tumours that manifest with reduced latency may add to the weight of evidence for a substance's carcinogenic potential. Reduced latency may be indicative of carcinogenic potential even in cases where tumour incidence is not statistically elevated, e.g., due to high spontaneous tumour incidence.

e. Whether responses are in single or both sexes:
In standard studies for carcinogenicity, both male and female animals are tested. However, tumours may be observed in one sex only, which may be caused by two broad reasons. The tumour may develop in a gender-specific tissue, such as the uterus or testes (sex-specific tissue), or it may develop in a non-sex-specific tissue, yet in one gender only. In the latter case, gender- or sex-specific mechanisms may promote tumorigenesis, such as hormone-mediated mechanisms or mechanisms involving gender- or sex-specific differences in toxicokinetics.

f. Whether responses are in a single species or several species:
According to the criteria, carcinogenicity in a single animal species (both sexes and ideally in a GLP study) could qualify as sufficient evidence and could therefore result in a Category 1B classification if no other evidence exists. However, it is possible to consider a single study in one species and sex as sufficient evidence of carcinogenicity if malignant neoplasms occur in an unusual manner in terms of incidence, site, type of tumour, or ages of onset, or when there is strong evidence of tumours at multiple sites. Furthermore, in case positive in vivo mutagenicity data in combination with a single study in one

species and sex exist, this would be considered sufficient evidence of carcinogenicity. The presence of positive responses in several species adds to the weight of evidence that a substance is carcinogenic.

g. Structural similarity to a substance(s) for which there is good evidence of carcinogenicity:
   In some cases, chemical substances that have not been tested for carcinogenicity might be classified as carcinogens based on tumour data from structurally similar chemicals that are already classified for carcinogenic activity. A robust and transparent argument must always support this assumption. Evidence of similarity can also be derived from data on toxicokinetics, physico-chemical properties or mutagenic activity.
h. Routes of exposure:
   Carcinogenicity classification does not take into account the specific routes of exposure. Unless there is a robust justification for dismissing the findings from a particular route, a chemical that has found to cause tumours will be classified, regardless of how it is administered.
i. Comparison of absorption, distribution, metabolism and excretion between test animals and humans:
   For classification, it is important to consider the physico-chemical, toxicokinetic, and toxicodynamic properties of the substance, as well as any information available on their chemical analogues i.e., structure activity relationship.
j. The possibility of a confounding effect of excessive toxicity at test dose:
   Carcinogenic responses in bioassays can be affected by excessive toxicity, such as toxicity at doses above the maximal tolerated dose (MTD). In addition to cell death (necrosis) and associated regenerative hyperplasia, such toxicity may cause tumour development as a secondary consequence, unrelated to the intrinsic ability of the substance itself to cause tumours at lower levels.
   In general, tumours that are only found at excessive doses that are severely toxic are more doubtful carcinogens in humans. Furthermore, tumours that occur only at sites of contact or at excessive doses need to be evaluated carefully for their carcinogenic potential in humans
k. Mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression:
   When a mechanism of tumour formation that is not relevant to humans is identified, then classification may not be appropriate. The carcinogenic evidence for a tumour can only be rejected if the mechanism of the tumour development is determined not to be relevant to humans.

3.3 Classification table for the assessment of human and experimental animal data

To assess the overall evidence of the available studies, the Committee defined four categories of evidence (see below). The evidence for human and experimental animal studies is first evaluated separately.

Assessment of results from epidemiological studies

++ Sufficient evidence of carcinogenicity. It is anticipated that a causal relationship exists between exposure to an agent and the development of cancer. That is to say, more than one study in human subjects has found a positive association between exposure and cancer, in which chance, bias and confounding can reasonably be excluded.

\+ Limited evidence of carcinogenicity. Studies in human subjects have established that there is a positive association between exposure and cancer. However, the possibility that chance, bias and confounding may play an important part in this cannot be excluded with any certainty.

? Inadequate evidence regarding carcinogenicity. There is little or no data to support statements concerning an association between exposure to an agent and cancer.

– Evidence suggesting lack of carcinogenicity. Sufficient evidence for the absence of carcinogenicity in more than one human study.

Assessment of results from experimental animal studies

++ Sufficient evidence of carcinogenicity. A causal relationship has been established between exposure to an agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in:

a. two or more animal species, or
b. in two or more independent studies using a single animal species carried out at different times or in different laboratories or under different protocols.

\+ Limited evidence of carcinogenicity. Experimental animal data suggests the presence of a carcinogenic effect, but no definitive conclusion can yet be drawn, as:

a. all of the available data comes from just a single animal study;
b. there are unresolved questions regarding the adequacy of design, conduct or interpretation of the studies;
c. the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential without evidence for oncogenic mutations; or
d. the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

? Inadequate evidence regarding carcinogenicity. There is little or no data to support statements concerning an association between exposure to an agent and cancer.

– Evidence suggesting lack of carcinogenicity. Sufficient evidence for the absence of carcinogenicity in experimental animal studies.

Based on the evaluation of the epidemiological data and experimental animal studies, the Health Council recommends a classification into one of the four categories. The Committee applies a decision chart to combine the evidence from both epidemiological studies and animal experiments.

Table 3 Classification table on assessment of human and experimental animal data

Animal experiments[a]	Epidemiology			
	++	+	?	–
++	1A	1A	1B	2
+	1A	1B	2	3
?	1A	2	3	4
-	1A	3	3	4

[a] This is an overall assessment of the animal experimental data taking into account whether or not the tumours observed are relevant to humans.

It is noted that this decision chart provides a general classification frame-work, the Committee deviates from this decision chart when supported by scientific data.

With regard to substances classified into Category 1A and Category 1B, the Committee also assesses which genotoxic mode of action is involved in the development of cancer to decide whether the health-based OEL for the substance should be risk- or threshold based (see Chapter 4).

04 determination of genotoxic mode of action for guidance value derivation

4.1 Introduction

For substances that are recommended to be classified as known to be carcinogenic to man or presumed to be carcinogenic to man, the Committee determines whether a genotoxic mode of action is involved. The Committee distinguishes non-genotoxic, genotoxic substances and substances for which genotoxicity has been insufficiently investigated. Also, the DECOS can request the Committee to judge the mode of action as this determines the type of health-based recommended occupational exposure limits (OELs) that the DECOS should derive. These OELs are average concentrations of a substnce in the air of a workplace that should cause no harm.

For a non-genotoxic carcinogen, it is assumed that a safe threshold can be determined, and the DECOS can derive a health-based recommended occupational exposure limit (HBR-OEL).

Genotoxic carcinogens can act by a direct or indirect mode of action.

For direct-acting genotoxic carcinogens, a safe threshold cannot be determined. Therefore, for these substances, Health-Based Calculated Occupational Cancer Risk Values (HBC-OCRVs) are derived by the DECOS. These are exposure levels (concentration in the air) that correspond to a predefined accepted level of risk of death from cancer that is set by the government.

For indirect genotoxic carcinogens, it is assumed that there is a safe limit (similar to non-genotoxic carcinogens). For these substances, the DECOS can derive a HBR-OEL (See Section 06 health-based occupational exposure limits of the *Guidance for recommending classifications and health-based occupational exposure limits* for more details).

In case the exact mechanism of action cannot be determined for a substance, the Committee takes a cautious approach. In this case, the Committee views the substance in question as a direct genotoxic carcinogen until new data proves otherwise. In such cases, the Committee will proceed on the basis of a worst-case scenario, which is that the substance in question is capable of initiating the cancer process. With this in mind, if a given substance has multiple carcinogenic mode of actions, at least one of which involves a direct genotoxic mode of action, then the Committee will recommend to use the direct genotoxic mode of action as a basis for deriving an occupational limit value. In exceptional and specific cases, the Committee retains the option of deviating from this principle, for

example, if data becomes available that indicates that other mechanisms play a dominant role.

4.2 Proposed categorisation based on genotoxic mode of action

In view of the above, the Committee uses the following categorisation based on mode of action:

Mode of action	Limit value based on
Genotoxic - direct	HBR-OCRV
Genotoxic - indirect	HBR-OEL
Non-genotoxic	HBR-OEL

The Committee uses one of the following standard phrases:

- The substance acts by a direct genotoxic mechanism.
- The substance acts by an indirect genotoxic mechanism.
- The substance acts by a non-genotoxic mechanism.
- Its genotoxicity has been insufficiently investigated. Therefore, the mode of action is not known.

4.2.1 Genotoxic carcinogens

Carcinogens that act by a direct genotoxic mechanism

This group includes substances that (either in their unchanged form or as reactive metabolites) interact directly with DNA, causing damage (adducts, single- and double-strand breaks). If this damage is not repaired quickly or adequately, gene mutations and chromosome abnormalities can occur at sites that are associated with carcinogenesis. Some examples are benzo[a]pyrene (DNA alkylation), methyl methane sulphonate (DNA alkylation) and reactive oxygen radicals (DNA breaks).

DNA changes caused by interactions with genotoxic carcinogens are known as hits. Even at the lowest possible exposure (which, in theory, could involve just a single molecule), genotoxic carcinogens can still initiate the cancer process, although the risk is very small. This line of reasoning clearly indicates that when two molecules of carcinogen are present the risk involved is increased accordingly. In this way, a linear relationship could be created between exposure and the risk of a hit. This is also referred to as one-hit kinetics.

The concept of linearity at low exposures also involves a number of assumptions. One of these is that the occurrence of DNA damage is a direct process, in which the state variable is DNA damage or no DNA damage, and in which the risk or probability of this resulting from exposure to a carcinogen is determined by chance and not by the degree of DNA damage. Another assumption is that there is no threshold value below which a substance that causes hits can be considered inactive. In other words, there is no dose at which the risk of a relevant effect is equivalent to zero. The one-hit kinetics at low exposure means that, no matter how low the exposure involved, there is always an elevated risk of cancer.

Accordingly, safety considerations dictate that it would be best to derive a HBC-OCRV.

DNA changes are continuously being induced by genotoxic carcinogens that are either naturally present in food or in the environment, or that are generated by normal metabolic processes and inflammatory responses in the body, such as various reactive oxygen radicals (background effects). Much of the DNA damage is corrected by efficient DNA repair enzymes (defence mechanisms). These background effects and defence mechanisms might explain, for instance, why DNA damage and mutations caused by direct genotoxic substances only become apparent at higher exposures. More information about background effects and defence mechanisms can be found in Annex A.

Carcinogens that act by an indirect genotoxic mechanism

These include substances that do not interact directly with DNA, but which can ultimately damage DNA indirectly. Various mechanisms are involved in this process, some of which are described below.

Inhibiting DNA repair

Cells possess DNA repair mechanisms that are capable of correcting many types of damage to their DNA. Accordingly, substances capable of inhibiting DNA repair mechanism can cause permanent DNA damage. [3,4]

Salts of cadmium, arsenic and nickel are examples of substances that act in this way.[5]

Effects on the spindle apparatus

The spindle apparatus plays a major part in cell division, by controlling the segregation of chromosomes. The spindle apparatus, which is a complex of centrosomes and microtubules, is part of the cytoskeleton. Substances such as vincristine and vinblastine sulphate,[6,7] which interact with spindle apparatus structures, can cause chromosomal aberrations.[3,4]

Topoisomerase inhibitors

Topoisomerases are enzymes that change the supercoiling of double-stranded DNA, by cutting and re-ligating one or both of its strands. They play an essential part in DNA transcription and replication, hence also in cell division. Topoisomerases are classified into a range of different types, depending on the exact nature of their function. These enzymes can be inhibited by cytostatic drugs, such as topoisomerase I inhibitors, camptothecin, *irinotecan and topotecan*, and topoisomerase II inhibitors, etoposide and doxorubicin, resulting in breaks in DNA, chromosomal aberrations and cell death (apoptosis).[3]

These are examples of indirect genotoxic carcinogens that interact with proteins involved in DNA repair, DNA replication, and chromosome segregation. As they can ultimately damage the DNA or chromosomes,

this group of substances are classified as genotoxic substances. However, they do not function in accordance with direct principles, as relevant DNA damage only occurs when the carcinogen inhibits the activity of the enzymes in question, or that of other proteins, to such an extent that their capacity for repair can no longer meet the requirement. Only then can sufficient damage accumulate for its effects to become significant and visible. Hence a threshold is involved.[8,9]

4.2.2 Non-genotoxic carcinogens

These carcinogens are capable of promoting various phases of the cancer process without damaging DNA, either directly or indirectly. Such substances are known as tumour promoters. Various non-genotoxic mechanisms contribute to cancer processes, of which some are described below.

Regulation of gene expression

Some processes affect gene expression without changing the DNA sequence, nevertheless these changes in expression are transmissible to daughter cells. One example of such an effect is the hyper- or hypo-methylation of gene promoter sequences, i.e. the C5 position of cytosine in a CpG sequence.[10,11] Changes in DNA methylation can cause genes to be activated or turned off, which can dramatically change a cell's behaviour. Substances suspected of causing cancer in this way include arsenite, dichloroacetic acid and trichloroacetic acid.[12]

Disruption of hormonal balance

Some substances can disrupt the hormonal balance and the functions of some hormones, either by accelerating the breakdown of hormones (e.g. thyroxine in rats) or because the substances themselves exert a powerful hormonal effect (e.g. oestrogenic substances). This increases the risk of tumour development, especially in hormone-sensitive organs.[13]
One example is the induction of thyroid cancer by polychlorinated biphenyls (PCBs).[14]

Inhibition of gap junctional intercellular communication

The inhibition of intercellular communication adversely affects the differentiation, proliferation and migration of cells, as well as programmed cell death (apoptosis).[15] Some substances can affect the expression of the numerous genes involved in intercellular communication, as well as the activity and function of the proteins involved. Such substances include phorbol esters (12-O-tetradecanoylphorbol-13-acetate (TPA / PMA)) and fluoranthene.[16-18]

Regulation of growth factors and steroid hormones

Cell proliferation, cell differentiation, and programmed cell death are regulated and controlled by a range of factors that stimulate or inhibit growth, such as cytokines. The inhibition or stimulation of such factors can promote the cancer process. Some examples are the use of steroid hormones such as oestrogen and progesterone in hormone therapy, and

possibly some phytoestrogens (at high intake levels).[19-23] Another example is the protein hormone insulin, which can contribute to tumour growth in breast cancer.[24]

Immunosuppression
Substances such as cyclosporin and purine analogues can suppress the immune system.[14,25] Any weakening of the immune system can lead to the uncontrolled growth of cancer cells.

Chronic tissue damage
Some carcinogens induce cancer by causing chronic tissue damage in organs. An example of this is the chronic renal toxicity caused by chloroform.[26] The body responds either with massive regeneration or with an inflammatory reaction, involving inflammatory cells such as macrophages. This is basically a normal biological reaction that ends with the recovery of the affected tissue. However, chronic exposure and toxicity may well lead to the development of cancer by promoting proliferation of cells suffering from endogenous DNA damage.

Accordingly, as shown above, the mechanisms underlying non-genotoxic effects are many and varied. Non-genotoxic mechanisms can promote the growth of DNA-damaged cells by stimulating cell proliferation while inhibiting the immune response to initiated cells, for example, which may ultimately lead to detectable tumours and even metastases. However, this tumour-growth-promoting effect does not appear until exposure levels reach a threshold at which the mechanisms that promote such growth become manifest.

4.3 Endpoints of carcinogenic modes of action

There is a wide range of test systems for identifying a carcinogen's mode of action. Table 4 contains a list of measurable endpoints and of the carcinogenic modes of action that may be associated with them.
In practice, the results of several types of tests are needed to arrive at a decision. In addition, it is quite likely that a given carcinogen may have several different modes of action.

The Committee uses the results of these tests to determine whether a substance has a genotoxic effect, and whether this effect is direct or indirect in nature. In this connection, it works on the assumption that substances that damage DNA indirectly by their interaction with proteins (e.g. by inhibiting DNA repair) theoretically involve a threshold. Substances that damage DNA directly have no threshold as theoretically they can damage DNA at very low concentrations. In addition, pragmatic decisions will be taken in some cases if the available experimental data so requires. In cases where experimental data is ambiguous, pragmatic decisions need to be taken.

Table 4 Indicators of carcinogenic mode of action

Endpoint	Mechanism(s)	Genotoxic - Direct	Genotoxic - Indirect	Non-genotoxic
DNA-adducts	• direct interaction with DNA	+	–	–
	• inhibition of DNA-repair enzymes	–	+	–
DNA-breaks	• direct interaction with DNA	+	–	–
	• replication of damaged DNA	+	–	–
	• inhibition of DNA-repair enzymes	–	+	–
Gene mutations (mutations, deletions, amplifications, breaks, translocations)	• replication of damaged (alkylation) DNA	+	–	–
	• inhibition of DNA-repair enzymes	–	+	–
Structural chromosome aberrations (translocations, deletions, sister chromatid exchanges)	• replication of damaged DNA (alkylation, intercalation, cross-links)	+	–	–
	• inhibition of DNA-repair enzymes	–	+	–
	• inhibition of topoisomerases	–	+	–
Numerical chromosome aberrations (aneuploidy, polyploidy; micronuclei)	• replication of damaged DNA (alkylation, intercalation, cross-links)	+	+	–
	• inhibition of topoisomerases	–	+	–
	• disturbance of spindle apparatus	–	+	–
Changed gene expression[a]	• epigenetic: DNA hypo- of hyper-methylation of cytosine	–	–	+
	• epigenetic: disturbance of (de)acetylation of histones	–	–	+
	• disturbance of receptor-directed regulation of gene transcription	–	–	+
Changes in normal cell proliferation and differentiation, and cell transformation	• disturbance of hormone equilibrium	–	–	+
	• immune suppression	–	–	+
	• cytotoxicity and chronic irritation	–	–	+
	• disturbed activity of growth factors and signaling factors	–	–	+
	• disturbed receptor mediated regulation of cell division	–	–	+
	• disturbed intracellular communication (via *gap junctions*)	–	–	+

[a] Gene mutations, structural and numerical chromosome aberrations can result ultimately in changed gene expression and cell proliferation and differentiation, and cell transformation.

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annex A
defence mechanisms and background effects

Defence mechanisms

Various defence mechanisms have been identified at the molecular, cellular and organism level that are capable of preventing or halting the genotoxic cancer process:

- harmful metabolites, such as oxygen radicals, are eliminated by antioxidants and radical scavengers
- carcinogens are converted into harmless metabolites by biotransformation
- DNA is repaired by various DNA repair pathways
- damage slows down cell division, allowing more time for DNA repair
- the immune system recognises tumour cells and their precursors, and eliminates them

Ultimately, it is the balance between exposure and defence mechanisms that determines the risk of DNA damage and the subsequent development of a tumour cell.

Background effects

Background effects can be caused by genotoxic carcinogens that are always present in people's surroundings, for example in the environment and in food. They can also be caused by carcinogens that are formed by normal metabolic processes and by inflammatory responses in the body. One example is reactive oxygen radicals, which are known to be carcinogens with a direct genotoxic mechanism. Another example is the existence of certain naturally occurring DNA damage in cells, such as methylation or deamination of DNA bases.[27,28] Accordingly, endogenous processes can also contribute to the risk of cancer.

Reactive oxygen species (ROS) are normally produced in large amounts by metabolic processes and by inflammatory reactions in the body. This may lead to oxidative DNA damage (oxidative stress). Ames and Gold (1991) estimated that – under steady state conditions – rat cells each contain about $1x10^6$ (one million) oxidative DNA adducts, and that about $1x10^5$ new oxidative DNA adducts are formed each day.[27] At the cellular level, however, there is a powerful antioxidant defence system that eliminates ROS. There is also a DNA repair system, which ensures that ROS-induced damage to DNA is rapidly repaired. It is these highly efficient defence and repair systems that make it possible for man to live in an oxygen-rich environment.

Certain xenobiotic substances that have been shown to be carcinogenic in animal studies – albeit at high exposure – are capable of producing ROS. It is suspected that this is the mechanism by which they cause cancer. The observation that there is no increase in tumours at low exposure can be explained by the fact that the amount of ROS produced by the carcinogen was negligible compared to the quantity of oxygen radicals generated by normal cellular processes. This means that there is no significant increase in the amount of ROS in the cell. At the lowest possible exposure (a single molecule) to xenobiotic carcinogens that produce one or several ROS, one or several hits fade into insignificance compared to the number of hits caused by normal biological processes. Accordingly, there will be no increased risk of DNA damage at low exposure, which means that there is a threshold below which no significant effects are observed. Pyrocatechol and cadmium are examples of carcinogens that generate ROS.

In practice, however, it will not be easy to demonstrate the existence of such thresholds for substances of this kind. This would involve carrying out a broad-based experimental animal study to obtain data on the status of oxidative stress at the cellular level in relation to tumour incidence as a function of exposure to the substance in question. This study is necessary to determine whether there is a causal link between that oxidative damage and carcinogenicity, and whether the existence of a threshold can be demonstrated experimentally. Where such data is unavailable, the Committee assesses genotoxic carcinogens that generate ROS as direct genotoxic carcinogens, given the genotoxicity of the ROS that is produced. This implies that linear extrapolation is the appropriate risk assessment methodology. However, if the exposure-response data unequivocally indicates the existence of a threshold and if it is also shown that no increase in oxidative stress occurs beneath that threshold then, according to the Committee, the cancer risk can be estimated using a threshold value.

In addition, with regard to the quantitative risk analysis of direct genotoxic carcinogens, it is important to determine the scope of any background effects involved. In the case of oxidative DNA damage, they would be high and might even be measurable using current technology. In other situations, that is not necessarily the case. Methyl methane sulphate, for example, induces characteristic DNA damage involving the creation of DNA adducts of the same type that occur naturally, albeit at a very low frequency. So low, in fact, that in everyday situations the natural background level is barely measurable with current technology. In situations like this, linear extrapolation is the only option.

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